THE DISTRIBUTION OF PHOSPHORUS IN SOME BONES OF THE WHITE RAT (RATTUS NORVEGICUS ALBINUS) WHOSE GROWTH HAS BEEN ACCELERATED BY GROWTH HORMONE. II. 80 HOURS AFTER A SINGLE INJECTION OF RADIOACTIVE PHOSPHORUS

by

VICTOR KAUFMAN

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INTRODUCTION

With the advent of the atomic age has come an abundance of radioactive isotopes of elements of biological importance. Many experiments have been devised and completed with the aid of these isotopes. Among the more common of the isotopes used as tracers is phosphorus, atomic weight 32.

One of the important characteristics of radioactive phosphorus is that it has a half life of 14.3 days. This is long enough to enable the researcher to carry out his experiment without due haste and short enough that the disposal problem is not difficult. Another characteristic of the isotope is that it decays by pure beta-ray emission with an energy of 1.7 Mev. Beta-rays of this energy are absorbed in a fairly thin layer of bone or any metal and do not create a hazard.

Phosphorus is found combined in all parts of the body with other elements to form both organic and inorganic compounds. However approximately eighty per cent of it is found in the bones and teeth in combination with calcium.

The bones serve not only as structural elements but also as storehouses of calcium and phosphorus which may be mobilized at times when the assimilation of these minerals is inadequate to meet bodily 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.

The growth of bone is markedly influenced by growth hormone (4, 14, 15). Administration of the hormone reinitiates growth in "plateaued" animals (5). Pecher (11) in experiments on the deposition of radioactive isotopes of calcium and strontium has shown that the distribution of these two elements in body tissues is similar. In the experiment of Marx and Reinhardt (10) it was found that growth hormone did not affect the total amount of strontium deposited in the femur and mandible of hypophysectomized rats.

Growth hormone produces an increase in the inorganic phosphorus of the blood and hypophysectomy causes a sharp reduction of this level (1, 8, 12, 16).

Manly and Bale (9) found that normally a rapid deposition of blood phosphorus takes place in the bones. The epiphysis acquired about twice as much of the marked phosphorus per gram of inorganic tissue as the diaphysis in the first day following administration. This extends and confirms the work of Hahn, Hevesy, and Lundsgaard (6). The former workers also showed that the diaphysis had greater retention of acquired phosphorus after the marked phosphorus in the blood had fallen to negligible amounts.

The literature cited above brings out the possibility that the growth hormone may be influential in the metabolism of inorganic phosphorus in the bone. This experiment was devised and conducted with the idea of finding the relationship, if any, between growth hormone and inorganic bone phosphorus deposition.

The radioactive isotope and autographic techniques were used to determine the location of phosphorus.

This problem was performed in co-operation with Mr. Robert H. Buchholz, Department of Zoology, Kansas State College.

METHODS AND MATERIALS

In the experiments white Wistar rats were used. These rats were interbred for several generations. This gave some assurance that unknown factors from different hereditary background would not cause discrepancies in the final results.

The rats were divided into four groups, A, B, C, and D, for the purpose of injection with the substances used in the experiment. Groups A and B were injected with growth hormone. Groups A and C were injected with the radioactive isotope of phosphorus. This type of subdivision gave four distinct groups. (1) Group A (rats 1, 2, and 3) – Injected with both the hormone and the phosphorus. (2) Group B (rats 4, 5, and 6) – Injected with growth hormone. (3) Group C (rats 7 and 8) – Injected with the phosphorus. (4) Group D (rats 9 and 10) – Injected with nothing and therefore used as a control. Care was taken to have the males and females classified in these groups in such a way that comparison could be made among individuals of the same sex.

The rats of groups A and B were injected daily except Sunday with growth hormone from March 2, 1950 until they were sacrificed. In order to observe the effect of the hormone, dosages

were increased in the following manner. One rat unit per rat per day was injected for thirteen days. The dosage was increased to two rat units per rat per day for three days and was then doubled to four rat units. Fifty two days later the dosage rate was increased to twenty rat units per rat per day. This was continued until the date of sacrifice.

Weight and tail measurements were taken twice a week of all the animals of these four sections. Buchholz (3) shows graphic representations of the average weights of all injected males, normal males, injected females, normal females, and of the average weight of the hormone injected rats and the normal rats.

The rats of groups A and C were injected with approximately twenty microcuries of radioactive phosphorus, atomic weight 32, which was prepared in the following fashion:

Four millicuries of radioactive phosphorus was obtained from the Isotopes Division of the Atomic Energy Commission, Oak Ridge, Tennessee. The chemical form of this was phosphoric acid. An initial dilution was made 1.41×10^6 seconds after the original assay, the concentration at this time being 679.5 microcuries per milliliter. To one-half of a milliliter was added one milliliter of a 1.25 milligrams per milliliter solution of sodium carbonate and two and one-half milliliters of distilled water. This gave four volumes of disodium monohydrogen phosphate with a concentration of 84.9 microcuries per milliliter. This compound is more easily absorbed into the body than phosphoric acid.

This was further diluted to the required activity by adding distilled water to bring the concentration to 20 microcuries per one-half milliliter.

All further dilutions were made in a similar manner to that mentioned above.

In preparing the dilution and all other handling of the radioactive material, all of the precautions prescribed and recommended by the Atomic Energy Commission were taken. Plate I indicates the techniques that were used. Where activity warranted, all dilutions were made with remote controlled instruments from behind shields. Survey meters were used at all times to keep the radiation level below the safety level which is approximately twelve milliroentgens per hour.

The rats of groups A and C were injected with approximately twenty microcuries of radioactive phosphorus. All injections were subcutaneous.

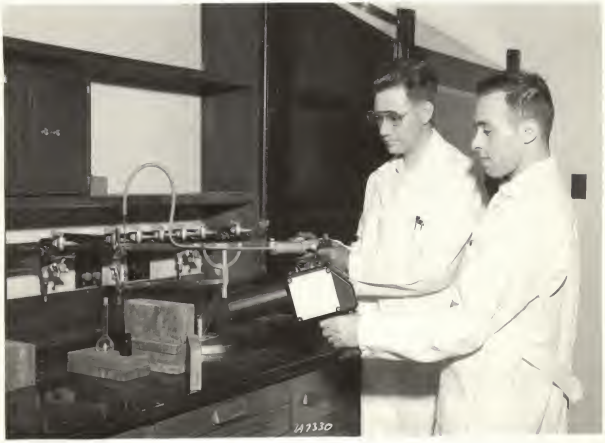
The animals were sacrificed by placing them in a large glass jar in which there was chloroform. The legs of the rat were removed and cooked in boiling water for a few minutes. This made it possible to remove all the meat and to obtain clean bones. The femora and the humeri, being the ones that interested the author, were the only ones saved for further use. It was found that simple dehydration by a single immersion in a fifty per cent alcohol solution was all that was necessary before imbedding the bones.

The femora and the humeri were imbedded in Ward's Bioplastic.

EXPLANATION OF PLATE I

Necessary precautions when working with radio isotopes showing the use of shields, remote control units, and survey meters.

PLATE I



This was accomplished by first preparing a mixture of the bioplastic and the proper amount of the catalyst. This mixture was poured into a small rectangular glass dish. Air bubbles were removed from the mixture by placing the glass dish in a bell jar vacuum system. The bioplastic was caused to set partially by placing it for a few minutes under an ultraviolet sun-lamp. The bones were imbedded in such a way that it would be possible to make sections of the left femora and humeri and of the right sagittal. Following this placement an additional layer of the bioplastic-catalyst mixture was added.

Five hours treatment of this bone-containing plastic in vacuum and under radiation from an ultraviolet lamp produced a product which could be cut and sanded. Such a procedure eliminated the long curing process normally used in this work. The specimens were further prepared for the microtome by sawing and sanding away the solid plastic, leaving a thin shell around the bone.

It is impossible to cut bone sections with the ordinary knife-edge microtome. Ordinary methods of bone sectioning produce sections too thick for optimum resolution of radioactive materials. Consequently, the microtome used was specially adapted for bone sectioning according to the method described by Roofe, Hoecker and Vorhees (13).

This conversion of the microtome consisted of removing the blade and fastening to the carriage an assembly holding a fractional horsepower motor with a circular saw mounted on its shaft.

The circular saw, containing 120 teeth with no set, was one-fourth inch in diameter and 240 microns in thickness. A variac was used to control the speed of the motor. With such an instrument sections varying in thickness from 60 to 250 microns were produced. Plate II is a photograph of the converted microtome.

The bone sections were washed in a very dilute solution of hydrochloric acid so as to remove any radioactive dust particles which might have been smeared over the section during the sectioning process or immediately thereafter. This in no way discernably effected the phosphorus previously deposited in the bone.

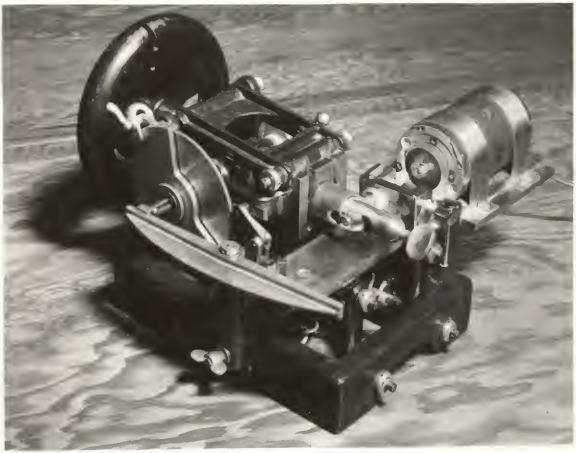
These washed sections were then immersed in a solution of acetone and placed at the end of celluloid strips which had been cut to a size one inch by three inches. Two, and sometimes three sections were placed in this manner on each of the celluloid strips. The acetone dissolved some of the plastic which 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 firmly attached to the celluloid strip. This proved to be a rapid, bubble-free method of mounting the specimens. The absence of air bubbles in the mounting and the absence of their images in the photomicrographs was a distinct advantage.

These bone sections which were now firmly attached to the celluloid sheets were placed in contact with emulsion on film strips one inch by three inches. One end of each of the film

EXPLANATION OF PLATE II

A photograph of the adapted microtome used in this experiment showing the position of the motor and saw blade.

PLATE II



strip and the celluloid strip was then cemented together with Carter's airplane glue. This joint withstood the stresses set up during the development of the exposed film. This development was accomplished without immersion of the celluloid and the bone sections.

The bone sections were pressed into close contact with the emulsion during exposure. This was accomplished by placing the film-bone-celluloid strips between pieces of one-fourth inch bakelite sheets in a small wooden box. A few layers of these were put into each box. On top of these layers, a few pieces of lead were used as weights. The boxes were closed and made light-tight by sealing with gum-paper.

Eastman Kodak No-Screen X-Ray Film was used in early experiments. It was possible to determine the time necessary for exposure of this film with the aid of a Geiger-Muller tube and a Scalar in the following fashion: The geometry of the tube was first determined. This was done with the aid of a Radium D and E standard from the National Bureau of Standards. The standard underwent five hundred disintegrations per second. This standard was placed in the container below the tube, and the number of counts per second recorded on the Scalar was taken. A background count was also taken. The bone section was then placed in exactly the same position originally occupied by the standard and the number of counts per second was again recorded. The area of the bone section was next determined. With these numbers available, the number of disintegrations per second per square centimeter of

bone could be calculated. Hamilton (7) has estimated that a total density of approximately 5,000,000 disintegrations per square centimeter are required for sufficient blackening of this photographic emulsion to yield a satisfactory radioautograph. Having a measure of the number of disintegrations per second per square centimeter of bone it was then possible to calculate the time for the required number of beta particles to strike the emulsion. The exposure time for this film was in general about twelve hours.

In later work Eastman Radioautographic Plates Type No-Screen and Type A and Eastman Portrait Panchromatic Film were also tried. The speeds of these films were unknown. A number of samples were prepared and developed at intervals of twelve hours for the first few days and every two days thereafter until the best exposure time for each of the films was determined. The radioautographic plates Type No-Screen gave a clear image in five days. The radioautographic plates Type A were sufficiently exposed in one week, and two weeks were necessary for sufficient blackening of the Portrait Panchromatic film emulsion. This means that approximately 5×10^7, 7×10^7 and 1.4×10^8 disintegrations per square centimeter, respectively, are required by these films for a satisfactory radioautograph.

Eastman Kodak Portrait Panchromatic Film was found to give the best results for the materials used in this experiment. The small grain of this emulsion gave a very high resolution to the radioautograph.

EXPLANATION OF PLATE III

Some instruments for the measurement of radioactivity radiation levels.

A – Portable Gamma Survey Meter.

B – Beta Gamma Survey Meter.

C – Lead chamber enclosing a Geiger-Muller tube.

D – A Scalar.

E – An amplifier and loud-speaker.

F – Brass chamber enclosing a Geiger-Muller tube.

PLATE III

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.

The instruments used in obtaining the above information are shown in Plate III. A and B of the plate are survey meters for measuring the intensity of radiation present in a given area. C is a lead chamber enclosing a Geiger-Muller tube and F is a brass chamber enclosing a similar tube. D is the Scalar previously mentioned and gives a numerical value to the radioactivity present in one of the chambers. E is a loud-speaker which may be attached to the scalar to make the counting audible.

After preparing many bone sections and radioautographs, typical results were selected for further study. Photomicrographs were taken of both the radioautograph and the bone section. This allowed a more detailed study of the two and made it possible to determine more exactly the localization of the radioactive phosphorus.

EXPERIMENTAL RESULTS

I. The hormone injected males used in this experiment exhibited a 196 per cent average increase in weight. Injected females increased on the average 189 per cent in weight. Normal males increased on the average 170 per cent while the normal females had an average weight increase of only 110 per cent. This gives an overall average weight increase of growth hormone injected rats of 192.5 per cent as against an overall average

EXPLANATION OF PLATE IV

Positives of photomicrographs of bone sections and radioautographs made from rats sacrificed 80 hours after injection of radiophosphorus.

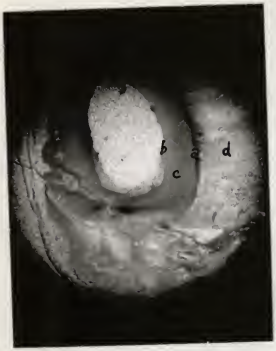
A – Cross section of the distal half of the diaphysis of the left femur of a growth hormone injected rat. (Rat 3).
  a. The periosteum.
  b. The endosteum.
  c. Osseous tissue.
  d. Bioplastic.

B – The radioautograph of the bone section shown in A above.

C – Cross section of the distal half of the diaphysis of the left femur of a normal rat. (Rat 8).
  a. The periosteum.
  b. The endosteum.
  c. Osseous tissue.
  d. Bioplastic.

D – The radioautograph of the bone section shown in C above.

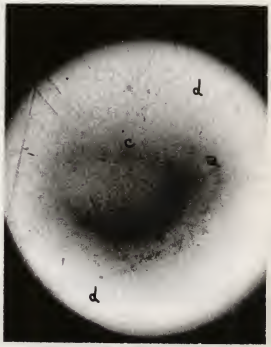
PLATE IV



A

B

C



D

EXPLANATION OF PLATE V

Positive of photomicrographs of bone sections and radioautographs.

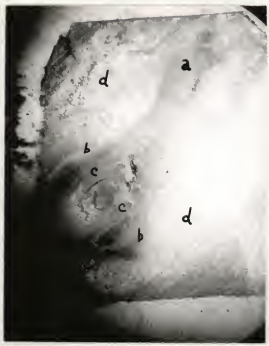
A – Cross section of the proximal half of the diaphysis of the left humerus of a growth hormone injected rat. (Rat 3).
   a. Inter-tubercular crest.
   b. The periosteum.
   c. The endosteum.
   d. Bioplastic.

B – The radioautograph of the bone section shown in A above.

C – Cross section of the proximal half of the diaphysis of the left humerus of a normal rat. (Rat 7). (Note: Rat 7 was actually sacrificed at 19 hours instead of 80 hours as was rat 3. However this section of rat 7 more closely conformed to that used in rat 3 than any section prepared of rat 8 and showed to an advantage the difference in deposition in the inter-tubercular crest. If a comparable section had been taken from rat 8, our experience has shown that its radioautograph would have exhibited a slightly more advanced state of metabolism than does D.)
   a. Inter-tubercular crest.
   b. The periosteum.
   c. The endosteum.
   d. Bioplastic.

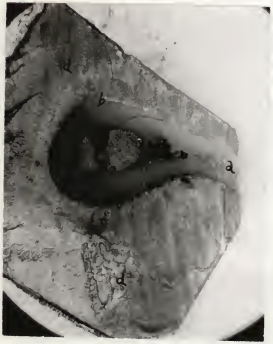
D – The radioautograph of the bone section shown in C above.

PLATE V



A

B



C

D

weight increase of 140 per cent in the normal rats.

II. The radioautographs of the bone sections of rats 2 and 3 were decidedly different from those of rat 8, as photomicrographs of representative sections shown in Plate IV and V.

A. In Plate IV-B the photomicrograph of the radioautograph of a cross section of the distal half of the diaphysis of the left femur of a growth hormone injected rat shows that there was a high concentration of the radiophosphorus in the periosteum. A similar bone section (Plate IV-C) from a non-growth hormone injected rat had more phosphorus deposited in the endosteum as shown in Plate IV-D.

B. A radioautograph (Plate V-B) of a cross section of the proximal half of the diaphysis of the left humerus of a growth hormone injected rat sacrificed 80 hours after phosphate injection shows an extremely high concentration of the isotope in the inter-tubercular crest and in the periosteum in comparison to a radioautograph (Plate V-D) of a similar section from a non-growth hormone injected rat. There is a small but still noticeable concentration of the phosphorus in the endosteum of the hormone injected rat. The distribution of the phosphorus in the normal rat is very diffuse with a moderately higher concentration in the endosteum.

III. Comparison of the radioautographs of hormone injected rats sacrificed 19 hours after injection of the labeled phosphorus (3) with those of hormone injected rats sacrificed 80 hours after injection (Plate IV-B and Plate V-B) shows that

the phosphorus has a greater density in growth areas in the 20 hour rats. This same trend is apparent in the non-growth hormone rats but to a lesser extent.

These results were representative of those encountered in the many radioautographs prepared for this experiment.

DISCUSSION

The greater difference between the per cent increased weight of the injected and non-injected females as compared to the per cent increased weight of the injected and non-injected males may be due in part to the fact that normal female rats mature and reach the "plateau" sooner in life.

The radioautographs extend and confirm what is known of bone phosphorus metabolism. Previous work has shown that phosphorus is first stored in the epiphysis and the endosteum. This phosphorus is then remobilized and deposited in the epiphyseal line, the diaphysis and the periosteum during bone growth.

Time appears to be an important factor in the distribution of phosphorus in growing rats. The phosphorus is first stored in the matrix of the epiphysis and in the endosteum. In time, this phosphorus is remobilized and deposited in the growth areas of the bone; i.e., in the periosteum and in the epiphyseal disk.

The results of this experiment appear to indicate that the growth hormone accelerated the bone phosphorus metabolism. The results are limited somewhat by the fact that too few animals were used and only a few hundred bone sections were made.

Marx and Reinhardt (10) as previously mentioned found that growth hormone had no effect on the quantity of radioactive strontium deposited in the femur. Although no actual quantitative measurements of radioactivity were taken of the bone in the experiment with phosphorus, it can be assumed from the density of the radioautographs that more radiophosphorus was present in the bones of growth hormone rats. It is entirely possible that in the experiment of Marx and Reinhardt an insufficient quantity of strontium was injected. In this case, all of the strontium would necessarily be taken up by the bones of both the normal and growth hormone injected rat.

Experiments to determine the effect of growth hormone on the total uptake of radioactive elements by the bones would materially aid the endocrinologist, the physiologist and the anatomist in their study of the human body.

SUMMARY

1. The radioactive isotope of phosphorus and the growth-stimulating hormone of the anterior pituitary gland were used in an experiment to determine the effect, if any, of the hormone on the deposition of phosphorus in the long bones of the white rat.

2. The rats were classified for the purposes of injection with the substances used.

3. Growth hormone was injected subcutaneously daily except Sunday for a period of eighty six days.

4. Radioactive phosphorus was injected as part of the molecule of disodium monohydrogen phosphate. The dilution and chemical transformation of the isotope as received from the Atomic Energy Commission were made with all of the prescribed precautions.

5. The animals were sacrificed 80 hours after injection with the radioactive substance.

6. The femora and the humeri were imbedded in bioplastic.

7. Cross-sections and sagittal sections were made with a modified microtome. The microtome was equipped with a circular saw blade in place of the customary knife.

8. The bone sections were mounted on celluloid and placed in contact with photographic emulsion for the production of radioautographs.

9. Photomicrographs of the bone sections and of the radioautographs were made.

10. Comparison of these photomicrographs and radioautographs indicates that growth hormone accelerates the remobilization and influences the deposition of the phosphorus. Time is an essential element in this remobilization and deposition.

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