THE RICE INSTITUTE

STUDIES ON THE RELATIONSHIP OF PLASMA TO BONE WITH
REFERENCE TO CALCIUM AND PHOSPHATE

by

Larry Thomas Wimer

A THESIS
SUBMITTED TO THE FACULTY
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF ARTS

Houston, Texas
May, 1959
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>2</td>
</tr>
<tr>
<td>The State of Calcium and Phosphate in Serum</td>
<td>5</td>
</tr>
<tr>
<td>Relationship of Tertiary Calcium Phosphate (TCP) to Bone Mineral and Serum</td>
<td>7</td>
</tr>
<tr>
<td>Relationship of Secondary Calcium Phosphate (SCP) to Bone Mineral and Serum</td>
<td>10</td>
</tr>
<tr>
<td>Occurrence of Colloidal Calcium Phosphate in Serum</td>
<td>14</td>
</tr>
<tr>
<td>Statement of the Problem</td>
<td>19</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>I. Incubation of Bone in Serum</td>
<td>20</td>
</tr>
<tr>
<td>A. Control Live Bone Experiments versus Dead Bone Experiments</td>
<td>21</td>
</tr>
<tr>
<td>B. Live Bone Experiments - Incubation of Successive Femurs in the Same Serum Sample versus Incubation of the Same Femur in Successive Serum Samples</td>
<td>22</td>
</tr>
<tr>
<td>C. Live Bone Experiments - Incubation of Femurs from Animals on a Calcium Free Diet versus Those Taken from Animals on a High Calcium Diet</td>
<td>23</td>
</tr>
<tr>
<td>D. Live Bone Experiments - Non-Scheduled Experiments: The Untoward Effect of Evaporation</td>
<td>24</td>
</tr>
</tbody>
</table>
II. Addition of Calcium and/or Phosphate Compounds to Plasma ........................................ 25
   A. High-Speed Centrifugation After the Addition of Tertiary or Secondary Calcium Phosphate ........................................ 26
   B. High-Speed Centrifugation of Plasma Augmented with Calcium and Phosphate ........................................ 27
   C. High-Speed Centrifugation of Plasma Augmented with Calcium or Phosphate ........................................ 28

III. Experimental Demonstration of the Presence of an Insoluble Calcium Phosphate Substance in the Blood in vivo ........................................ 28
   A. High-Speed Centrifugation of Plasma from Controls and from Animals Administered PTE ........................................ 29
   B. High-Speed Centrifugation of Plasma from Nephrectomized Controls and from Nephrectomized Animals Administered PTE ........................................ 29
   C. High-Speed Centrifugation of Plasma from Nephrectomized Animals on a Low Calcium or a High Calcium Diet ........................................ 30

IV. Calcium Analysis ........................................ 32
V. Phosphate Analysis ........................................ 33

RESULTS ........................................ 35

Loss of Calcium from Serum Incubated with Bone ........................................ 35
   A. Control Live Bone Experiments versus Dead Bone Experiments ........................................ 35
   B. Live Bone Experiments - Incubation of Successive Femurs in the Same Serum Sample versus Incubation of the Same Femur in Successive Serum Samples ........................................ 36
C. Live Bone Experiments - Incubation of Femurs from Animals on a Calcium-Free Diet versus Those Taken from Animals on High Calcium Diet .................. 38

D. Live Bone Experiments - The Effect of Evaporation on the Calcium and Phosphate Values of the Incubation Medium .......... 41

Changes in Calcium and Phosphate Level After the Addition of Calcium and/or Phosphate Compounds to Plasma .................. 43

A. High-Speed Centrifugation After the Addition of Tertiary or Secondary Calcium Phosphate .................. 43

B. Plasma Values After High-Speed Centrifugation of Plasma Augmented with Calcium and Phosphate (5 mg.% of Calcium and of Phosphorus as Phosphate) .......... 44

C. Plasma Values After High-Speed Centrifugation of Plasma Augmented with Calcium or Phosphate (5 mg.% of Calcium or of Phosphorus as Phosphate) .......... 45

Experimental Demonstration of the Presence of an Insoluble Calcium Phosphate Substance in the Blood in vivo .................. 46

A. High-Speed Centrifugation of Plasma from Controls and from Animals Administered PTE .................. 46

B. High-Speed Centrifugation of Plasma from Nephrectomized Controls and from Nephrectomized Animals Administered PTE .. 47

C. High-Speed Centrifugation of Plasma from Nephrectomized Animals on a Low Calcium or a High Calcium Diet .................. 48
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Follows Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Loss of Calcium from Serum Incubated with Bone</td>
<td>35</td>
</tr>
<tr>
<td>II. Incubation of Bone in Successive Serum Sample(s)</td>
<td>36</td>
</tr>
<tr>
<td>III. Incubation of Femurs Taken from Animals on a Calcium-Free Diet versus Those Taken from Animals on a High Calcium Diet</td>
<td>38</td>
</tr>
<tr>
<td>IV. The Effect of Evaporation on the Calcium and Phosphate Values of the Incubation Medium</td>
<td>41</td>
</tr>
<tr>
<td>V. Changes in Calcium and Phosphate Levels After the Addition of Tertiary or Secondary Calcium Phosphate to Plasma</td>
<td>43</td>
</tr>
<tr>
<td>VI. Plasma Values After High-Speed Centrifugation of Plasma Augmented with Calcium and Phosphate (5 mg.% of Calcium and of Phosphorus as Phosphate)</td>
<td>44</td>
</tr>
<tr>
<td>VII. Plasma Values After High-Speed Centrifugation of Plasma Augmented with Calcium or Phosphate</td>
<td>45</td>
</tr>
<tr>
<td>VIII. High-Speed Centrifugation of Plasma from Controls and from Animals Administered PTE</td>
<td>46</td>
</tr>
<tr>
<td>IX. High-Speed Centrifugation of Plasma from Nephrectomized Controls and from Nephrectomized Animals Administered PTE</td>
<td>47</td>
</tr>
<tr>
<td>X. High-Speed Centrifugation of Plasma from Nephrectomized Animals on a Low Calcium and a High Calcium Diet</td>
<td>48</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I wish to thank Dr. Roy V. Talmage for his encouragement and helpful criticisms while directing this research.

I also wish to thank Miss Sharon V. Brown for typing the rough draft of this thesis from illegible handwriting.
The relationship between bone and biological fluids can only be studied after one has a knowledge of their various components. The most distinguishing characteristic of bone is its hardness, brought about by the deposition of mineral substance within an organic matrix. The entire organic fraction of bone amounts to approximately 35 per cent of the total dry, fat-free weight. The greater percentage of this organic fraction consists of the bone matrix; however the cellular content of bone is an important part of this fraction. The organic matrix is divided into two primary components. By far the most prominent of the two is collagen, which makes up 90 to 96 per cent of the dry fat-free weight of the organic matter. Its composition is identical with or closely related to the collagens found in other connective tissues (McLean, 1957). The other component of the organic fraction is commonly known as the ground substance, a mucopolysaccharide characterized as condroitin sulfate and hyaluronic acid. Although the ground substance makes up only a small part of the organic fraction, its importance in calcification is out of proportion to its quantity. While under the light microscope it appears to be
amorphous, recent electron microscope studies indicate that it has a definite structure and organization. The ground substance and the tissue fluid of bone are considered to be coextensive and homogeneous, and interconnected throughout (McLean and Urist, 1955).

Bone salt has been shown by chemical analysis to contain calcium, phosphate, carbonate, citrate, water, sodium, magnesium, potassium, and chloride, fluoride and hydroxyl ions. The X-ray diffraction pattern of bone mineral has shown that it is very similar to hydroxyapatite with an empirical formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (Posner, 1957). The Ca:P (calcium:phosphate) molar ratio of hydroxyapatite is 1.66. The Ca:P ratio of bone salt varies from 1.33 to 2.0. Variation in this ratio can be explained by the substitution of hydrogen ions and water for calcium, and by the possible adsorption of calcium and/or phosphate ions on the crystal surface. Neuman and Neuman (1953) have postulated that normal bone is one of the apatite series with a Ca:P ratio of 1.5.

In discussing the ionic exchange process occurring in bone, it should be noted that different ions can be found in
various locations in bone crystal. These ions can be (1) in the bulk solution, (2) in the hydration layer which contains non-specific boundary anions in equilibrium with the bulk solution, (3) in the crystal surface containing more or less specific cations and anions in equilibrium with the hydration layer, and (4) in a number of positions in the lattice interior which has a slow but measurable equilibration with the outer layers (Weikel, 1954; Weikel, et al, 1954). Equilibration of the bulk solution with the hydration layer may be complete within two hours whereas the latter stage may not reach equilibrium for fifty days. Several hetero-ionic exchange reactions have been studied: uranyl, strontium, and hydronium ions can displace surface calcium (Neuman and Neuman, 1953); carbonate ions, citrate, and possibly hydroxyl ions as bicarbonate can displace surface phosphate (Neuman, et al, 1956c; Armstrong and Singer); and fluoride ions can displace hydroxyl and bicarbonate ions (Neuman, et al, 1950).
The State of Calcium and Phosphate in Serum

The total calcium content in the serum of most mammals is around 10 mg.% or 2.5 mM per liter. The state of calcium in serum can be divided into two main categories: (1) non-diffusible and (2) diffusible. The non-diffusible fraction is bound to protein and comprises about 35% of the total calcium. Of this fraction, 75% is bound to albumin, the remainder to globulins. Of the diffusible fraction (65% total), 80% is ionized and 20% complexed with bicarbonate (Greenwald, 1941; Neuman, et al., 1956b), phosphate (Greenwald, et al., 1940), and citrate. Therefore, about fifty percent of the total calcium is ionized (Neuman and Neuman, 1958). This agrees well with the earlier classic results of McLean and Hastings (1935) using the frog heart method.

The state of inorganic phosphate in serum is much less complicated than that of calcium. The inorganic phosphate of serum is presumably all diffusible. Although none has been shown to be non-diffusible, there is indirect evidence that some phosphate could be bound to protein. The serum phosphate value for man varies from 3.1 mg.% for the fasting adult to 6.2 mg.% for the infant. The inorganic phosphate
is basically divided into two fractions. The $\text{HPO}_4^{2-}$ represents about 81% of the total inorganic phosphate whereas the $\text{H}_2\text{PO}_4^-$ represents about 19%. Only a very small amount of $\text{PO}_4^{3-}$ (.008%) is present (Neuman and Neuman, 1958).
Relationship of Tertiary Calcium Phosphate (TCP) to Bone Mineral and Serum

In 1928 Shear and Kramer stated that the presence of tertiary calcium phosphate (TCP) in bone mineral had never been demonstrated. Nevertheless, prior to and since that time many investigators, knowing that TCP has a Ca:P ratio similar to that of bone mineral, have attempted to determine the solubility of bone by demonstrating a solubility product for TCP in biological fluids. Holt, et al (1925a) calculated a solubility product for TCP in a water solution and extended their work using serum (1925b). The solubility of TCP in serum was found to be greater than its solubility in cerebrospinal fluid (Holt, 1925). Levinskas (1953) using salt solutions arrived at the same value for the solubility products of hydroxyapatite as did Holt (1925) using TCP in cerebrospinal fluid. Holt, et al (1925b) came to the conclusion that serum was supersaturated with TCP to the extent of 200%.

calcium carbonate in their experiments. An interesting obser-
vation of Hastings in these experiments was that serum
did not change its concentration of calcium when equili-
brated with calcium carbonate after depletion or augmenta-
tion of its calcium concentration. In a later paper (1926-
27a) Sendroy and Hastings confirmed the results of Holt, et
al (1925b). They were unable to show a loss of phosphate
from serum but they did show a loss of bicarbonate indi-
cating that the calcium precipitated on the TCP as calcium
carbonate. Sendroy and Hastings (1926-27b) compared the
solubility of a crude bone preparation with a mixture of
calcium carbonate and TCP in an artificial edema fluid and
arrived at the same values. They therefore concluded that
bone was a mixture of calcium carbonate and TCP.

Logan and Taylor (1937) came to the conclusion "that
bone salts cannot precipitate spontaneously from the bone
plasma unless the ion product is increased. Once formed,
the bone salts cannot dissolve unless the concentration of
the ions composing them are decreased below the concentra-
tion found in the blood plasma." They found that the solu-
binity of TCP and powdered bone decreased with increasing
amounts of solid. Greenwald (1942a) criticized their data
and said that the reason they came to this conclusion was that they did not allow a long enough time for equilibration to occur and that when they used small amounts of solid, they actually had supersaturated solutions. Greenwald (1942a) repeated their experiments and came to the opposite conclusion, i.e., the solubility of the solid increases with the amount of solid phase. Levinskas (1953) came to the same conclusion as Greenwald (1942a) using hydroxyapatite in salt solutions.

In calcification experiments, Sobel and Hanok (1952) state that if the solubility of TCP determines calcification in bone, a given amount of calcium in the incubation medium would be more effective than a given amount of phosphate. However, by varying the amount of calcium and phosphate, keeping the \( Ca \times P \) product the same, they were unable to show any difference in the amount of calcification which occurred.

Although TCP is thought to be hydroxyapatite with phosphate chemisorbed (Fabry, 1958) the ion product of secondary calcium phosphate rather than TCP is thought to be the determining factor in solubility and calcification studies (Shear and Kramer, 1928; Logan, 1940; Sobel and Hanok, 1952; Strates, et al, 1957).
Relationship of Secondary Calcium Phosphate (SCP) to Bone Mineral and Serum

Strates, et al (1957) came to the conclusion that serum was supersaturated with calcium and phosphate with respect to bone mineral but undersaturated with these substances with respect to serum. The first evidence for the undersaturated condition of serum with respect to calcium and phosphate was that of Shear and Kramer (1928) who, after recalculating data of other investigators, came to the conclusion that serum was not saturated with secondary calcium phosphate (SCP). Shear, et al (1929) supported their conclusion with additional evidence concerning the solubility of SCP. They emphasized that the important substance in considering the solubility of calcium and phosphate in serum is secondary calcium phosphate and not tertiary calcium phosphate. One reason for believing that secondary and not tertiary calcium phosphate determines the upper limits of calcium and phosphate in serum is that when calcium and phosphate precipitate from a supersaturated solution, the precipitate has a Ca:P ratio which approximates that of SCP, and only later does the Ca:P ratio of the pre-
cipitate approximate that of TCP (Greenwald, 1942b; Holt, et al, 1925a).

According to Shear, et al (1929) inorganic serum solutions with an empirical Ca x P product of less than about 50 are undersaturated with respect to SCP. Those with a Ca x P product of about 50 are just saturated, and in only those solutions with a Ca x P product greater than about 50 does the supersaturated condition with respect to SCP become approached. Solutions having the inorganic composition of rickettic serum are decidedly undersaturated with calcium and phosphate. Solutions having the inorganic composition of normal serum are undersaturated with respect to SCP. If all of the calcium and phosphate in normal serum were ionized, the serum would be saturated with these substances (Ca x P product = 50).

Shear and Kramer (1930) added SCP to human, beef, and lamb serum and noted a final Ca x P (total) product of 84. The initial Ca x P (total) product in the serum of the latter two animals was 76 whereas in human serum it was 35. This clearly shows that human serum is greatly undersaturated with SCP, while the beef and lamb serum approach the upper limits of saturation.

According to Levinskas (1953) blood is normally undersaturated with respect to SCP. He states that "the
solubility of basic calcium phosphates are limited by some factor before reaching the solubility of secondary phosphates, for instance, the surface of the solid phase." He demonstrated that solutions containing calcium and phosphate were stable in the absence of bone mineral but when placed in a solution with bone mineral, rapidly lost calcium and phosphate to the solid. In extending this work, Strates, et al (1957) found that it was necessary to exceed the solubility product of SCP in order to initiate precipitation in the absence of a solid phase. Above pH 6.9 SCP is not stable and therefore hydroxyapatite governs the solubility equilibrium when the solid phase is present. Strates, et al (1957) therefore came to the conclusion that serum is normally undersaturated with calcium and inorganic phosphate in the absence of the solid phase but supersaturated with respect to hydroxyapatite.

Several attempts have been undertaken to determine a solubility product of substances similar to bone mineral both from supersaturated (Holt, et al, 1925b; Hastings, et al, 1926-27) as well as undersaturated (Levinskas, 1953; Nordin, 1957) solutions. These investigators also came to
the conclusion that biological solutions were supersaturated with respect to bone mineral. The solubility products which they found for TCP were inadequate to explain the levels of calcium and phosphate in normal serum. Nordin (1957) suggests that the tissue fluid concentration of calcium and phosphate could maintain an equilibrium with the bone mineral if the pH on the surface of the bone was about 6.6 to 6.8. Neuman, et al (1956a) have given a similar explanation. Results, such as reported here, have disproven the theory of Albright and Reifenstein (1948) who believed that serum was saturated with calcium and phosphate such that these substances held an inverse relationship to each other in serum.
Occurrence of Colloidal Calcium Phosphate in Serum

Grollman (1927) was probably the first to notice the presence of a non-ultrafilterable inorganic phosphate fraction in the blood indicating the formation of a colloidal phosphate substance. In the pig and dog, the inorganic phosphate was 100 per cent ultrafilterable, while in the chicken, frog, and terrapin only from 50 to 90 per cent was ultrafilterable.

Roepke and Hughes (1935) found a non-ultrafilterable inorganic phosphate fraction in the serum of cocks, non-laying and laying hens. Almost twice as much was present in the laying hens as in the cocks. They did not recognize that the non-ultrafilterable inorganic phosphate was probably bound to calcium. The calcium level in the laying hens was almost twice that of the cocks or the non-laying hens. The insoluble substance formed when the Ca x P (total) product was 60. This indicates that some condition other than solubility governs the presence of colloidal calcium phosphate in the blood at least in the fowl.

McDonald and Riddle (1945) could not detect a significant difference in either the ultrafilterable calcium or
phosphorus during the reproductive cycle or after the injection of estrogens in the pigeon; however, the non-ultrafilterable calcium and phosphorus increased markedly during the reproductive cycle and after the administration of estrogen in fasted, PTH, hypophysectomized, and normal animals. A direct relationship was found between the non-ultrafilterable calcium and non-ultrafilterable inorganic phosphorus in the plasma of estrogen-treated (endogenous and administered) pigeons. The non-ultrafilterable inorganic phosphorus was probably a colloidal form of calcium phosphate with the rest of the non-ultrafilterable calcium being bound to protein.

In goldfish, during the preovulatory state, there occurred a marked increase in the serum non-ultrafilterable calcium. The increase in calcium occurred in two distinct fractions. One fraction was in the form of a colloidal calcium phosphate and accounted for the increase in the non-ultrafilterable inorganic phosphorus. The other fraction was bound to a serum phosphoprotein, vitellin, and this complex accounted for the marked increase in protein phosphorus and total protein (Bailey, 1957).
Laskowski (1933), Smith (1934), and Hopkins, et al (1952) demonstrated the presence of colloidal calcium phosphate in vitro by augmenting serum with calcium. The Ca x P (diffusible) product at equilibrium was between 40 and 50. Laskowski (1933) and Smith (1934) used serum from animals with a high serum phosphate; therefore, they had to add only a small amount of calcium, whereas Hopkins, et al (1952) added up to 20 mg.% of calcium in human serum before they noticed the presence of colloidal calcium phosphate. Grollman (1927) added only .5 mg.% of calcium to pig serum in order to produce a non-ultrafilterable fraction of inorganic phosphate. Greenberg, et al (1935) augmented beef serum with calcium; however, the Ca x P (diffusible) product at equilibrium was only slightly higher than normal. In some instances, they were able to show the presence of the colloid in normal serum, but they failed to control the pH and this could lead to erroneous results. Greenberg (1932-33) and Greenberg and Larson (1939) augmented the serum calcium in vivo demonstrating a higher than normal Ca x P (diffusible) product at equilibrium.

Laskowski (1933), Masket, et al (1942), and Hopkins, et al (1952) produced a decrease in the diffusible calcium
by augmenting serum with inorganic phosphate. They had to add unusually high amounts of phosphate indicating that the presence of a colloidal calcium phosphate complex is not simply a supersaturation phenomenon when only phosphate is added. Greenberg and Larson (1939) noted that in conditions of hyperphosphatemia (in vivo) the $\text{Ca} \times P$ ratio was unusually high. McLean and Hastings (1935) found that when dog serum was augmented with phosphate a small amount of colloidal calcium phosphate formed at a $(\text{Ca}^{++}) \times P$ product of about 75. McLean and Hinrichs (1938) later found that when the phosphate was augmented in vivo and then let equilibrate in vitro, the $(\text{Ca}^{++}) \times P$ product was around 35. They measured $(\text{Ca}^{++})$ by the frog heart method (McLean and Hastings, 1934) and therefore they measured ionic calcium and not total diffusible calcium. The $(\text{Ca}^{++}) \times P$ product of 35 was somewhat higher than normal.

Gersh (1938a; 1938b) noticed that when the calcium or phosphate level of the blood was raised by an intravenous injection of these substances, an insoluble calcium phosphate substance appeared in the blood. He found that this substance was rapidly destroyed by the liver and spleen and
that almost all was removed from the blood within one and one-half hours after the injection. For comprehensive reviews on the relationship between bone and biological fluids the reader is referred to the following papers: Schmidt and Greenberg, 1935; Eisenberger, et al, 1940; Logan and Taylor, 1940; and Neuman and Neuman, 1953.
STATEMENT OF THE PROBLEM

It is the general opinion of recent investigators that serum is supersaturated with respect to bone but undersaturated with respect to secondary calcium phosphate. One method of studying this postulate is to approximate the \textit{in vivo} system employing an \textit{in vitro} system. Once the relationship between fresh bone and serum is established, with reference to calcium and phosphate, a technique must be devised for determining the saturated condition of serum with respect to calcium and phosphate. By adding various amounts of calcium and/or phosphate to serum one can establish the relative saturated condition of serum with respect to these substances. These latter studies can be extended by experimentally changing the level of calcium and/or phosphate in the blood \textit{in vivo}. When these studies are complete, the relationship between bone and serum calcium and phosphate will be more clearly elucidated.
MATERIALS AND METHODS

I. Incubation of Bone in Serum

Holtzman rats from which the femurs were taken were maintained on Purina lab chow with water \textit{ad libitum}. Each animal was sacrificed by a blow on the head, following which both femurs were removed and cleaned of muscles and tendons. Each femur was divided into five parts by the following method: the epiphysis was separated from the shaft and a cross section of the shaft made equidistant from the ends. These sections were cut longitudinally. All portions were cleaned of marrow before being placed in the incubation medium. This operation was completed within fifteen minutes after the death of the animals.

The source of the serum was male Holtzman rats weighing over 400 grams. Blood was obtained from etherized animals by heart puncture with a heparinized syringe, and immediately centrifuged at 800 \textit{x G} for twenty minutes. The plasma then was drawn off, pooled, and stored at -18°C. Fibrin which formed while thawing prior to use was removed by centrifugation and the resulting serum apportioned to
the beakers used as incubation vessels.

Six vessels were placed in a Dubnoff apparatus kept at a temperature of 37°C. Maintenance of serum pH between the limits of 7.35 and 7.55 was accomplished by enclosing the beakers in an atmosphere saturated with a 90% oxygen and 10% carbon dioxide gas mixture. This condition was obtained by covering the partially immersed beakers with a specially designed stainless steel cover through which the gas mixture was piped. The cover was provided with six hinged lids to enable the experimenter to sample each of the containers without exposing the others.

A. Control Live Bone Experiments versus Dead Bone Experiments

The procedure described below was that of a typical experiment. Male Holtzman rats weighing from 225 to 275 grams were used. The femurs of two animals were used in each experiment. One femur was allotted to each experimental vessel. Two additional beakers containing only serum served as controls. Each incubation vessel contained 4 ml. of serum which
was given a fifteen minute equilibration period with
the gas mixture before receiving the femur prepara-
tion. Samples for analyses were taken at intervals
of 30 minutes, 1 hour, and 2 hours after the addi-
tion of bone. In the control live bone experiments
.9 ml. samples were taken for the calcium and phos-
phate analyses; however, in the rest of the experi-
ments only .7 ml. were taken. The dead bone experi-
ments deviated from this procedure only in that the
femurs were heated to 150°C, for one hour prior to
incubation.

B. Live Bone Experiments - Incubation of Successive

Femurs in the Same Serum Sample versus Incubation

of the Same Femur in Successive Serum Samples

Samples were taken at two hours and four hours.
In the experiments which consist of the incubation
of successive femurs in the same serum sample, the
femurs from half the beakers were replaced with
femurs from freshly killed animals after the two
hour samples had been taken. The control beakers
initially contained 7 ml. of serum for those experiments in which the same femurs were incubated in successive serum samples. After the two hour samples had been removed, 4 ml. of serum were taken from the control beakers and placed in a new beaker to which was added a femur which already had been incubated for the first two hour period. The beakers which were not changed at two hours had a volume of 3.5 ml. at the termination of the experiment because samples were taken only for calcium analyses.

C. Live Bone Experiments - Incubation of Femurs From Animals on a Calcium-Free Diet versus Those Taken From Animals on a High Calcium Diet

Half of the animals were placed on the Talmage (1958) calcium-free diet (see below) four days prior to being sacrificed, and were given distilled water ad libitum. The animals on the high calcium diet were given Purina lab chow and drinking water containing 5 grams of calcium lactate per 100 ml. of water four days prior to being killed. Twenty-four
hours before being sacrificed the animals were placed on metabolism cages to determine their rate of calcium excretion.

**Talmage Calcium-Free Diet**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphacel</td>
<td>110 grams</td>
</tr>
<tr>
<td>Dextrose</td>
<td>100 grams</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 grams</td>
</tr>
<tr>
<td>Lard (Crisco)</td>
<td>80 grams</td>
</tr>
<tr>
<td>Wesson Oil</td>
<td>12 grams</td>
</tr>
<tr>
<td>Wheat Germ Oil</td>
<td>5 grams</td>
</tr>
<tr>
<td>Cod Liver Oil</td>
<td>3 grams</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>115 grams</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>465 grams</td>
</tr>
</tbody>
</table>

D. Live Bone Experiments - Non-Scheduled Experiments: The Untoward Effect of Evaporation

When the experiments were continued in the recently completed Anderson Biological Laboratories, it was found necessary to make changes in the procedure in order to control the effects of tempera-
ture and humidity on evaporation. Fresh plasma was used in these and all succeeding experiments so as to eliminate the evaporation which occurred during freezing, thawing, and recentrifugation of the plasma. Incubation was carried out in 10 ml. Erlenmeyer flasks instead of using the stainless steel cover and beakers. Two syringe needles were inserted through rubber stoppers. A mixture of 90% O₂:10% CO₂ and 95% O₂:5% CO₂ was allowed to flow in one syringe needle with the other serving as an outlet. The mixture was allowed to flow continuously during the incubation period. Samples were taken after the pre-incubation period to determine the amount of evaporation which occurred from zero time to thirty minutes.

II. Addition of Calcium and/or Phosphate Compounds to Plasma

The Erlenmeyer flask apparatus was used in all of the experiments of this section. The blood preparation used was that described in section I with the modification
made in section I-D. The initial centrifugation was carried out for thirty minutes in order to more completely remove the white blood cells. The incubation period was carried out in a Precision Thelco incubator. A water bath was maintained at 37°C, inside the incubator. The flasks were not agitated.

After the equilibration period the incubation medium, contained in capped tubes, was centrifuged at 14,000 x G for thirty minutes in an International Portable Refrigerated Centrifuge, model PR-I. The temperature of the plasma solution was maintained between 34°-36°C, during centrifugation. The supernatant was drawn off with a syringe and duplicate samples for calcium and phosphate analyses were taken. pH was determined using a Beckman Zeromatic pH Meter and ranged from 7.40 to 7.90.

A. High-Speed Centrifugation After the Addition of Tertiary or Secondary Calcium Phosphate

The procedure described below was that of a typical experiment. Triplicate samples for calcium (.5 ml.) and phosphate (.4 ml.) analyses were taken
from the pooled plasma before it was added to the individual flasks. To two flasks were added 8 ml. of pooled plasma followed by 240 mg. of tertiary calcium phosphate. Two flasks containing 8 ml. of serum served as controls. The flasks were gassed for eight minutes using a mixture of 90% O2 and 10% CO2. The flasks then were sealed off and allowed to equilibrate for two hours. After the equilibration period the contents were subjected to high-speed centrifugation. The supernatant was drawn off and duplicate samples taken for calcium (.5 ml.) and phosphate (.4 ml.) analyses. The experiments employing secondary calcium phosphate deviated from this procedure in that 180 mg. of this substance were added to 6 ml. of plasma.

B. High-Speed Centrifugation of Plasma Augmented With Calcium and Phosphate

To 5.7 ml. of plasma were added .15 ml. of a calcium chloride solution containing .30 mg. of calcium and .15 ml. of a dibasic sodium phosphate solution
containing .30 mg. of phosphorus. An incubation period of thirty minutes was employed. Samples for calcium and phosphate analyses were taken immediately before and after high-speed centrifugation.

C. High-Speed Centrifugation of Plasma Augmented With Calcium or Phosphate

To 7.8 ml. of plasma were added either .2 ml. of a calcium chloride solution containing .40 mg. of calcium or .2 ml. of a dibasic sodium phosphate solution containing .40 mg. of phosphorus. The procedure was the same as that described in the above section.

III. Experimental Demonstration of the Presence of an Insoluble Calcium Phosphate Substance in the Blood in vivo

Male Holtzman rats weighing 225 - 285 grams were used for these experiments with the exception of the controls in section A which were over 400 grams. The procedure through the initial centrifugation was basically the same as that described in section II. Parafilm was
placed over the tops of the centrifuge tubes immediately after the blood was taken. Duplicate samples for calcium and phosphate analyses were taken after the initial centrifugation. The plasma was not pooled but was transferred directly to lusteroid centrifuge tubes with caps. Final centrifugation was carried out using the high-speed apparatus described in section II. The supernatant was drawn off and duplicate samples were taken for calcium and phosphate analyses. The pH of the rest of the supernatant was taken and ranged from 7.40 to 7.90.

A. High-Speed Centrifugation of Plasma From Controls and From Animals Administered PTE

Animals were given 100 units of PTE (Parathyroid Extract, Lilly) intraperitoneally every other day for ten days. The experiments were terminated on the tenth day and the blood was taken and treated in the usual manner.

B. High-Speed Centrifugation of Plasma From Nephrectomized Controls and From Nephrectomized Animals Administered PTE

Bilateral nephrectomy was performed through a
midventral incision while the animals were under ether anesthesia. The animals were divided into two groups, with one group serving as controls and the other group receiving 250 units of PTE (Lilly), intraperitoneally, six hours and eighteen hours after nephrectomy. Blood was taken from all animals thirty hours after nephrectomy when the experiment was terminated.

C. High-Speed Centrifugation of Plasma From Nephrectomized Animals on a Low Calcium or a High Calcium Diet

The animals used in the experiments were divided into two groups. Nephrectomy was performed on each of the animals in the usual manner. All the animals had been placed on a calcium-free diet (modified from section II-E, see below) 24 hours prior to nephrectomy. The first group was given distilled water, and the second group was provided a solution of calcium lactate containing 15 mg. of calcium per 100 ml. of water, ad libitum. The animals maintained on the calcium lactate water were adminis-
tered, by stomach tube, .2 ml. of a solution containing 20 mg. of calcium, as calcium chloride, four hours after nephrectomy and then .1 ml. of the same solution every four hours up to 48 hours at which time all animals were sacrificed. The blood was taken and treated in the usual manner.

**Modified Talmage Diet**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphacel</td>
<td>110</td>
</tr>
<tr>
<td>Dextrose</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40</td>
</tr>
<tr>
<td>Lard (Crisco)</td>
<td>80</td>
</tr>
<tr>
<td>Wesson Oil</td>
<td>20</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>115</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>465</strong></td>
</tr>
</tbody>
</table>
IV. Calcium Analysis

The calcium determination used was that described by Elliott and Talmage (1957) with minor modifications. The total calcium in the samples was determined using the following procedure: to the .5 ml. samples contained in centrifuge tubes were added 2 ml. of a 4% ammonium oxalate solution. The contents of the tubes were then mixed thoroughly and allowed to stand for at least four hours. Then the tubes were centrifuged at 840 x G for twenty minutes. After this procedure the supernatant was decanted and replaced with 2 ml. of 2% ammonium hydroxide which was allowed to stand for twenty minutes without mixing of the precipitate. The contents then were centrifuged for twenty minutes at 840 x G and the supernatant decanted. The tubes were left inverted while the precipitate was allowed to dry overnight. This precipitate was dissolved using .1 ml. of 70% perchloric acid, after which the centrifuge tubes were agitated. Then 1.9 ml. of 10% isopropyl alcohol were added and the contents mixed thoroughly until dissolved.

The amount of calcium in this solution was determined using a Beckman model DU Spectrophotometer with flame at-
The wavelength was set at 623 μ with a slit width of .3 mm.

The stock standard calcium solution was prepared by dissolving 1.575 grams of monocalcium phosphate-monohydrate in 1.5 ml. of 70% perchloric acid and then diluting with 10% isopropyl alcohol to 500 ml. A calibration curve was prepared using standards varying from 10 μg.m. - 100 μg.m. of calcium per ml. All of the unknown values were read from this curve. The 50 μg.m. standard was set at a transmittance of 50%. The final dilution was such that the readings fell between 20-40% transmittance.

V. Phosphate Analysis

In the initial group of experiments the method of Gomori (1942) was used for the phosphate determinations. However, the method of Ammon and Hinsberg (1936) was found to be more consistent and sensitive and was used in the remaining experiments.

The phosphate analyses were run on either .1, .2, or .4 ml. samples. Ten per cent TCA was added to bring the volume up to 2.5 ml. The contents were mixed thoroughly,
allowed to stand for fifteen minutes to insure complete precipitation of the protein, and then filtered. To 1 ml. of the filtrate were added 4 ml. of glass distilled water and 1 ml. of an acid-molybdate solution which was prepared immediately before use. After the addition of the acid-molybdate, .5 ml. of a .5% ascorbic acid solution were added, following which the contents were mixed thoroughly by inversion. The tubes were let stand for twenty minutes to allow for color development, and then read with the aid of a Bausch and Lomb "Spec 20" colorimeter at 660 µ using photocell number 1P40 and a red filter. Optical density was set at zero using a reagent blank.

The stock standard phosphate solution was prepared by dissolving .4390 gm. of dry monopotassium phosphate in water and diluting to one liter. A standard curve was drawn up using phosphate solutions containing from 0-15 µgm. of phosphorus per ml.
RESULTS

Loss of Calcium From Serum Incubated With Bone

A. **Control Live Bone Experiments versus Dead Bone Experiments, See Table I.**

A significant loss of calcium from solution was noted when bone was placed in serum. The greatest drop occurred within the first thirty minutes of incubation. A continuous drop in serum calcium was noted at the one hour and two hour periods, though to a lesser extent. The loss appeared to be exponential. Reference will be made to these experiments as the control live bone experiments.

When bone was heated to 150°C. for one hour prior to incubation, a loss of calcium from serum was noted and the mode of loss was similar to the loss observed in the control live bone experiments. However, the rate of loss of calcium appeared to be affected by heating. There was a loss of 1.83 mg.% of calcium from serum after thirty minutes of incubation in the control live bone experiments and a loss of 1.62 mg.% with dead bone. The serum in which the dead bone was incubated showed a much greater loss of calcium after
TABLE I. Loss of Calcium from Serum Incubated with Bone

A. Incubation of Live Bone in Serum

<table>
<thead>
<tr>
<th></th>
<th>Time in Serum</th>
<th>Serum Value (With Bone)</th>
<th>Number of Analyses</th>
<th>Serum Value (Without Bone)</th>
<th>Number of Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>30 min.</td>
<td>8.89±.24*</td>
<td>14</td>
<td>10.72±.20</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>8.46±.20</td>
<td>16</td>
<td>10.55±.22</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2 hr.</td>
<td>7.48±.22</td>
<td>14</td>
<td>10.56±.29</td>
<td>15</td>
</tr>
<tr>
<td>Phosphate</td>
<td>30 min.</td>
<td>7.36±.11</td>
<td>15</td>
<td>6.76±.09</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>7.36±.20</td>
<td>12</td>
<td>6.72±.19</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2 hr.</td>
<td>7.40±.22</td>
<td>14</td>
<td>7.22±.18</td>
<td>15</td>
</tr>
</tbody>
</table>

B. Incubation of Dead Bone in Serum

<table>
<thead>
<tr>
<th></th>
<th>Time in Serum</th>
<th>Serum Value (With Bone)</th>
<th>Number ofAnalyses</th>
<th>Serum Value (Without Bone)</th>
<th>Number of Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>30 min.</td>
<td>9.05±.12</td>
<td>12</td>
<td>10.67±.08</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>8.14±.10</td>
<td>12</td>
<td>10.91±.35</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2 hr.</td>
<td>6.97±.08</td>
<td>12</td>
<td>10.93±.19</td>
<td>6</td>
</tr>
<tr>
<td>Phosphate</td>
<td>30 min.</td>
<td>7.14±.09</td>
<td>12</td>
<td>6.90±.08</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>7.04±.07</td>
<td>12</td>
<td>6.76±.12</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2 hr.</td>
<td>7.07±.12</td>
<td>12</td>
<td>7.14±.13</td>
<td>5</td>
</tr>
</tbody>
</table>

* .24 = S.E. = ± \sqrt{\frac{\sum x^2 - \bar{X}^2}{N}} \div \sqrt{\frac{N}{N-1}}
one hour of incubation than did the live bone, the loss being 2.77 and 2.09 mg.% respectively. After two hours of incubation, the serum to which the live bone was added showed a loss of 3.08 mg.% as compared with 3.96 mg.% in the dead bone experiments. This problem of the increase in the rate of loss of calcium from the serum to which the dead bone was added will be dealt with more thoroughly in the discussion. The phosphate values in the control live bone or dead bone experiments did not appear to change during the course of incubation.

B. Live Bone Experiments – Incubation of Successive Femurs in the Same Serum Sample versus Incubation of the Same Femur in Successive Serum Samples. See Table II.

In experiments employing the incubation of successive femurs in the same serum sample, the initial bone preparation was replaced after two hours of incubation with a bone from a freshly killed animal in an attempt to determine if the effect on the rate of loss of calcium from serum was due to its concentration in serum and therefore independent of the bone itself. After two hours of incubation with the initial bone, a loss of 2.67 mg.% of calcium from the serum was not-
TABLE II. Incubation of Bone in Successive Serum Sample(s)

<table>
<thead>
<tr>
<th></th>
<th>Time in Serum</th>
<th>Serum Value (With Bone)</th>
<th>Number of Analyses</th>
<th>Serum Value (Without Bone)</th>
<th>Number of Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Incubation of Successive Femurs in the Same Serum Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium mg.%</td>
<td>2 hrs.</td>
<td>8.85±.14</td>
<td>28</td>
<td>11.52±.17</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4 hrs.</td>
<td>8.02±.13</td>
<td>12</td>
<td>11.86±.24</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4 hrs.*</td>
<td>7.70±.17</td>
<td>16</td>
<td>11.86±.24</td>
<td>16</td>
</tr>
<tr>
<td>B. Incubation of the Same Femur in Successive Serum Samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium mg.%</td>
<td>2 hrs.</td>
<td>8.58±.05</td>
<td>16</td>
<td>10.59±.19</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4 hrs.</td>
<td>7.38±.05</td>
<td>8</td>
<td>10.67±.10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4 hrs.*</td>
<td>8.83±.06</td>
<td>8</td>
<td>10.67±.10</td>
<td>8</td>
</tr>
<tr>
<td>Phosphate mg.% of P</td>
<td>2 hrs.</td>
<td>6.44±.16</td>
<td>8</td>
<td>6.48±.13</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4 hrs.</td>
<td>6.38±.18</td>
<td>8</td>
<td>6.48±.13</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>4 hrs.*</td>
<td>6.11±.18</td>
<td>8</td>
<td>6.48±.13</td>
<td>8</td>
</tr>
</tbody>
</table>

* Samples to which a new femur was added at two hours

* Samples to which a new serum sample was added at two hours
ed as compared with 3.08 mg.% in the control live bone experiments. However, the volume of the incubation medium at two hours in the control live bone experiments was less than that of the present experiments because of the removal of samples. This difference in volume can be shown to account for the differences in calcium removed in the two experiments. At the four hour period a loss of 3.84 mg.% was noted in the samples which were incubated with one femur continuously for this period as compared with a loss of 4.16 mg.% in those samples which had a new bone added. A slightly greater loss was noted in the samples to which a new bone was added; however, this loss was not significant. The phosphate data of these experiments were unable to be used due to technical difficulties.

Incubation of the same bone in successive serum samples was employed to emphasize further that the rate of loss of calcium was dependent upon the concentration of calcium in the serum and at the same time to show that a single femur was capable of initiating this response in successive serum samples. The drop produced by the bone in the first aliquot of serum was somewhat smaller than expected (2.01
mg.% as compared with 2.67 mg.% but was considered to be within the experimental error and due partially to the unusually lower initial calcium value of the incubation medium. The effect of this lower initial calcium value was also seen in the serum values of this series of experiments in those samples in which a single bone was incubated continuously for four hours. Those femurs which were transferred after two hours to a second serum sample reduced the serum value during the subsequent two hour period to 8.83 mg.% as compared with a level of 8.58 mg.% at the initial two hour period. These two values are not significantly different. The phosphate level of the serum aliquots used in these experiments could not be shown to change during the incubation period.

C. Live Bone Experiments - Incubation of Femurs From Animals on a Calcium-Free Diet versus Those Taken From Animals on a High Calcium Diet. See Table III.

It is impossible to correlate the exact figures of these experiments with those from previous experiments. Because of the interference due to evaporation (explanation given in section D) the control values were adjusted to a
### TABLE III. Incubation of Femurs Taken From Animals on a Calcium-Free Diet versus Those Taken From Animals on a High Calcium Diet

#### A. Calcium-Free Diet

<table>
<thead>
<tr>
<th>Time in Serum</th>
<th>Serum Value (With Bone)</th>
<th>Number of Analyses</th>
<th>Serum Value (Without Bone)</th>
<th>Number of Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium mg.%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>10.26±.20</td>
<td>12</td>
<td>Each Experiment</td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>9.52±.15</td>
<td>12</td>
<td>Was Adjusted to 10.80</td>
<td></td>
</tr>
<tr>
<td>2 hr.</td>
<td>8.53±.20</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate mg.% of P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>6.99±.16</td>
<td>6</td>
<td>Each Experiment</td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>7.09±.24</td>
<td>8</td>
<td>Was Adjusted to 6.70</td>
<td></td>
</tr>
<tr>
<td>2 hr.</td>
<td>7.12±.37</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### B. High Calcium Diet

<table>
<thead>
<tr>
<th>Time in Serum</th>
<th>Serum Value (With Bone)</th>
<th>Number of Analyses</th>
<th>Serum Value (Without Bone)</th>
<th>Number of Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium mg.%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>9.91±.19</td>
<td>12</td>
<td>Each Experiment</td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>9.60±.13</td>
<td>12</td>
<td>Was Adjusted to 10.80</td>
<td></td>
</tr>
<tr>
<td>2 hr.</td>
<td>8.17±.19</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate mg.% of P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>7.21±.21*</td>
<td>8</td>
<td>Each Experiment</td>
<td></td>
</tr>
<tr>
<td>1 hr.</td>
<td>6.90±.09</td>
<td>8</td>
<td>Was Adjusted to 6.70</td>
<td></td>
</tr>
<tr>
<td>2 hr.</td>
<td>6.38±.20*</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Probability of Null Hypothesis, P = .015
common number, the experimental values being adjusted accordingly. The control calcium values were adjusted individually by dividing the observed values by a fraction which would give 10.80. The experimental values were then individually divided by the same value as their matched controls. The phosphate values were adjusted to 6.70 the same way. These values were chosen because they were the mean values of several experiments.

To indicate the effectiveness of the two diets, excretion data were taken. Animals on the calcium-free diet had a calcium excretion rate of from .026 to .066 mg./24 hours, in contrast to a rate of from 1.72 to 4.88 mg./24 hours for animals on the high calcium diet. When the epiphysis of the femurs from the animals on the calcium-free diet was broken off the metaphysis also broke off. This indicates that there was a decrease in the deposition of calcium in the bone which caused it to become brittle. This was not observed in the normal animals or in the animals on the high calcium diet. In the latter group it was even more difficult to break off the epiphysis.

In comparing the two groups of experiments, it can be noted that the calcium values at subsequent periods were
similar. At thirty minutes and at two hours the calcium values of the serum, to which were added the femurs taken from animals on the low calcium diet, were slightly higher than the values of serum incubated with bone taken from animals on a high calcium diet. However, since the one hour values were the same, no significant difference could be shown. If any change could be shown, it was expected that the femurs from the animals on the low calcium diet would have taken up calcium more rapidly than the femurs from the animals on the high calcium diet.

It was interesting to compare the thirty minute and two hour phosphate values of the serum in which the femurs from the animals on the high calcium diet were placed. These values were significantly different ($P = .015$). In this group it appeared that the phosphate in the serum rose immediately and later fell to a value below normal. Because the standard error of the control values could not be calculated, one could not tell whether these figures were significant from normal; however, they probably were not. The phosphate values in the low calcium diet group remained unchanged.
D. Live Bone Experiments - The Effect of Evaporation on the Calcium and Phosphate Values of the Incubation Medium.

See Table IV.

One of the main problems of this type of experiment was controlling the evaporation. When this work was continued in the recently completed Anderson Biological Laboratories, a large rise in the plasma calcium values was noted in the control flasks. This was attributed to evaporation. The humidity and temperature in the Anderson Biological Laboratories were maintained considerably lower than the previous location and tended to cause a high amount of evaporation. Even though the media were contained in flasks, condensation which collected on the sides of the flasks caused the calcium and phosphate levels to rise. This was because the flasks were only partially immersed in the water bath and the difference in temperature between the water bath (37°C.) and room temperature (22°C.) caused this condensation.

The calcium values of the plasma of the control flasks increased from 10.40 to 12.79 mg.% during the experiment. The phosphate values showed a similar rise from 5.15 to 6.40
TABLE IV. The Effect of Evaporation on the Calcium and Phosphate Values of the Incubation Medium

<table>
<thead>
<tr>
<th></th>
<th>Time in Serum</th>
<th>Serum Value (With Bone)</th>
<th>Number of Analyses</th>
<th>Serum Value (Without Bone)</th>
<th>Number of Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium mg.%</td>
<td>0 min.</td>
<td>10.21±.13</td>
<td>20</td>
<td>10.40±.13</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>30 min.</td>
<td>10.21±.13</td>
<td>20</td>
<td>11.45±.26</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>9.83±.13</td>
<td>20</td>
<td>12.18±.40</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2 hr.</td>
<td>9.43±.17</td>
<td>19</td>
<td>12.79±.56</td>
<td>10</td>
</tr>
<tr>
<td>Phosphate mg.% of P</td>
<td>0 min.</td>
<td>6.07±.15</td>
<td>18</td>
<td>5.15±.06</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>30 min.</td>
<td>6.07±.15</td>
<td>18</td>
<td>5.75±.15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>6.22±.19</td>
<td>17</td>
<td>6.06±.21</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2 hr.</td>
<td>6.35±.18</td>
<td>18</td>
<td>6.40±.34</td>
<td>8</td>
</tr>
</tbody>
</table>
mg.%. The calcium values of the serum containing bone were not as low as noted earlier for the control live bone experiments, however the relative differences between the control values and the values of the serum containing bone in this series of experiments were approximately the same. The plasma phosphate values showed an increase during incubation indicating that evaporation had occurred with no apparent deposition of phosphate onto the bone. The standard error of the observed values was quite high showing the large variation in evaporation from experiment to experiment. In the few experiments where evaporation was a factor, corrections were made as described in C and the results used only to show differences between the groups of the individual experiments.
Changes in Calcium and Phosphate Levels After the Addition of Calcium and/or Phosphate Compounds to Plasma

A. High-Speed Centrifugation After the Addition of Tertiary or Secondary Calcium Phosphate. See Table V.

Tertiary or secondary calcium phosphate was added to plasma in order to compare the relative solubility of these two compounds. A loss of calcium and phosphate from solution was noted when tertiary or secondary calcium phosphate was added to plasma and the contents subjected to high-speed centrifugation. In the former experiments the calcium level dropped from 11.80 to 1.95 mg.%, and the phosphate level from 6.59 to 3.41 mg.%. It was interesting to note that after high-speed centrifugation the phosphate level was almost twice as high as the calcium level. Due to the predominance of \( \text{HPO}_4^- \) in plasma the phosphate level remained at a relatively high value.

When secondary calcium phosphate was employed the calcium level dropped from 11.66 to 7.64 mg.% and the phosphate level from 6.65 to 5.75 mg.% The difference between the latter figures was statistically significant \((P = .002)\).
<table>
<thead>
<tr>
<th></th>
<th>Plasma Value</th>
<th>Plasma Value AHSC (Without Calcium Phosphate)</th>
<th>Plasma Value AHSC (With Calcium Phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium (12)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg.%</td>
<td>11.15±.56</td>
<td>11.80±.25</td>
<td>1.95±.18</td>
</tr>
<tr>
<td><strong>Phosphate (12)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg.% of P</td>
<td>6.57±.33</td>
<td>6.59±.17</td>
<td>3.41±.21</td>
</tr>
</tbody>
</table>

**B. High-Speed Centrifugation After the Addition of Secondary Calcium Phosphate**

<table>
<thead>
<tr>
<th></th>
<th>Plasma Value</th>
<th>Plasma Value AHSC (Without Calcium Phosphate)</th>
<th>Plasma Value AHSC (With Calcium Phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium (8)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg.%</td>
<td>11.21±.15</td>
<td>11.66±.16</td>
<td>7.64±.15</td>
</tr>
<tr>
<td><strong>Phosphate (8)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg.% of P</td>
<td>6.47±.21</td>
<td>6.65±.16*</td>
<td>5.74±.17*</td>
</tr>
</tbody>
</table>

* Probability of Null Hypothesis - $P = 0.002$

(BHSC) Before high-speed centrifugation
(AHSC) After high-speed centrifugation
( ) Number of analyses
The calcium level did not fall below the phosphate level.
It is obvious that secondary calcium phosphate is considerably more soluble in plasma than tertiary calcium phosphate.

B. Plasma Values After High-Speed Centrifugation of Plasma Augmented With Calcium and Phosphate (5 mg.% of Calcium and of Phosphorus as Phosphate), See Table VI.

After the addition of 5 mg.% of calcium and of phosphate to plasma, the contents were subjected to high-speed centrifugation. The calcium level dropped from 16.52 to 11.40 mg.% and the phosphate level from 11.50 to 8.99 mg.%. The calcium level returned to normal whereas the phosphate value was a little over 2 mg.% higher than normal. Calcium and phosphate were lost from the plasma in a Ca:P weight ratio of 2:1. A calculated value of (Ca) x P of 51 was obtained from this data. The symbol (Ca) is used in these and all succeeding experiments to designate one-half of the total calcium value, which is usually considered to approximate that portion of serum calcium which is ionized. Low-speed centrifugation (840 x G) was performed on one series of these experiments after the addition of calcium and of phosphate with no apparent change in the calcium or phosphate levels.
TABLE VI. Plasma Values After High-Speed Centrifugation of Plasma Augmented With Calcium and Phosphate (5mg.% of Calcium and of Phosphorus as Phosphate).

<table>
<thead>
<tr>
<th></th>
<th>Plasma Value (BHSC)</th>
<th>Plasma Value (Without Calcium &amp; Phosphate)</th>
<th>Plasma Value (With Calcium &amp; Phosphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (8) mg.%</td>
<td>11.59±.28</td>
<td>11.30±.24 (BHSC)</td>
<td>16.52±.29 (BHSC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.30±.24 (AHSC)</td>
<td>11.40±.23 (AHSC)</td>
</tr>
<tr>
<td>Phosphate (8) mg.% of P</td>
<td>6.52±.26</td>
<td>6.79±.17 (BHSC)</td>
<td>11.50±.26 (BHSC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.67±.24 (AHSC)</td>
<td>8.99±.27 (AHSC)</td>
</tr>
</tbody>
</table>

( ) Number of analyses
C. Plasma Values After High-Speed Centrifugation of Plasma Augmented With Calcium or Phosphate (5 mg.% of Calcium or of Phosphorus as Phosphate), see Table VII.

When only calcium was added to serum and the procedure followed as in B above, the calcium level dropped from 17.05 to 15.02 mg.% and the phosphate from 6.62 to 5.93 mg.% The final (Ca) x P product was 45. Calcium and phosphate were lost from solution in a Ca:P weight ratio of 3:1.

When only phosphate was added and the contents treated as above, the calcium level dropped from 10.36 to 9.74 mg.%, whereas the phosphate appeared to remain unchanged. Assuming the drop in calcium and phosphate to be in a Ca:P weight ratio of 2:1, the phosphate value, even if it had dropped, would have remained within the range of experimental error. This very small loss of calcium with no apparent loss of phosphate was to be expected since the (Ca) x P product was 50.
TABLE VII. Plasma Values After High-Speed Centrifugation of Plasma Augmented With Calcium or Phosphate

### A. The Addition of 5mg.% of Calcium to Plasma

<table>
<thead>
<tr>
<th>Plasma Value (Without Calcium)</th>
<th>Plasma Value (With Calcium) (BHSC)</th>
<th>Plasma Value (With Calcium) (AHSC)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (8) mg.%</td>
<td>Each Analysis Was Adjusted to 11.30</td>
<td>17.05±.24</td>
<td>15.02±.23</td>
</tr>
</tbody>
</table>

### B. The Addition of 5mg.% of Phosphate to Plasma

<table>
<thead>
<tr>
<th>Plasma Value (Without Phosphate) (BHSC)</th>
<th>Plasma Value (With Phosphate) (BHSC)</th>
<th>Plasma Value (With Phosphate) (AHSC)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (8) mg.%</td>
<td>10.81±.12</td>
<td>10.80±.14</td>
<td>10.36±.11</td>
</tr>
<tr>
<td>Phosphate (8) mg.% of P</td>
<td>5.61±.13</td>
<td>5.61±.11</td>
<td>10.27±.09</td>
</tr>
</tbody>
</table>

* Probability of Null Hypothesis

( ) Number of analyses
Experimental Demonstration of the Presence of an Insoluble Calcium Phosphate Substance in the Blood in vivo

A. High-Speed Centrifugation of Plasma From Controls and From Animals Administered PTE, See Table VIII.

Calcium and phosphate levels in plasma taken from the control animals were not affected by high-speed centrifugation even though the pH rose to 7.90. The (Ca) x P product before and after high-speed centrifugation was 37.

A loss of calcium and phosphate from solution was noted when the plasma from animals receiving PTE was subjected to high-speed centrifugation. The calcium level dropped from 10.92 to 9.16 mg.% and the phosphate level from 7.82 to 6.79 mg.% The latter figures were statistically significant (P = .013). The loss of calcium and phosphate from the solution was in a Ca:P weight ratio of 1.7:1.

The PTE was administered in an attempt to raise the calcium level of the plasma. If the calcium level had been raised, one would have expected the formation of an insoluble substance in the plasma. However, this substance appeared to be formed even though the calcium level was not altered. The phosphate level was a little over 1 mg.% higher than normal.
<table>
<thead>
<tr>
<th></th>
<th>Plasma Value (Without PTE) (B)</th>
<th>Plasma Value (With PTE) (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium</strong> mg.%</td>
<td>11.67±.15</td>
<td>10.92±.04</td>
</tr>
<tr>
<td><strong>Phosphate</strong> mg.% of P</td>
<td>6.42±.26</td>
<td>6.79±.13</td>
</tr>
</tbody>
</table>

Number of animals

Probability of Null Hypothesis - P = .013
B. High-Speed Centrifugation of Plasma From Nephrectomized Controls and From Nephrectomized Animals Administered PTE, See Table IX.

When the plasma from the nephrectomized controls, or from the animals receiving PTE, was subjected to high-speed centrifugation there occurred a loss of both calcium and phosphate from the plasma. The calcium level in the former experiments was lowered from 11.07 to 8.03 mg.% whereas the calcium level in the latter experiments was lowered from 11.07 to 7.32 mg.%. The PTE did not raise the calcium level but it did appear to cause an increase in the loss of calcium from the solution. This increased loss of calcium from solution in the nephrectomized animals which had received PTE parallels the situation which occurred in the normal animals which received PTE.

In the nephrectomized controls the phosphate level after high-speed centrifugation dropped from 17.29 to 14.88 mg. % (P = .02), while the level in those animals receiving PTE dropped from 18.19 to 15.86 mg.% (P = .007). The magnitude of loss of phosphate from solution was the same in both of these experiments. In the former experiments the calcium
TABLE IX. High-Speed Centrifugation of Plasma From Nephrectomized Controls and From Nephrectomized Animals Administered PTE

<table>
<thead>
<tr>
<th></th>
<th>Plasma Value (Without PTE)</th>
<th>Plasma Value (With PTE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(BHSC)</td>
<td>(AHSC)</td>
</tr>
<tr>
<td>Calcium (4) mg.%</td>
<td>11.07±.37</td>
<td>8.03±.36</td>
</tr>
<tr>
<td>Phosphate (6) mg.% of P</td>
<td>17.29±.60</td>
<td>14.88±.64</td>
</tr>
</tbody>
</table>

(P) = .022

(P) = .007

( ) Number of animals

(P) Probability of Null Hypothesis
and phosphate were lost from solution in a Ca:P weight ratio of 1.3:1 with a (Ca) x P product of 60. In the latter experiments the calcium and phosphate were lost from solution in a Ca:P weight ratio of 1.6:1 with a (Ca) x P product of 58.

C. High-Speed Centrifugation of Plasma From Nephrectomized Animals on a Low Calcium or a High Calcium Diet. See Table X.

A loss of calcium and phosphate from solution was noted when the plasma of the nephrectomized animals on a calcium-free diet was subjected to high-speed centrifugation. The calcium level dropped from 9.25 to 7.54 mg.% and the phosphate level from 18.95 to 18.08 mg.%. Due to the high standard error of the latter figures, they were not significantly different. This was due to the variability in the plasma phosphate level of the different animals after nephrectomy. There was a loss of phosphate from the plasma in six of the eight animals. Assuming this loss to be significant, calcium and phosphate were lost from solution in a Ca:P weight ratio of 2:1 with a (Ca) x P product of 68. Because of the lack of calcium in the diet the calcium
TABLE X. High-Speed Centrifugation of Plasma From Nephrectomized Animals on a Low Calcium and a High Calcium Diet

<table>
<thead>
<tr>
<th></th>
<th>Plasma Value (Low Calcium Diet) (BHSC)</th>
<th>Plasma Value (Low Calcium Diet) (AHSC)</th>
<th>Plasma Value (High Calcium Diet) (BHSC)</th>
<th>Plasma Value (High Calcium Diet) (AHSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (8) mg.%</td>
<td>9.25±.24</td>
<td>7.54±.24</td>
<td>11.16±.12</td>
<td>11.06±.10</td>
</tr>
<tr>
<td>Phosphate (8) mg.% of P</td>
<td>18.95±.77</td>
<td>18.08±.97</td>
<td>8.89±.54</td>
<td>9.00±.54</td>
</tr>
</tbody>
</table>

( ) Number of animals
level in the plasma was lower than normal before high-speed centrifugation. This allowed the phosphate level to increase considerably as compared with those animals receiving calcium.

The plasma calcium and phosphate level of the nephrectomized animals on a high calcium diet appeared to remain unchanged after high-speed centrifugation. The administration of calcium to these animals did not appear to increase the amount of calcium in the plasma, however, it greatly suppressed the amount of phosphate in the plasma. The (Ca) x P product before high-speed centrifugation was 50, therefore one would not have expected the formation of an insoluble substance in the plasma.
DISCUSSION

I. Loss of Calcium From Serum Incubated With Bone

When bone was placed in serum, a significant loss of calcium from solution was noted irrespective of the previous treatment of the bone. When dead bone was placed in serum, the loss of calcium from solution was greater than that observed for the control live bone experiments. Kunin, et al., have observed a loss of hydronium ions from bone when heated over 110°C. This loss of hydronium ions could account for the increased loss of calcium from solution in the dead bone experiments as compared with the control live bone experiments. The experiments employing the incubation of successive femurs in the same serum sample as well as incubation of the same femur in successive serum samples have demonstrated conclusively that, for a given amount of solid and solution, the rate of loss of calcium from solution is dependent upon its concentration in serum and independent of the bone itself. The excretion data shows that the diets were effective in those experiments in which the animals were placed on a high or a low calcium diet; however, the
femurs from both groups did not respond differently when placed in the serum.

When bone was placed in serum, the phosphate value of the solution appeared to remain unchanged except in those experiments in which the femurs from the animals on the high calcium diet were employed. In the high calcium diet experiments, the phosphate level appeared to rise at the thirty minute period and then fall below normal at the two hour period. Although these two values were significantly different, one must be cautious at coming to any definite conclusions. When Sendroy and Hastings (1926-27a) placed tertiary calcium phosphate in serum, they could not detect a loss of phosphate from solution; however, they noticed a loss of bicarbonate ions indicating that calcium was incorporated into the solid as calcium carbonate. Neuman and Neuman (1956a) have shown that carbon dioxide is fixed by hydroxyapatite accompanied by a displacement of phosphate from the solid to the solution. Since a continuous supply of carbon dioxide was used in the present experiments to control the pH, it is likely that this exchange reaction would cause the deposition onto the bone of calcium as the
carbonate rather than the phosphate. Citrate (Armstrong and Singer) and possibly hydroxyl ions as bicarbonate (Neuman and Neuman, 1953) also can displace phosphate.

Logan and Kane (1939) placed glycerol-KOH ashed bone and fetal bone in serum and noticed that the latter preparation was more soluble than the former. Glycerol-KOH ashed bone has the organic material removed and this procedure probably causes a marked alteration in the structure and nature of the bone mineral (Neuman and Mulryan, 1950). Greenwald (1938) found that the solubility of tertiary calcium phosphate is greatly increased by the presence of organic material. Dallemagne, et al (1956) have recently shown that chemical bonding between the bone mineral and the organic fraction of bone definitely exist. The relationship between the organic fraction of bone and the bone mineral itself must account for the differences among investigators using bone containing the organic fraction (Logan and Kane, 1939) and bone with the organic fraction removed (Levinskas, 1953; Dallemagne, et al, 1956).

An attempt was made to demonstrate the level of calcium in solution when bone was equilibrated with serum.
Falkenheim, et al (1951) have shown that equilibration of a bone preparation with a calcium chloride solution occurs in two to four hours. Equilibrium between the solid and solution essentially must have been reached in the present experiments. After serum was pre-reduced with tertiary calcium phosphate to a calcium level of 2 mg.% and a phosphate level of 3.5 mg.%, bone was added to this serum and in two hours the calcium level rose to 6 mg.% and the phosphate to 4.7 mg.%.

Levinskas (1953) found that when hydroxyapatite was equilibrated with a salt solution, the calcium level was 1.2 mg.% and the phosphate level .4 mg.% as compared with a calcium level of approximately 7.5 mg.% and a phosphate level of approximately 6.5 mg.% in the present investigations. Levinskas (1953) has shown that when hydroxyapatite was placed in a salt solution a colloid suspension formed. This is insufficient in explaining the high levels of calcium and phosphate present as compared with the experiments using hydroxyapatite because several serum samples which had been incubated with bone were subjected to high-speed centrifugation with no apparent loss of calcium or phosphate from
solution.

McLean and Urist (1955) and Talmage (1956) have postulated that bone mineral is composed of a labile and a stable portion. The labile portion of bone will maintain the serum calcium level at 7 mg.% and the parathyroid glands by acting on the stable portion will maintain the calcium level around 10 mg.% In a parathyroidectomized rat the calcium level falls to about 7 mg.% (Talmage and Kraintz, 1954). This level was approximately the same as that observed in the in vitro experiments reported herein supporting the postulate of McLean and Urist (1955) and Talmage (1956).

II. High-Speed Centrifugation After the Addition of Calcium and/or Phosphate Compounds to Plasma

A loss of calcium and phosphate from solution was noted when tertiary or secondary calcium phosphate was placed in plasma and the contents subjected to high-speed centrifugation. High-speed centrifugation was employed because the colloidal substance formed does not precipitate due to the protective action of the protein (Csapo, 1927). When tertiary calcium phosphate was employed, the calcium and phosphate levels were lowered to approximately 2 mg.% and 3.5
mg.\% respectively. Holt, et al (1925b) and Sendroy and Hastings (1926-27a) obtained similar calcium values; however, their phosphate values at equilibrium were 1.5 mg.\%.

Sendroy and Hastings (1926-27b) employing a mixture of calcium carbonate and tertiary calcium phosphate in an artificial edema fluid demonstrated a calcium value of 1.2 mg.\% and a phosphate value of 1.7 mg.\% at equilibrium. Levinskas (1953) obtained the same calcium level as did Sendroy and Hastings (1926-27b) using hydroxyapatite but observed a level of 0.4 mg.\% for the phosphate. Tertiary calcium phosphate behaves very similar to hydroxyapatite except for the phosphate value. The carbon dioxide atmosphere may account for this difference; however, equilibration may have been incomplete. Levinskas (1953) used a carbon dioxide free atmosphere whereas the other investigators did not.

When secondary calcium phosphate was employed, the calcium and phosphate levels at equilibrium were 7.6 mg.\% and 5.7 mg.\% respectively. Shear, et al (1929) observed that when secondary calcium phosphate was placed in an inorganic serum solution the calcium and phosphate equilibrated at a Ca x P product of slightly over 50. Shear and
Kramer (1930) found that when secondary calcium phosphate was added to serum the \((\text{Ca}) \times P\) product at equilibrium was 42. This product was attained even though the initial product in human serum was 17.5. Strates, et al. (1957) have demonstrated that secondary calcium phosphate is unstable at pH 7.4 and rapidly hydrolyzes to hydroxyapatite. This could possibly affect the results found by the present investigator but since the solubility product of secondary calcium phosphate acts as a limiting factor in the relationship between solid bone and serum it is not unreasonable to suspect that bone and secondary calcium phosphate would behave similarly in serum.

A loss of calcium and phosphate from solution was noted when calcium and phosphate were added to plasma and the contents subjected to high-speed centrifugation. Similar changes were observed when only calcium was added; however, when only phosphate was added, the calcium level was lowered with no apparent effect on the phosphate. Since the latter change was relatively small it possibly could be due to the change in pH. Calcium and phosphate were lost from solution in a Ca:P weight ratio of approximately 2:1 indi-
cating the precipitate was tertiary calcium phosphate as postulated by Greenberg, et al (1935). The precipitate first formed should have been secondary calcium phosphate (Greenwald, 1942b), however enough time must have elapsed for tertiary calcium phosphate to be formed. Smith (1934) augmented dog serum with calcium and phosphate and observed a Ca x P (diffusible) product higher than normal at equilibrium. Laskowski (1933) and Hopkins, et al (1952) have shown that when calcium was added to serum, the Ca x P (diffusible) product was between 40 and 50 irrespective of whether the initial product was 20 or 40. Laskowski (1933) noted that when only phosphate was added to serum, unusually high amounts had to be added in order to decrease the amount of diffusible calcium. Hopkins, et al (1952) produced a decrease in the diffusible calcium when the phosphate level was raised to 17 mg.%. Results such as reported by Shear, et al (1929); Shear and Kramer (1930); Laskowski (1933); Smith (1934); Hopkins, et al (1952); Levinskas (1953); and Strates, et al (1957) as well as the data reported herein, demonstrate conclusively that serum is undersaturated with respect to secondary calcium phosphate.
The change in pH was thought to have some effect on the final amount of calcium and phosphate in solution. Some investigators have demonstrated a decrease in the diffusible calcium by increasing the pH from 7 to 8 (Sendroy and Hastings, 1926-27b; Dillman and Visscher, 1933; Smith, 1934; and Howard, 1957) while others could not detect a change (Greenberg and Gunther, 1929-30; McLean and Hastings, and Ludewig, et al., 1942). Although some effect may have occurred, it would have been relatively small.

III. Experimental Demonstration of the Presence of an Insoluble Calcium Phosphate Substance in the Blood in vivo

The presence of colloidal calcium phosphate in the normal animal has been indicated in all classes of vertebrates except mammals (Grollman, 1927; McDonald and Riddle, 1945; and Bailey, 1957). When plasma from normal rats was subjected to high-speed centrifugation the calcium and phosphate levels remained the same. While centrifuging the pH rose from 7.40 to 7.90. This change in pH apparently did not cause the spontaneous formation of colloidal calcium phosphate at least in the time allowed for centrifugation,
in contrast with the work of Howard (1957) mentioned previously.

Parathyroid extract was administered to rats in an attempt to elevate the calcium level of the blood and as a result cause the production of colloidal calcium phosphate. Colloidal calcium phosphate apparently formed even though the calcium level was unchanged. The phosphate level was somewhat higher than normal but was not high enough to produce a supersaturated solution of secondary calcium phosphate. Collip, et al. (1925), Grollman (1927), and Greenberg and Larson (1939) administered PTE to dogs over a short period of time and observed a slightly elevated phosphate level, however the calcium level was approximately 17 mg.%. Dickens (1941) and Elliott and Freeman (1956b) administered PTE to dogs over a much longer period of time. The calcium level was 14 mg.% while they failed to measure the phosphate. Handler, et al. (1951) observed an increase in the plasma inorganic phosphate when PTE was administered unaccompanied by a change in plasma calcium. They stated that this effect may be due to more than one principle in the commercial extract; however, in a later publication Handler and
Cohen (1952) found that the results reported by Handler, et al. (1951) were due to an error in technique. It appears that in the experiments reported herein PTE caused the production of colloidal calcium phosphate from a solution undersaturated with secondary calcium phosphate.

A loss of calcium and phosphate from the plasma taken from nephrectomized controls and from nephrectomized animals administered PTE was observed after high-speed centrifugation. In both groups the initial calcium level remained the same whereas the phosphate level was increased to approximately 18 mg.%. PTE was administered to nephrectomized animals at the critical time when citric acid and calcium were considerably higher than normal (Freeman and Chang, 1950; Elliott and Freeman, 1956a; and Talmage and Elliott, 1956) in an attempt to maintain a high calcium level in the blood. Although the calcium level was not affected by the PTE, the phosphate level was sufficiently high to cause the production of colloidal calcium phosphate. The \( (\text{Ca}) \times P \) product after high-speed centrifugation was somewhat higher than that found by the addition of calcium \textit{in vivo} but supports the work of Greenberg and Larson (1939) who observed
a much greater increase in the Ca x P (diffusible) product in conditions of hyperphosphatemia.

When calcium was administered by stomach tube to nephrectomized animals the calcium level in the blood remained unchanged. However, the phosphate in the blood was suppressed by the administration of calcium. Since the plasma was not saturated with secondary calcium phosphate, colloidal calcium phosphate did not form. Binger (1917-18) and Salvesen, et al (1924) suppressed the calcium level in the blood by administering phosphate; therefore, it is not surprising that the administration of calcium would suppress the phosphate. The control animals to this series of experiments showed a much greater increase in the plasma phosphate level and a consequent production of colloidal calcium phosphate even though the calcium level was lower than normal.
SUMMARY

When bone was placed in serum, calcium was removed from the solution with no detectable change in the phosphate level. The stable phosphate level indicated that calcium precipitated onto the bone as calcium carbonate. At equilibrium the calcium level in the serum was approximately 7.5 mg.\% and the phosphate level 6.5 mg.\%. The decrease in the calcium level indicated that serum was supersaturated with respect to bone.

The solubility of tertiary calcium phosphate was inadequate in explaining the level of calcium and phosphate in normal plasma. Secondary calcium phosphate appeared to act as the limiting factor in the relationship between solid bone and plasma. When plasma was augmented with calcium and/or phosphate, precipitation of a calcium phosphate substance occurred. This was detected by high-speed centrifugation at an RCF of 14,000. The precipitate appeared to be colloidal tertiary calcium phosphate and did not form below a (Ca) x P product of between 45 and 50. Normal rat plasma had a (Ca) x P product of 37. These relationships indicated that rat plasma was undersaturated with respect
to secondary calcium phosphate in the absence of the solid phase.

Spontaneous precipitation of calcium phosphate did not occur in normal rat plasma when the pH was elevated to 7.90. When parathyroid extract was administered to normal animals the calcium level in the plasma did not change; however, colloidal calcium phosphate formed and the \( (\text{Ca}) \times P \) product was 42.

Colloidal calcium phosphate appeared in the plasma when the phosphate level was elevated by bilateral nephrectomy. The \( (\text{Ca}) \times P \) product at equilibrium was somewhat higher than that observed after the addition of only calcium or calcium supplemented with phosphate in vitro. When calcium was administered to nephrectomized rats, the phosphate level was suppressed below the level which would cause the production of colloidal calcium phosphate. The data reported herein supports the conclusion that rat serum is supersaturated with respect to bone but undersaturated with respect to calcium and phosphate in the absence of the solid phase.
BIBLIOGRAPHY


Smith, R. G. 1934. Non-diffusible serum calcium at various
calcium, phosphate, and hydrogen ion concentrations.

Sobel, A. E. and Hanok, A. 1952. Calcification, VII. Re-
versible inactivation of calcification in vitro and

The solubility of bone mineral. II. Precipitation of
near-neutral solutions of calcium and phosphate. Jour.

Talmage, R. V. 1956. Studies on the maintenance of serum
calcium levels by parathyroid action on bone and kid-

Talmage, R. V. and Elliott, J. R. 1956. Changes in extra-
cellular fluid levels of calcium, phosphate and
citrate ions in nephrectomized rats following para-

Talmage, R. V. and Elliott, J. R. 1958. Removal of calcium
from bone as influenced by the parathyroids. Endo-

Talmage, R. V. and Kraintz, F. W. 1954. Immediate changes
in phosphate excretion following parathyroidectomy
416-419.

Weikel, J. H., Jr. 1954. Investigations into the dynamics
of skeletal metabolism. Doctoral Thesis, University
of Rochester.

Weikel, J. H., Jr., Neuman, W. F. and Feldman, I. 1954. The
surface chemistry of bone. VIII. On the mechanism of
ionic exchange. Jour. Amer. Chem. Soc. 764: 5202-
5207.