PLASMA CALCIUM REGULATION ASSOCIATED WITH INDUCED HYPOCALCEMIA AND HYPERCALCEMIA

by

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ABSTRACT

The plasma calcium level is one of the most precisely regulated constants of the internal environment, and the large reservoir of calcium in the skeleton is primarily responsible for this homeostasis.

The experiments presented in this thesis were designed to study quantitatively the regulation of plasma calcium. Acute hypocalcemia was induced by continuous intravenous EDTA infusion (a calcium chelating agent) at a known rate, and hypercalcemia was induced by intravenous calcium gluconate infusion. The rate used in most cases was 10 mg. calcium per kg. for one hour. Both mobilization and storage of calcium appeared to depend on an equilibrium with a labile calcium storage pool in bone. The rate of storage or mobilization was shown to be proportional to the amount of blood coming in contact with this labile pool in bone (bone blood flow), and the plasma/bone difference in Ca^{++} activity. Bone blood flow was measured using the Fick Principle for calcium storage, and it was calculated to be 6.46 $\pm$ 0.60% of the cardiac output (14 dogs). The extracellular fluid calcium was also estimated and found to be 15.73 $\pm$ 0.72 mg/kg (14 dogs), corresponding to an extracellular fluid volume of approximately 20% of body weight. Less than 5% of the injected calcium was excreted in the urine.

The labile calcium storage pool in bone was estimated

from the changes in the bone-blood equilibrium after calcium was injected, and was found to be 2 - 5 times greater than the extracellular calcium. The net loss of calcium from the plasma after calcium injection, which is assumed to equal the rate at which calcium is used for bone mineralization less calcium released by resorption, was estimated as 1 - 2 mg. Ca/kg/hr. or 0.15 - 0.35% of the total bone calcium per day.

The methods described provide a means of assessing quantitatively the factors involved in acute regulation of the plasma calcium level.

(Anthony Head)

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TABLE OF CONTENTS

I. INTRODUCTION

A. General scope of problem.

The homeostasis of the calcium concentration in blood is very important physiologically in order to maintain normal function of all tissues of the body, especially nerves and muscles.

This thesis is concerned with acute restoration of plasma calcium to equilibrium when there is rapid addition to or removal of calcium from the blood stream. This is achieved by intravenous injections of calcium gluconate, or of a calcium chelating agent, EDTA.

The role of the skeleton in this acute regulation of blood calcium has been studied. Also the rates of restoration of blood calcium to equilibrium have been estimated.

B. The Importance of the Skeleton in the Homeostasis of Calcium.

The body contains approximately 15 g. of calcium per kg. body weight. There is about ½ g. in the entire extracellular compartment, and 11½ g. in soft tissue (intracellular) in a 70 kg. man.(15)

The skeleton contains 99% of the body's calcium, and is primarily involved in the homeostatic regulation of the serum calcium. The kidneys and intestinal tract are important in normal calcium balance, but are of minor

importance in comparison to the large calcium reservoir of bone, in restoring equilibrium during rapid removal or addition of calcium to the extracellular fluid.

C. The Structure of Bone.

Bone salt is a compound of calcium, phosphate, and hydroxyl ions, and water, with a characteristic apatite pattern. The crystals are described as flat tablets or rods a few hundred angstroms in length and breadth and only a few unit cells thick (30 - 70 A^{o}). The findings most commonly reported, from electron microscopy, are that the crystals are rod- or needle-shaped. It has also been suggested by Finean and Engstrom (10), that the tablets appear to be aggregations of rods. In intact bone, these crystals are found to be closely associated with collagen in an organized fashion, with the long axes of the crystals oriented in the long axes of the collagen fibres. Because of the minute size of the crystals, the specific surface area of bone mineral is so large, that surface phenomena dominate the chemical behaviour of the bone mineral(22).

The structural units of bone are the osteone (for lamellar or "hard" bone), and the trabecula (for soft and cancellous type). There is a minute artery and vein within the osteone. The trabeculae are completely surrounded by extracellular fluid of the highly vascular marrow. In both

types, throughout the tissues, there are spaces (canaliculi and lacunae) through which tissue fluids flow. (14)

Bone cells, or osteoblasts, produce a matrix of collagen and polysaccharide, along which are deposited the bone salts in a normal environment. Bone mineral is a quite impure hydroxy apatite, having as its principal impurities, carbonate, 6%; citrate, 1%; sodium, 0.7%; and magnesium, 0.7% with traces of fluoride.

The impurities in bone are there as a passive physicochemical consequence of the presence of these ions in the fluids in which the crystals form. (22)

D. The Growth of Bone.

During growth of bone, a newly formed matrix is added to the surface just under the layer of osteoblasts. When this calcifies, a new layer of matrix is laid upon the older, pushing it back farther from the site of most active exchange with the extracellular fluids. However, these layers are still a store-house that could become available if exposed. Bone is living tissue, and therefore is constantly being destroyed, and newly formed. These processes occur over and above the pure exchange reactions between atoms of the crystals and the extracellular fluids. (15)

The bone mineral participates in electrolyte metabolism throughout the lifetime of the animal because of the constant "turnover".

Neuman classifies the osteone maturation and reactivity in three ionic processes:

(1) diffusion into the hydration shell

(2) ion-exchange or ion-displacement at the crystal surface

(3) thermal recrystallization within the cell.

These are modified under the influence of physiological conditions.

Because the serum is supersaturated with respect to calcium and phosphate, the tendency is for all bone structure gradually to attain maximal mineralization. Thus the crystals grow slowly, to the complete or near complete exclusion of water, and the more fully mineralized the structure, the more restricted are the circulation, diffusion, and exchange of ions. From this it follows that the age of the bone is the primary determinant of its chemical reactivity. This applies to any bone structure, such as trabecula, a Haversian system, an interstitial or subperiosteal lamella.

Young bone is more vascular and has a higher water content, so that this bone remains in equilibrium with the body fluids. The crystals are small and exhibit rapid surface exchange, recrystallization and intracrystalline exchange. As the water content falls to its minimal value, with advancing age, there results an ever increasing proportion of the skeleton that is old, fully mineralized, and non-reactive. However, all the osteones in the adult skeleton are not inert.

There is a continuous pattern of Haversian remodelling by which erosion cavities are continually forming, and new Haversian systems developing. Thus, there results a constant supply of active exchangeable bone mineral throughout the life of the animal. (22)

Three mechanisms for the process of calcification have been suggested by Neuman.

"(1) That the collagen fibres of bone specifically possess the chemical property of inducing the production of crystal nuclei perhaps through the presence in the molecule of a phosphorylated amino acid.

(2) That in cartilage, the protein is rendered "active" by the enzymatic transfer of a pyrophosphate group from ATP to the protein.

(3) That the active nucleation centre in cartilage involves a complex between collagen and the mucopolysaccharide, chondroitin sulphate."

The first deals with mineralization in forming bone, and the last two with the mineralization of cartilage. (21)

McLean defines resorption as "the putting into solution of a complicated structure in such a fashion that it disappears, its end-products entering the blood stream. Resorption also progresses inward from the surfaces of bone; it never arises within the deeper layers of the structure."

One small fraction of bone is already in fluid

form, so it may be easily removed. The remainder is in solid form and insoluble in aqueous fluids, so that it must be rendered soluble in water before it can be transferred to the body fluids. Koelliker in 1873 (16) suggested that the osteoclasts eroded bone by chemical means, but did not specify the nature of the chemical action.

An assumption is now made that the bone salt may be dissolved whenever another substance having a stronger affinity for calcium is in a solution in contact with bone. It is assumed that there is continually applied to the surface of bone, a solution that will depolymerize mucopolysaccharides, digest collagen and hold calcium in a firm combination. Such a mechanism would require only certain enzyme systems and an organic substance to combine with calcium. (20)

E. Transport of Calcium.

The transport of calcium to and from bone is accomplished by the blood stream, extracellular fluid, and lymph.

Ca-45 studies have demonstrated the enormous surface area of bone on which active ionic exchange processes can occur, and an immediate transfer between bone and blood can take place because of this active exchange. In this type of reaction the ions participating in the exchange are present on the surfaces of the bone salt crystals. (2)

It is well established, that most of the injected calcium is carried by the blood to the bone, but the extent to which organs or secretions may influence the removal of calcium from the circulation is poorly understood. (13)

F. Calcium Balance.

The calcium content of the serum is dependent on the balance between how much enters and how much leaves the blood stream.

Calcium enters by two sources; from the intestine by absorption, and from the skeleton by resorption. Calcium leaves the plasma by excretion in the urine, in the feces (being derived from intestinal juice calcium which may remain unabsorbed) or it may be deposited in bone salt. (7)

The normal total serum calcium level is 10 mgm.%, or 2.5 mM/liter. Some of this is bound to protein, and the rest, 6.5 mg. per cent (1.62 mM/l) is ultra-filtrable and freely diffusible across the normal capillary membrane. Of this diffusible calcium, a small fraction is in the form of soluble complex ions. The ionized calcium is estimated at 1.33 mM/l (Fig. 1A)

The relationship of calcium to plasma protein has been incorporated in a formulation that describes the plasma as a solution of a partially ionized electrolyte, calcium proteinate, the ionized and unionized fractions being in

equilibrium with each other.

G. Homeostatic Regulation of Plasma Calcium.

The calcium in solution in the plasma is in constant exchange with the calcium of extracellular fluid of the bones. This exchange is present at all levels of calcium ion concentration in the fluids of the body.

The parathyroid glands function to maintain the constancy of the calcium concentration in the plasma at 10 mg.% (2.5 mM/liter).

Experiments of Copp et al show that turnover of calcium in blood and bone may occur independently of parathyroid activity.

So, whatever may be the true chemical and physical nature of the skeleton, some of the calcium present in the skeletal tissues is available for mobilization in time of need, and some of the skeletal tissue is available to store excess calcium. The parathyroids are a dominant factor in the ability of bones to provide calcium to the extracellular fluids when necessary.

McLean makes a distinction between :

(1) A mechanism acting in one direction only, i.e. mobilization of calcium from the bones under the influence of the parathyroid glands.

(2) The transfer of calcium between blood and bone, in both directions, independent of parathyroid function.

This is thought of as a dual mechanism, one part acting by diffusion equilibrium between the plasma and the labile fraction of the bone mineral. The parathyroid hormone causes destruction of both the mineral and organic components of bone.

According to his interpretation, the labile fraction of the calcium of the mineral is easily accessible to ionic exchange with the fluids of the body. On the other hand, the stable fraction of calcium does not dissolve readily and requires the action of the parathyroid hormone for its liberation. (20)

There has also been found a relationship between citrate and calcium metabolism, which indicates that the metabolic reactions which influence the calcium of the body can affect the accumulation of citrate in the tissues. The bone is relatively rich in citrate which cannot be eluted from the bone by water. This citrate is thought to be held on the surface of the bone crystals by virtue of its property of complexing with calcium.

In vitro studies have shown the presence of citrogenase and aconitase activity in bone tissue, and it has been suggested that citrate accumulation in the skeleton could result from metabolic activity of bone cells.

If serum is supersaturated, some cellular mechanism must be postulated by which this supersaturated state can be maintained. (12)

H. The Kidneys in Calcium Homeostasis.

Normally more than 99% of the filtered calcium is reabsorbed by the renal tubules. In experiments of Poulos, where calcium chloride and calcium gluconate infusions were carried out, tubular reabsorption increased nearly in proportion to the increased rate of delivery of calcium into the glomerular filtrate, and no tubular reabsorptive maximum was observed. (24)

Wolf performed experiments where dogs received steady intravenous infusions of calcium chloride or calcium gluconate for five hours. There was no effective threshold for retention of calcium, and plasma concentrations of this ion are apparently not regulated extensively by renal function. (26)

I. Plan for Experiments.

1. The physiological significance of processes governing the rapid interchange between the bone crystals and the circulating fluids.

The extent to which the available skeleton mineral can participate in buffering against shifts in the calcium composition of the extracellular fluids is poorly understood. The importance of the skeleton in the homeostatic regulation of the calcium-ion concentration in the blood was investigated. This was accomplished by estimating acute storage of calcium or acute mobilization of calcium by bone, after intravenous injections of calcium gluconate or EDTA. These injections were performed under normal conditions, and in dogs following

removal of the thyroid and parathyroid glands. Intravenous injections were given following removal of the kidneys, or following ligation of the ureters to compare the removal of injected calcium with that of the normal animal. In other experiments the excretion of calcium in the urine was measured during calcium gluconate infusions. The effect of calcium infusions in raising the bone-blood calcium equilibrium or increasing skeletal activity was also studied.

Arterial, venous, and marrow samples were simultaneously taken during calcium gluconate or EDTA infusions to demonstrate the difference in plasma calcium concentration.

Methods for calculating the following are outlined (in the appendix).

(1) the rate of acute calcium storage in bone.

(2) the rate of mobilization by bone.

(3) bone-blood flow.

(4) the amount of calcium in various extracellular fluid compartments.

(5) the volume of various extracellular fluid compartments.

(6) the amount of available calcium in bone.

(7) the rate at which calcium is used for bone mineralization.

II. MATERIALS AND METHODS

A. Analytical.

1. Plasma calcium determination with EDTA titration.

The plasma calcium was determined by a rapid titration with EDTA. This determination can be done within ten to fifteen minutes after the blood sample is taken from the animal. The method is a modification of Lehmann's. (18)

0.2 ml. of plasma is diluted with 5 ml. of distilled water. The pH is adjusted to 12 with concentrated NaOH, and ammonium purpurate is used as an indicator. The photometric titration method described by Campbell (—) and Fales (9) was adapted so that a single Klett colorimeter could be used.

Because there is only a small difference in the maximum light absorption of the ammonium purpurate as compared to calcium purpurate, the end point must be determined photometrically, and a monochromatic light source is essential. A second order interference filter with transmission at 500 Lambda is used to obtain this. (6)

To determine serum or plasma calcium the following procedure was employed:

(1) 0.2 cc. plasma is placed in a 10 cc. Klett tube.

(2) 5 ml. distilled water is added.

(3) the pH is adjusted to 12 with concentrated NaOH (1-2 drops).

(4) one drop of caprylic alcohol is added to prevent foaming while mixing.

(5) enough ammonium purpurate is added so the reading for calcium purpurate is about 200 on the Klett colorimeter (2-5 drops).

(6) the mixture is mixed up by a jet of air from a polyethylene tube attached to an aquarium aerator.

(7) the titration is done with 0.01% sodium versenate from a 2 ml. burette.

As dilute EDTA is added, complexing with calcium, the calcium purpurate is changed to ammonium purpurate and the light absorption rapidly decreases. When all of the calcium is complexed with EDTA, any further decrease in light absorption is due to simple dilution of the ammonium purpurate, which occurs very slowly as more versene is added.

The light absorption is plotted against the mls. of 0.01% versene added. The end point is the point of intersection of the steepest slope and the dilution curve (Fig. 1B). Table I shows typical values for a standard. The titration is reproducible within $\pm$ 0.05 mg.% to 0.1 mg.%.

2. Urine calcium determination. (5)

Take urine samples into small Erlenmeyer flasks. Add one to two cc. of concentrated nitric acid and boil until the solution is colourless. Dissolve precipitate by adding 2 - 3 cc. of water.

Add: 1 cc. ammonium oxalate; 1 drop methyl red; 2 cc. 20% acetic acid; 20% ammonium hydroxide until the colour

is salmon pink. Leave overnight.

Then: centrifuge; remove supernatant; wash in 2% ammonium hydroxide; recentrifuge; remove supernatant. Add 2 cc. 20% sulfuric acid and heat on hot plate to 90°C. Titrate with 0.0126 N $KMnO_4$ until the colour is pink.

Standard: Take 2 cc. of 10 mg.% calcium standard into centrifuge tube. Add 1 cc. ammonium oxalate; 1 drop methyl red; 2 cc. 20% acetic acid. Proceed as above.

B. Solutions used:

1. For plasma calcium determination with EDTA titration.

(a) Ammonium purpurate (0.1%).

400 ml. of distilled water are boiled to 200 ml. to remove the oxygen, which oxydizes ammonium purpurate[1], especially in light. 200 mg. of ammonium purpurate are added and the solution is immediately covered with mineral oil, and the flask stored in a cardboard container in the refrigerator. Only 3 or 4 ml. of the solution are required per day for plasma calcium determinations.

(b) Disodium Versenate.

A stock solution of 0.1% sodium versenate is prepared from regular sodium versenate.[2] The 0.1% solution is diluted to 0.01% for plasma calcium determinations.

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1. Calcium indicator reagent, procured from Hagan Corporation, the Buromin Company of Calgon Inc., P.O, Box 1346, Pittsburgh,Pa.,U.S.A

2. Supplied by Beresworth Chemical Company,Framingham,Mass.,U.S.A.

C. Infusion Solutions.

1. EDTA (about 6%).

Disodium ethylene-diamine-tetracetate (EDTA) is a calcium chelating compound. From the physiological point of view, the important characteristic of a chelate is that its ability to ionize in solution is very low. (20) EDTA forms a soluble, non-toxic and very stable complex with calcium at the pH of blood, and removes calcium as far as biological activity is concerned, as effectively as if it were actually extracted from blood. (23) The reaction is immediate and quantitative. When Ca-EDTA was infused intravenously at the same rate as the original EDTA, no effect was observed on titrable calcium level in plasma, in blood pressure, or in the general condition of the animal. (6) A stock solution for infusions of approximately 6.0% is prepared, with the pH adjusted to 7.4% with NaOH. When standardized, one ml. of this solution should chelate 5-7 mg. of calcium. This solution is diluted with 5% glucose in water (Abbott) when infused into an animal.

With the rate of the infusion pump calibrated, the rate of calcium chelation can be controlled to equal 10 mg/kg/hr. or any other rate required.

2. Calcium gluconate.

Some of the calcium gluconate used was obtained in 10 cc. ampoules (No.239, Parke Davis and Co. Ltd.) of 10% calcium gluconate. They were found on analysis to contain

8-10 mg. calcium per ml. This was diluted with 5% glucose in water to the calculated concentration for infusion. (10 mg./kg/hr). Other calcium gluconate solutions were prepared from calcium gluconate powder (about 8% calcium). This powder was dissolved in warm water and warm 5% glucose in water to give a concentration of 5% glucose and 5-7% calcium. This mixture was heated until the powder dissolved. Thus one ml. of solution would contain 5-7 mg. calcium. This was again diluted to give the appropriate concentration for infusion. All of the calcium gluconate infusion solutions were analyzed by the EDTA titration method for their exact calcium content.

3. Parathyroid Extract.

Parathormone was the material used (Parathyroid extract), supplied through the courtesy of E.Lilly and Co. Ltd., and was from Lot #2126 - 678089, expiration date Aug. 1, 1958, and Lot #7148 - 687712, expiration date Aug. 1, 1958.

The extract was prepared from beef glands by the method of Collip. The parathyroid extract was diluted with 5% glucose in water to give the appropriate concentration.

D. Infusion Apparatus.

1. The first type of infusion pump used gives a continuous delivery (Figure 2). It is a "Motor Driven Compensator", produced by the American Instrument Company. A syringe is driven by a constant rate motor, the rate can be

adjusted by the selection of several gears, and different sizes of syringes.

2. The second type does not give a continuous flow although the mls. delivered per hour can be varied to a much greater extent (10-2000 ml./hr). A rubber tube is attached to a piece of flat metal which lies parallel to a cam shaft. The cams on the shaft are set at different angles so that each compresses the tube in succession, in such a manner that the solution is sucked up and pushed through the tubing (Figure 3).

E. Blood Sampling Apparatus.

2 ml. blood samples were taken with dry or moist heparinized 5 ml. syringes, so that no clotting or dilution of plasma would occur. The blood was delivered from the syringes into dry heparinized hematocrit tubes through pieces of dry polyethylene tubing. The tubes were centrifuged for 10-15 minutes and the plasma removed with a Pasteur pipette.

F. Animal Procedures.

Most of the animals used were adult mongrel dogs, fasted overnight before use. They were anaesthetized and maintained under anaesthetic with nembutal during the experiment. Dogs used for survival experiments were given an injection of 1 ml. of penicillin in oil (300,000 units) intramuscularly at the conclusion of the operation. Antiseptic technique was

attempted during experiments. Complete bilateral thyroparathyroidectomy was performed, and these animals were given a maintenance dose of ¼gr. dessicated thyroid every other day. Blood samples were obtained from the jugular vein and arterial samples from the femoral artery or carotic artery. Most of the infusions were carried out through the large superficial veins of the front and hind legs.

Infusions of 5% glucose in water were performed when no other infusion was carried on, to keep the animal well hydrated.

Nephrectomy and ureteral ligation was done using the retroperitoneal approach. The incision was made just in front of the anterior superior iliac spine and extended to a point below the base of the last rib.

The animals were kept warm by blankets and heat lamps.

G. Experimental Procedures.

1. Calcium gluconate infusions.

Calcium gluconate was infused intravenously at a continuous rate of 10 mg/kg/hr, usually for a period of one hour, although duration of infusions range from ½ to 2½ hours. Control samples were taken before the infusion, during the infusion, and for several hours after the infusion stopped (until the plasma calcium returned to normal, or to some new level).

This procedure was carried out on the normal dogs,

thyroparathyroidectomized dogs, nephrectomized dogs, and dogs in which the ureters were ligated. During all of the experiments, blood samples were taken for calcium determination. During several experiments, urine samples were taken for calcium analysis.

Stat (instantaneous) doses of calcium gluconate from 3-25 mg/kg were injected intravenously in thyroparathyroidectomized dogs. Also, long infusions of 9-12 hours at 2-4 mg. calcium/kg/hr were given to control and thyroparathyroidectomized animals.

2. EDTA infusions.

EDTA was injected intravenously at a continuous rate, so that 10 mg. calcium/kg/hr would be chelated, and thus removed from the plasma. The infusion continued ½-1 hr. Blood samples were taken before the infusion, during the infusion, and several hours after the infusion.

3. Calcium gluconate and EDTA infusions during maintenance dose of parathyroid extract.

Immediately after bilateral thyroparathyroidectomy, parathyroid extract was infused at 0.1 units/kg/hr. This appeared to be the maintenance dose of parathormone for the majority of the dogs used, and maintained the blood level for at least 10 hours. EDTA was infused for one half hour at a rate to chelate 5 mg. calcium/kg/hr. Time for recovery was allowed, then 5 mg. calcium/kg/hr. were infused over ½ hour to

replace that calcium removed with EDTA. The maintenance dose of parathormone was simultaneously proceeding with the EDTA and calcium gluconate infusions. The EDTA and calcium gluconate infusions were repeated on the same dog, as above, with additional parathormone in the EDTA and calcium gluconate solutions.

4. Calcium gluconate infusion after a stat dose of parathormone.

A thyroparathyroidectomized dog was given a stat dose of parathormone (10 units/kg.). The plasma calcium rose and levelled off. At this point calcium gluconate was given continuously at a rate of 10 mg./kg./hr. for one hour.

5. Measuring arterio-venous difference of plasma calcium during calcium gluconate and EDTA infusions.

Arterial, venous, and marrow blood samples were taken simultaneously at the end of both calcium gluconate and EDTA infusions. The arterial sample was taken from the femoral artery or the carotid artery; venous sample from the jugular or femoral vein; and the marrow blood sample from the femurs.

III. RESULTS

A. Calcium gluconate and EDTA infusions.

1. In the normal animal.

(a) In over 200 control determinations in 62 dogs, the mean control level of plasma calcium was found to be 10.08 mg.% (standard deviation ± 0.27 mg.%; standard error 0.03 mg.%).[1] By the end of the calcium gluconate infusion, the plasma calcium had risen to 12-15 mg.%. After the infusion was stopped, the plasma calcium returned to the control level or to some new level, above the control level. (Fig. 4 & 5).

An irregularity in the descending curve as shown in Fig. 5 occurred in most of the animals. The overall rapid fall or loss of calcium from the circulation is due to the disappearance of calcium from the arterial plasma into the bone. Thus circulatory mixing is complicated by the fact that the injected calcium does not remain within the blood system. The irregular rises in the descending curve might be explained by the return of calcium to the central circulation from extra-skeletal areas of the body, such as the viscera. (17) Fig. 4 shows the rapid return of the blood calcium to the control level. In Fig. 5 the plasma calcium returned to a new level, representing a rise in the equilibrium between bone

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[1] Statistical analysis by Dr. L.W.E. Flather in summer of 1957.

and blood.

(b) Comparison of calcium gluconate and EDTA infusion curves with the infusion sequence (Fig. 6 & 7).

The blood calcium returned to the control level after both infusions in Fig. 6. In Fig. 7 the bone blood equilibrium has been lowered after EDTA infusion, presumably due to the initial infusion of calcium gluconate.

(c) Calcium gluconate was infused at a rate of 4 mg./kg./hr. for 9.5 hours, into a normal dog. The plasma calcium level rose above the control level, then levelled off for several hours. The plasma calcium level fell promptly to a little below control after the infusion was stopped. (Fig. 8). The calcium excreted in the urine was 5.26% of the calcium infused intravenously. The urine was collected by canulation of one ureter.

2. Effect of calcium gluconate infusions in thyroparathyroidectomized dogs.

Calcium gluconate was infused at 10 mg./kg./hr. for different time intervals. (Fig. 9). The equilibrium level rose in proportion to the total amount of calcium infused. The first infusion was a total of 5 mg./kg; the second 10 mg./kg; and the third a total of 25 mg./kg. (see also Table X).

In one parathyroidectomized dog the plasma calcium

level returned rapidly to the pre-injection level after calcium infusion. (Fig. 10). In all other dogs under the same conditions, the plasma level came back to a new and higher level, and then fell at a very gradual rate.

Calcium gluconate was also given in single or stat doses of 5-20 mg./kg./hr. The plasma calcium level rose sharply after the injection and then fell to a new equilibrium level (Fig. 11).

Calcium infusions at slow rates of 1-3 mg./kg./hr. were done on thyroparathyroidectomized dogs (Fig. 12). The plasma calcium rose immediately after the calcium infusion started. The rise was not linear, and the plasma calcium fell immediately after the infusion was stopped. The time index (abcissa) in Fig. 12 was shortened, and the rates of disappearance of calcium from the blood stream during and after the infusion were calculated from the resulting curve. (Fig. 13)

3. When calcium gluconate was infused after nephrectomy, the plasma calcium rose and then fell to a new level above control, which was maintained for a few hours. There was no immediate gradual fall in the new calcium level as soon as an equilibrium was reached. The mobilization curve was similar to those obtained in a normal dog. (Fig.14)

4. Immediately after ureteral ligation, and in some cases both thyroparathyroidectomy and ureteral ligation, calcium gluconate and EDTA were infused. (Fig. 15) The curves were

similar to those obtained in a normal dog. Thus, disappearance of injected calcium from the blood stream in this experiment cannot be attributed to excretion in the urine.

5. Calcium gluconate and EDTA infusions of one half hour duration each were carried out during a maintenance dose of parathyroid extract (0.1 units/kg./hr. in experiment presented). The plasma calcium did not return to normal after the EDTA infusion. Thus the maintenance dose of parathyroid extract is enough to maintain the blood calcium at the normal level when the parathyroids are removed, but is not enough to bring the blood calcium back to the normal level after removal of calcium with EDTA. The amount of calcium that was removed from the blood stream during the one half hour infusion of EDTA was replaced by a one half hour infusion of calcium gluconate. This restored the plasma calcium level to normal. Additional parathyroid extract in the EDTA solution restored the plasma calcium closer to normal. (Fig. 16)

B. Urine calcium excretion.

The method for analysis was not accurate in the majority of experiments. Urine was collected by catheterization in several experiments, and small portions of urine were spilt while ashing. More accurate urine analysis was accomplished when the urine was collected by canulation of one ureter. The ashing method was also improved. But adequate allowances

were made for these sources of error, and the determination is expressed as an approximation. The accurate analyses are indicated in the tables (accurate). Tables II and III present urine calcium excretion in the normal dog and in the parathyroidectomized dog. Since not much more than 5% of the injected calcium was excreted in the urine, very little disappearance of injected calcium from the blood stream could be attributed to excretion in the urine.

C. Results showing the difference between arterial, venous and marrow blood samples during calcium gluconate and EDTA infusion. (Fig. 17)

Prior to the calcium infusion, the plasma calcium was 5 mg.%. The plasma calcium was in equilibrium with the calcium on the surface of the bone crystals, and the extracellular fluid around it. The plasma calcium was suddenly raised by calcium infusion, so that it was no longer in equilibrium with the labile bone calcium. Eventually this equilibrium was re-established at a higher level, due to storage of calcium in the bone storage pool. Thus at the end of the infusion, the carotid artery blood sample (assumed to be mixed arterial sample) had more calcium than the marrow sample, and also more than the femoral vein blood sample, which had just returned from the bone.

In other experiments the difference in plasma calcium between the femoral artery, femoral vein, and jugular vein was measured, during calcium gluconate and EDTA infusions. (Fig.18)

Five dogs showed similar results.

During EDTA infusions the femoral vein sample had the highest plasma calcium because it had just returned from the long bones of the leg where calcium mobilization was taking place. The jugular vein sample was lower since the blood was coming chiefly from the brain. The arterial sample was the lowest for it contained a mixture of blood from all parts of the body. The reverse was true during calcium gluconate infusions.

D. Methods for calculating calcium storage and mobilization by bone; bone blood flow; extracellular fluid spaces; calcium storage pool in bone; and rate at which calcium is used for bone mineralization, are found in the appendix.

The summary of the results is presented in the discussion.

IV. DISCUSSION

A. Acute Storage by Bone.

When calcium is injected intravenously, it disappears rapidly from the blood, moving into bone and extracellular space. The assumption is made that the blood passing through the bone in contact with bone mineral is restored to its pre-injection level during one passage. (Fig. 19) On the basis of this assumption, the rate at which calcium is stored in bone, and the bone blood flow may be estimated by the methods given in the appendix, p. 40 . By these methods, the calcium storage may be calculated in mg./min. for various plasma calcium levels. The storage rate was found to be directly proportional to the increase in plasma calcium above the control level, for when each storage rate is divided by the increase in the plasma calcium level, a constant is obtained. This constant is expressed in mg. Ca/min./mg.% rise in the plasma calcium. (See Appendix for details on calculations.)

The first part of the curve in Fig. 23 shows a rise during the infusion, and then a rapid fall at the end of the infusion to the previous level, or a new equilibrium level. This represents the rapid movement of the injected calcium into interstitial space and bone. The rate of this movement is directly proportional to the bone blood flow measured as % of cardiac output. The bone blood flow ranged from 3-8% of the cardiac output in 14 adult dogs. 80% ranged between 5-8% of

the cardiac output. (Table IV) The second part of the curve which shows a more gradual slope, represents the fall of the new equilibrium level between the blood and the labile portion of bone. The rate of this fall depends on the rate of net removal of calcium. (accretion or irreversible uptake in bone mineralization, less bone resorption).

The injected calcium is chiefly stored in bone, since only a relatively small proportion (5% or less) is excreted in urine. This increase in stored calcium may increase the equilibrium level.

Two kinds of storage curves are shown in the normal and parathyroidectomized dogs. (Figs. 4 and 5) The plasma calcium level returns to the normal control level in some dogs, and in others it returns to a new and higher level. This difference may depend on the rate at which calcium is leaving the plasma to be used for bone mineralization. The difference may also depend on the size of the calcium storage pool in bone. This has been expressed as a ratio of bone storage calcium/ extracellular fluid calcium. If the calcium in the bone storage pool is large enough in comparison to the extracellular fluid calcium, a rise in the equilibrium between the two, due to storage of calcium, might not be noticeable.

B. Calcium storage compared with bone clearance of Ca-45.

When radiocalcium is injected intravenously, it

disappears rapidly from the blood, moving into interstitial space and bone. The kinetics of the blood disappearance curves have been analyzed for rats, rabbits, cattle and humans. Armstrong found that approximately half the blood calcium in the rat exchanged with calcium in the interstitial fluid per minute, so that the two may be considered a single compartment. There is also very rapid movement of Ca-45 into bone, the limiting factor being bone blood flow. Frederickson, Honour and Copp determined the initial bone clearance of Ca-45 from blood in the rat and obtained a value of 5-8% of the cardiac output. Similar values have been obtained with Sr-90 and P-32. (11)

C. Storage vs. Mobilization.

When calcium is removed from the blood stream by EDTA infusion at 10 mg./kg./hr. a mirror image of the storage curve is obtained, and in most animals the specific mobilization equals the specific storage as defined in the appendix. (Tables IV and V) This indicates an equilibrium between the blood calcium and a labile calcium pool in bone, which can be approached from either the hypocalcemic or hypercalcemic side. It suggests the presence of a labile reservoir in bone available for calcium storage, or calcium release in times of calcium stress. The rate at which mobilization or storage occurs is proportional to the amount of blood coming in contact with the bone mineral or

calcium reservoir (i.e. bone blood flow), and the difference in Ca++ activity between the blood and bone pool. The bone blood flow was calculated by using both calcium storage curves and calcium mobilization curves. The results obtained were almost identical.

By calcium storage calculations BONE BLOOD FLOW

$= 6.46 \pm {}^{*}0.60$ % of cardiac output.

By calcium mobilization calculations BONE BLOOD FLOW

$= 6.37 \pm {}^{*}0.61$ % of cardiac output

*standard error

1. EDTA infusion prior to calcium gluconate infusion (Fig. 6)

If the EDTA infusion is carried out first and followed by a calcium infusion, the rate of calcium mobilization is not significantly different from the rate of calcium storage. (Table VI) When the specific storage and specific mobilization was expressed graphically, the storage and mobilization tended to fall along the same line running through the origin. (Fig.20). Using Dog 7-45 as an example, the results indicate the presence of an equilibrium between the labile fraction of bone and the blood. Whether calcium is removed from the blood stream with EDTA or added by calcium infusion, the equilibrium is regained at the same rate. (Table VII).

2. Calcium gluconate infusion prior to EDTA infusion. (Fig. 7)

There is evidence that hypercalcemia depresses

parathyroid gland function. (8) This in turn will lower the calcium equilibrium between bone and blood. Inversely, a low blood calcium is thought to stimulate parathyroid function, thus raising the equilibrium.

During these experiments, hypercalcemia was produced first and seemed to have some definite effect on the mobilization rate of calcium, and also on the bone-blood equilibrium level. Table VIII shows calculations of calcium storage and calcium mobilization when calcium gluconate was infused before EDTA. If this table is compared with Table VI, no significant difference between the two can be seen.

When mobilization and storage is plotted against the difference in calcium level, the slopes are similar, but in certain animals there is a marked difference in the intercept. (Fig. 21&22) The slope of the line represents the bone blood flow, which should be constant. Most of the graphs in Fig. 21 show the storage line passing through the origin. The mobilization line may cut the x-axis, giving a negative mobilization rate, at 0 mg.% depression in the plasma calcium. This negative mobilization indicates some upset in the normal plasma calcium balance. The rate of this negative mobilization would depend on the rate of net removal of calcium, which is the accretion or irreversible uptake in bone mineralization less bone resorption.

D. Changes in the Bone-Blood Calcium Equilibrium.

Changes in the bone-blood calcium equilibrium occur after calcium infusion. This change was more clearly demonstrated if EDTA was infused after calcium gluconate. (Fig. 22A) The plasma calcium did not return to normal after EDTA infusion in the majority of animals. This also may indicate that hypercalcemia due to the calcium infusion suppressed the parathyroid function. The result was a lowered bone-blood calcium equilibrium or an impaired mobilization mechanism. This lowered equilibrium was noticed in some dogs as early as four hours after calcium infusion. Table IX gives a few examples of changes in the equilibrium.

1. Increasing the skeletal activity of bone by calcium infusion in the parathyroidectomized dog.

Radioactive studies indicate that the reservoir of available calcium in the bone is the calcium ions on surface crystals available to the blood circulation. This reservoir of available calcium or the skeletal activity of bone can be increased by adding calcium to the reservoir. Since an equilibrium is maintained between the calcium in the reservoir and the calcium in blood, any rise in the equilibrium level indicates a rise in skeletal Ca++ activity (a Ca++)

Calcium infusion experiments (continuous and single injections) were done on parathyroidectomized dogs. Results indicated that the increase in the bone-blood calcium equilibrium

level was proportional to the amount of calcium infused, and therefore to the amount of calcium stored in the bone. If mobilization from bone occurred, brought about by EDTA infusion, the equilibrium level dropped. The deficit or drop was proportional to the amount of calcium removed from the blood stream by EDTA, and therefore proportional to the amount of calcium mobilized from the calcium reservoir in bone. Table X shows a relationship between the amount of calcium added intravenously, and the rise in the bone-blood equilibrium. If the rise in the equilibrium level is measured from the beginning of the continuous infusion, there is a slightly higher rise in the bone-blood equilibrium level as compared to the same amount injected in a single dose. Therefore the rise in the equilibrium level was measured from the end of the infusion, for the purpose of calculations. (intercept of extrapolated line at "t", Fig.23)

E. <u>The Size of the Calcium Storage Pool in Bone and Rate at which Calcium is used for Bone Mineralization (see Fig.23)</u>

The total amount of calcium infused intravenously is known. By measuring the rise in the bone-blood equilibrium ("x" mg.%), and knowing the extracellular fluid calcium, the size of the calcium storage pool in bone can be estimated. The slope of the gradual line is used to estimate the net rate at which calcium is lost from blood for bone mineralization. (accretion or irreversible uptake in bone mineralization, less

bone resorption.) The calculations are shown in detail in the Appendix.

Table XI shows the estimated calcium storage pool in bone and the estimated accretion rates of several dogs. Table XII shows results of Bauer, Carlsson and Lindquist in humans. (4) Bauer, Carlsson and Lindquist have also studied the kinetics of turnover of Ca-45 and P-32 in bone, in rats. In the young rats, they estimated for tibia an accretion rate of 6.2% of the bone Ca/day; a resorption rate of 4.7% of the bone Ca/day; and exchangeable fraction equivalent to 3.0% of the total calcium of the bone. (3)

F. Effect of Slow Calcium Infusion.

1. Effect of slow calcium infusion in a normal dog.

When calcium was infused at 4 mg./kg./hr. for 9.5 hours, the plasma calcium rose from 8.5 mg.% to 10 mg.% in approximately an hour, and maintained this level until the infusion stopped. (Fig.8). The calcium excreted in the urine was 5.25% of the total amount injected. That is, approximately 0.2 mg./kg./hr. of the total 4 mg./kg./hr. was excreted in the urine. Since an equilibrium was reached and maintained, a balance of approximately 3.8 mg. Ca/kg/hr. may have been used for net bone mineralization.

2. Effect of slow calcium infusion in a parathyroidectomized dog.

During long calcium infusions in two parathyroidectomized

dogs, the excretion of calcium was not measured, but the plasma calcium did not reach renal threshold. During one experiment, the infusion rate was 2 mg. Ca/kg/hr., and the plasma calcium rose 3.5 mg.% in seventeen hours, then promptly fell when the infusion stopped. (Fig. 12) The time scale was shortened (Fig. 13), and the net loss of calcium from the blood was measured at various plasma calcium levels. The rate of disappearance of calcium was found to be proportional to the rise in plasma calcium. Between plasma calcium levels of 8.2 - 9.2 mg.%, the rate of disappearance of calcium was up to 2.8 mg. Ca/kg/hr. Between plasma calcium levels of 6.2 - 7.2 mg.% the disappearance rate was as low as 0.7 mg. Ca/kg/hr.

Calcium was infused at 3 mg./kg/hr. into the other dog. The plasma calcium level rose 2 mg.% and levelled off. Since the plasma calcium was below renal threshold, the entire 3 mg. Ca/kg/hr. may have been used for net bone mineralization. This falls within the range of estimated rate of bone mineralization. (Tables XI and XII)

G. Calcium Excretion in the Urine.

Very little calcium is lost through the urine normally, for 98-99% of the calcium filtered in the kidney by the glomerulus is reabsorbed by the tubules. The calcium excreted in the urine was measured before and after calcium infusion, in both the intact and parathyroidectomized dogs.

After calcium infusion in the normal dog, approximately

5% of the injected calcium was lost in the urine (Table II), so that only a small fraction of the rate of disappearance of calcium from the blood stream can be attributed to excretion in the urine. The parathyroidectomized dogs showed a calcium excretion of much less than 5% of the injected dose. (Table III)

H. Effect of Ureteral Ligation and Nephrectomy on Removal of Injected Calcium from the Blood Stream.

When the ureters are ligated the urine production is terminated, but the kidney may still carry on its metabolism, which is not the case after nephrectomy, or ligation of the renal arteries. The relation between kidney metabolism and calcium metabolism is considered important. One theory claims that calcium citrate is carried from the bone into the blood pool. As it passes through the kidney, the citrate is metabolized and the calcium is let free into the plasma. Also, the concentration of citrate is thought to have some direct effect on the amount of calcium lost in the urine. After ureteral ligation (Fig. 15) EDTA and calcium gluconate solutions were infused. Both curves were similar to those obtained in the normal animal. All of the infused calcium must have gone into extracellular fluid and the storage pool of bone, since none could escape in the urine. In the nephrectomized dog, after calcium infusion, the plasma calcium returned to a new and higher level, and did not show the gradual fall, but maintained the new level for

several hours. (Fig. 14) This seems to indicate some change in the state of calcium in the circulation, or may be an impairment of the removal mechanism. The change in the state of calcium in the circulation is thought to parallel citrate accumulation after nephrectomy. However, the method of citrate analysis had not been established at the time of these experiments.

I. Effect of Parathyroid Extract on Calcium Storage, Calcium Mobilization, and Bone-Blood Equilibrium Level. (Fig. 16)

A maintenance dose (0.1 units/kg./hr. for this dog) was administered immediately after parathyroidectomy. EDTA and calcium infusions were done during this maintenance dose. The equilibrium level was lower after EDTA infusion, showing that a maintenance dose of parathyroid extract is not sufficient to raise the storage pool of calcium in bone back to the normal level. The equilibrium level was returned to normal with the addition of calcium by infusion equal to that removed with EDTA, thus restoring the storage pool to its previous size. EDTA and calcium infusions were repeated with extra parathyroid extract. (1.0 units/kg./hr. in each infusion solution) Calculations showed no change in calcium storage or calcium mobilization rates with extra parathormone. However, the equilibrium level was restored almost to normal after EDTA infusion. Thus additional parathyroid hormone was required to increase the storage pool in bone to its previous size after calcium was removed by acute hypocalcemia.

V. SUMMARY AND CONCLUSIONS

1. When calcium was injected intravenously, it disappeared rapidly from the blood, moving into bone. Similarly when calcium was removed from the blood stream by EDTA infusion, the calcium was restored rapidly to the blood by mobilization from the bone.

2. The rate at which the calcium was stored by the bone agrees closely with the rate at which calcium was mobilized from bone.

Calcium storage = 1.20 ± 0.11 mg/min/mg% rise in plasma calcium/M^2

Calcium mob. = 1.08 ± 0.10 mg/min/mg% depression in plasma calcium/M^2

This data indicates the presence of an equilibrium between the extracellular calcium, and the labile calcium in bone. The rate at which this equilibrium is regained is proportional to the amount of blood circulating through the bone and coming in contact with the labile calcium fraction of bone.

3. Bone blood flow as measured from calcium infusion = 6.46 ± 0.60% cardiac output

Bone blood flow as measured from EDTA infusion = 6.37 ± 0.61% cardiac output.

This estimated bone blood flow is in general agreement with values derived from clearance studies made with radioactive isotopes.

4. The extracellular fluid calcium was estimated as 15.73 ± 0.72 mg/kg, and the extracellular fluid volume as 203.3 ± 10.4 ml/kg.

5. The quantity of Ca^{++} in the labile fraction of bone could be increased by calcium infusion. This Ca^{++} activity in bone was increased in proportion to the amount of calcium infused, and therefore to the amount of calcium stored in the bone.

The labile calcium storage pool in bone was estimated as 2-5 times greater than the extracellular calcium, or approximately 0.2 - 0.5% of the total bone calcium.

6. The net rate of disappearance of calcium from the blood after calcium infusion was estimated at 1-2 mg Ca/kg/hr., or 0.15 to 0.35% of the total bone calcium per day. These results are similar to values reported for calcium accretion by bone in humans.

VI. APPENDIX

METHODS USED FOR CALCULATING THE QUANTITATIVE ASPECTS OF CERTAIN FACTORS INVOLVED IN REGULATION OF PLASMA CALCIUM.

A. RATE OF CALCIUM STORAGE IN BONE FOLLOWING INTRAVENOUS INFUSION

The calculations below are based on the following assumptions:

(a) It is assumed that there is an equilibrium between plasma calcium and a labile calcium pool in bone adjacent to the circulation.

(b) Since no appreciable increase in soft tissue calcium content of muscle and skin was observed during hypercalcemia (except that due to increase in extracellular fluid calcium), and since less than 5% of the injected calcium appeared in urine, it is assumed that most of the calcium which disappears from blood following intravenous infusion has been taken up by the skeleton.

(c) Since 50-80% of the plasma calcium exchanges across the capillary wall per minute (1), it is assumed that the interstitial and plasma calcium act as a single pool (extracellular calcium pool) with respect to processes occurring over a period of 1-6 hours.

(d) Because of the rapid movement of calcium across the capillary wall and the vast excess of the calcium in the labile bone pool (as compared to the calcium in the bone capillary) it is assumed that the activity of calcium ion leaving the capillary in the bone venules will not differ significantly from that in

the labile bone storage pool.

This situation would be analogous to the unloading of CO_2 in the lungs, and it would be anticipated that the amount of calcium taken up by bone would then be proportional to blood flow and the A-V difference.

i.e. Calcium uptake by bone in mg/min = k (bone plasma flow in ml/min) x (A-V)

where A and V are the arterial and venous concentrations of plasma calcium in mg/ml. A is obtained from the arterial or mixed blood sample; V is assumed to correspond to the plasma in equilibrium with the labile bone storage pool.

In some animals, this bone-blood equilibrium appears to correspond to the pre-injection plasma calcium level; in others, the level is raised or lowered during the infusion.

To test this hypothesis, the rate of Ca storage or mobilization has been determined as described below, and plotted against the A-V difference in calcium level as defined above. In almost all cases a linear relationship is observed, confirming the hypothesis. The slope gives a clearance value which, according to Fick Principle, should be the functional bone blood flow. This blood flow may be expressed directly; in terms of body surface area; or as a per cent of the average resting cardiac output for a dog of the same surface area.

METHOD OF CALCULATION (Fig. 24)

1. SPECIFIC CALCIUM STORAGE AND MOBILIZATION

(a) An equilibrium line representing the assumed bone-blood equilibrium is drawn (usually 10 mg% in the normal dog). Several lines at each 0.5 mg% level or 0.25 mg% level are drawn above the equilibrium line.

(b) For each level the following time intervals are measured:

t_{Ca} = time of infusion (A M)

t_R = time of recovery to same level (M C)

$t_{Storage}$ = time of storage = $t_{Ca} + t_R$ (A C)

The storage rate may be calculated using two assumptions:

(1) Extracellular calcium at A = extracellular calcium at C.

(2) Mg calcium stored in $t_{Storage}$ = calcium infused in t_{Ca}

Since the infusion rate was 10 mg/kg/hr.

$$\text{the Storage rate} = \frac{t_{Ca} \times \frac{10 \text{ mg}}{60 \text{ min}} \times \text{body wt.(kg)}}{t_{Storage}} = \text{mg/min}$$

The storage rate of calcium can be calculated in mg/min by the above method for any plasma calcium level above the equilibrium calcium level. When this storage rate is divided by the A - V difference in plasma calcium, a constant

is obtained. If the storage rate is plotted against the A - V difference, a straight line running through the origin is usually obtained. (Fig. 20) The <u>average A - V difference</u> in plasma calcium is calculated by the following method:

(a) The area BAC is divided by $t_{Storage}$ to determine the <u>average plasma Ca level</u> over the period A C.

(b) Thus the <u>average A - V difference (mg%)</u> over the period of A to C = average plasma Ca level (over A to C) minus the control equilibrium calcium level

When there is a rise in the bone-blood equilibrium level, the <u>A - V difference in plasma calcium</u> is determined as following:

An assumption is made that the dotted line drawn in free-hand (Fig.25) corresponds to the Ca++ of the venous blood returning from the labile calcium pool in bone.

Area PQRS is divided by AC. The answer in mg.% is added to the SR mg.% level. This is subtracted from the average plasma calcium level during AC.

Thus the <u>Calcium Storage Clearance</u> (in mg/min/ A - V diff.Ca++ conc.)

$$= \frac{\text{Storage rate (mg/min)}}{\text{Average A - V diff. } Ca^{++} \text{ conc.}} = \text{CONSTANT}$$

If this ratio is expressed graphically, a straight line through the origin is usually obtained.

The SPECIFIC CALCIUM STORAGE CLEARANCE is determined by dividing the CONSTANT by the surface area of the animal.

2. BONE BLOOD FLOW

The bone blood flow may be determined by multiplying the CONSTANT by 100, and expressed as mls/min.

The bone blood flow may also be expressed as per cent of the resting cardiac output by the following fraction:

$$\frac{\text{Specific Calcium Storage Clearance}}{\text{Cardiac Index (for plasma)}}$$

The cardiac index for plasma = 1.6 liters/M^2 (25)

3. THE EXTRACELLULAR CALCIUM SPACE

The extracellular calcium space is estimated by the following method:

(a) The average A - V plasma Ca difference (mg.%) is determined by dividing area BDE by the total time of infusion.

(b) The calcium stored during the infusion
= the A - V plasma Ca difference during Ca infusion x the average storage (mg/min/mg.% A - V diff.) x infusion time (min.)

(c) The net addition of calcium to the extracellular pool will equal the calcium added during t_{Ca} minus the calcium stored during t_{Ca} .

The assumption is made that there is a linear relationship between the increase in plasma calcium and extracellular fluid

calcium during the infusion. The net addition of calcium to the extracellular fluid pool is therefore responsible for the rise in the plasma calcium at the end of the infusion.

Thus the total extracellular calcium (mg/kg)

$$= \text{Net addition of calcium during the infusion} \times \frac{\text{control plasma Ca level}}{\text{rise in plasma calcium by the end of the infusion}}$$

(d) Assuming the plasma calcium is 5 mg/kg (5% body wt. at 10 mg.%) the calcium in the interstitial fluid may be calculated.

Thus interstitial fluid Ca = total extracellular fluid calcium - 5 mg/kg

(e) Assuming the interstitial fluid is 7 mg.%, the interstitial fluid volume may be calculated.

Thus the interstitial fluid volume = $\frac{\text{interstitial fluid Ca}}{7 \text{ mg.}} \times 100$ ml.

(f) The extracellular fluid volume = interstitial fluid volume + plasma volume

4. EXAMPLE OF THE ABOVE CALCULATIONS(Fig. 26 and Table XIII)

Dog 7-17 is used as an example. The dog's weight was 20.86 kg; the surface area was 0.765 M^2. The control calcium level (determined over a one and one-half year period) was 10.3 mg.%. Calcium was infused for one hour at a rate of 212.5 mg. of calcium per hour. (10.17 mg/kg/hr.)

(a) Determination of Specific Calcium Storage

(1) The equilibrium line was drawn at 10.3 mg.% calcium

(2) Lines were drawn at each 0.5 mg.% level (i.e. 14.0, 13.5, 13.0, 12.5, 12.0, 11.5 mg.%)

(3) For each level t_{Ca}, t_R, and $t_{Storage}$ were determined.

e.g. at plasma calcium level of 14.0 mg.%

t_{Ca} = 4 min., and $t_{Storage}$ = 6 min.

Infusion rate of calcium was 212.5 mg/hr. = 3.55 mg/min.

Therefore storage rate at 14.0 mg.% =

$$\frac{4 \text{ min.} \times 3.55 \text{ mg.}}{6 \text{ min.}} = 2.365 \text{ mg/min.}$$

(4) Determination of average A - V plasma calcium difference

The average plasma calcium level above 14.0 mg.%

$$= \frac{17}{6} \times 0.05 = 0.1415 \text{ mg.\%}$$

$$14.0 + 0.1415 = 14.1415 \text{ mg.\%}$$

The average A - V plasma calcium difference

= 14.1415 - 10.3000 = 3.8415 mg.%

(5) The calcium storage clearance

$$= \frac{2.365 \text{ mg/min.}}{3.842 \text{ mg.\%}}$$

$$\text{The Specific Calcium Storage clearance} = \frac{.666}{.786 \text{ M}^2}$$

$$= .870 \text{ mg/min/mg.\% A - V diff./M}^2$$

(b) Estimation of Bone Plasma Flow

$$\text{Bone plasma flow} = \frac{2.365}{3.842} \times 100 = 61.3 \text{ ml/min.}$$

$$\text{or bone plasma flow} = \frac{.870}{1.6} = 5.43\% \text{ cardiac output}$$

(c) Estimation of Extracellular Calcium Space

(1) $\frac{2383}{60} \times 0.05 = 1.96$ mg.%

(2) Calcium stored during infusion = 1.96 x 0.666 x 60 min.

= 78.6 mg.

(3) Net addition of calcium to E.C.F. pool

= 212.5 mg. - 78.6 mg. = 133.9 mg. = 6.43 mg/kg

(4) Total extracellular calcium pool = $6.43 \times \frac{10.30}{4.00}$

= 16.55 mg/kg.

(5) Plasma calcium = 5 mg/kg.

(6) Interstitial fluid calcium = 16.55 - 5.0 = 11.5 mg/kg.

(7) Interstitial fluid volume = $\frac{11.5}{7} \times 100 = 164$ ml/kg

or

Interstitial fluid volume = $\frac{164}{10}$ = 16.4% body weight

(8) Total extracellular fluid calcium space

= 16.4% + 5% = 21.4% body weight

B. ESTIMATION OF LABILE CALCIUM STORAGE POOL IN BONE

When the plasma calcium level is raised by intravenous infusion most of the excess calcium is taken up by bone, and there is a rapid fall to a new equilibrium level. It is assumed that at this time, the calcium in plasma is once more in equilibrium with the labile bone storage pool. In most cases (and invariably in the parathyroidectomized dog) this new equilibrium level is above the original control value. At this new level, some of the injected calcium will be accounted for by the increase in extracellular calcium corresponding to the higher plasma calcium level; however, most will be accounted for by storage in bone. Assuming that the increase in extracellular calcium and storage pool calcium is directly proportional to the increase in the plasma calcium level, it is possible to estimate the dimensions of the latter in the following manner:

Assuming an extracellular calcium pool of 15 mg/kg at 10 mg.% plasma calcium, an increase of 1 mg.% will be associated with an increase in 1.5 mg/kg in the extracellular calcium. Since the quantity of calcium stored is known, the difference will represent calcium taken up by bone. If it is assumed that this is all in the labile storage pool, and that the increase is directly proportional to the increased plasma calcium, the size of this pool can be estimated, and equals

$$\frac{\text{Ca injected - increased Ca in extracellular fluid}}{\text{NEW equilibrium plasma Ca(mg.\%) - control plasma Ca(mg.\%)}} \times 10$$

This may be expressed as mg. Ca/kg or as a per cent of the total bone calcium.

METHOD OF CALCULATING THE NET LOSS OF PLASMA CALCIUM AFTER CALCIUM INFUSION

The net loss of calcium from the plasma is assumed to equal the rate at which calcium is used for bone mineralization less calcium released by resorption. The rate of the fall in the equilibrium level after calcium infusion is used to estimate this net loss. From the above calculations, the slope of the equilibrium line is used to measure the net loss of calcium in mg/kg/hr., or as % of total bone calcium per day.

EXAMPLE OF CALCULATIONS (Fig.23)

1. Estimation of labile calcium storage pool in bone

Dog 7-07 is used as an example. The dog had been parathyroidectomized several days.

(a) 9.4 mg. Ca/kg were infused.

(b) Rise in equilibrium level was 1.2 mg.%

(c) 1 mg.% rise in equilibrium level represents 7.75 mg. Ca/kg added intravenously.

(d) The extracellular calcium in Dog 7-07 was 17.5 mg. Ca/kg
(10 mg.% Ca in blood represents 17.5 mg.Ca/kg in extracellular fluid)
(1 mg.% Ca in blood represents 1.75 mg.Ca/kg in extracellular fluid)

(e)

Then 7.75 mg/kg
\- 1.75 mg/kg
6.0 mg/kg

The labile calcium storage pool in bone
= 6.0 mg/kg x 10 = 60 mg/kg

(f) Ratio of $\frac{\text{bone storage calcium}}{\text{E.C.F. calcium}} = \frac{60}{17.5} = 3.4$

That is, the bone storage pool or the amount of available calcium in bone is 3.4 x greater than the E.C.F. calcium.

(g) Assume calcium in bone is 15 gm/kg (19)

The labile calcium storage pool = $\frac{60 \times 100}{15000}$ = 0.40% of the total bone calcium

2. Estimation of net loss of calcium from the plasma

(a) Fall in the equilibrium level = $\frac{5.65 \text{ mg.\%} - 4.40 \text{ mg.\%}}{5.0 \text{ hours}}$

= 0.25 mg.%/hr.

(b) 1 mg.% rise in equilibrium level represents 7.75 mg.Ca added to blood.

0.25 mg.% fall in equilibrium level represents 1.94 mg.Ca/kg/hr. removed from the blood.

(c) $\frac{1.94 \times 24}{15000} \times 100$ = .31% of the total bone calcium/day.

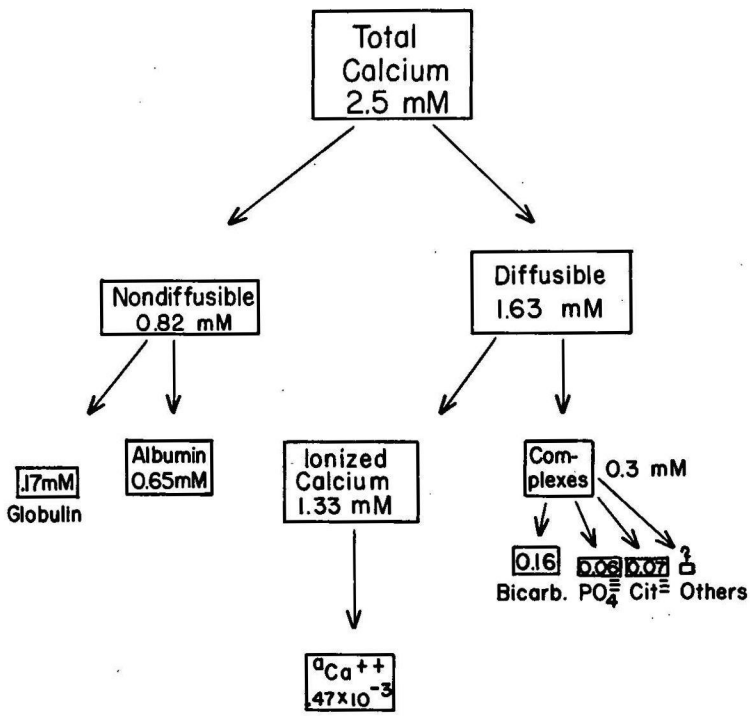

Figure 1A. The state of calcium in normal serum as calculated from ultrafiltration data and formation constants (22)

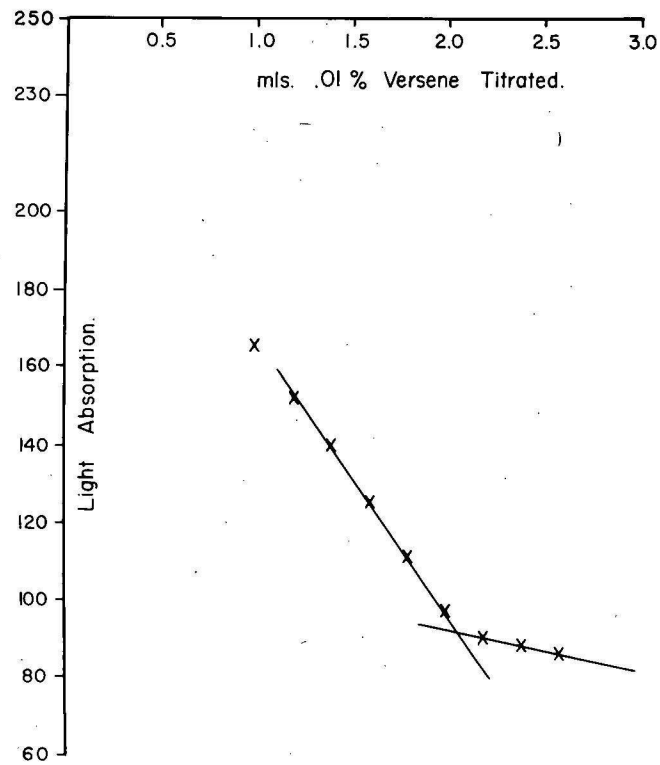

Figure 1B. Photometric titration of calcium using Klett Colorimeter and 500 Lambda Filter. (values taken from Table I)

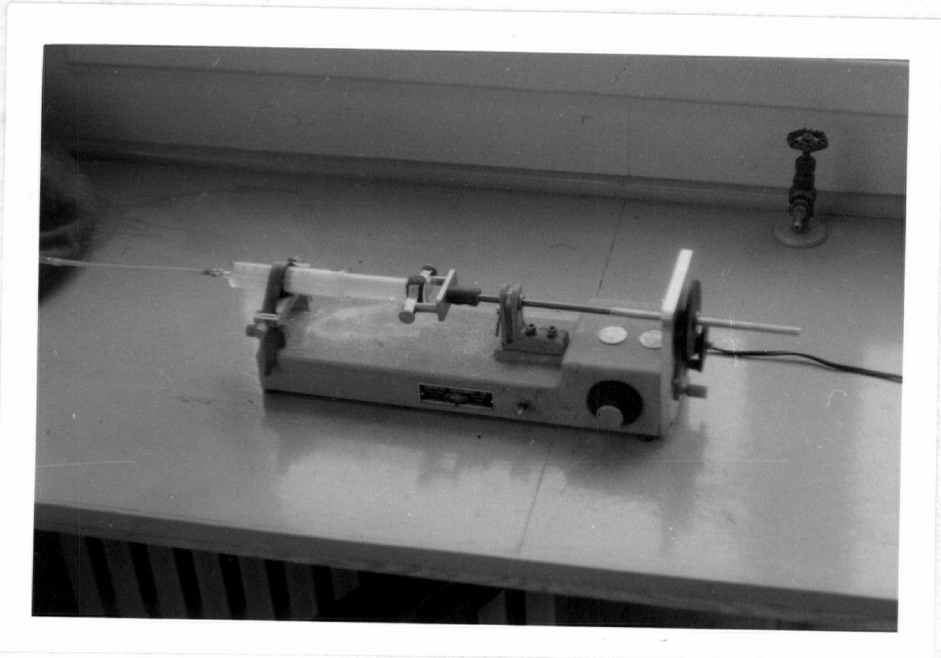

Figure 2. Continuous delivery infusion machine.

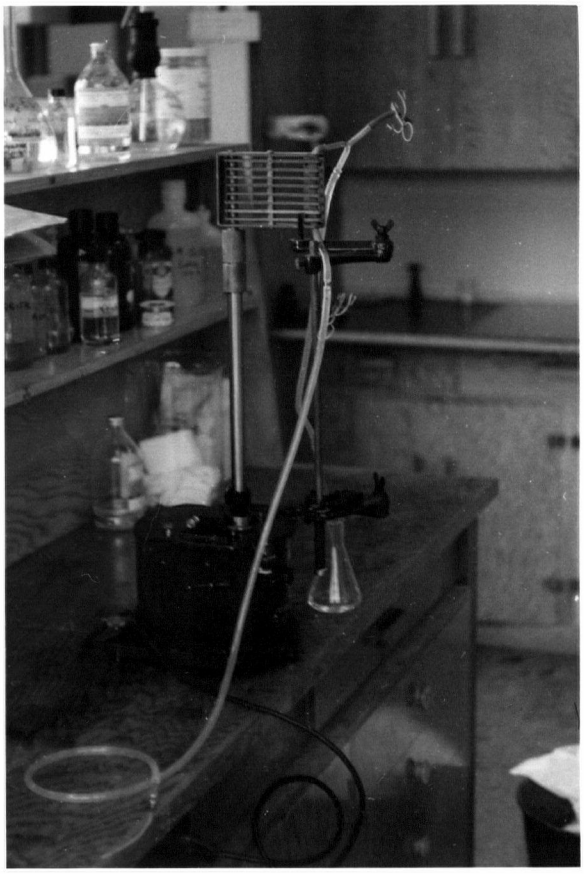

Figure 3. Kymagraph infusion machine.

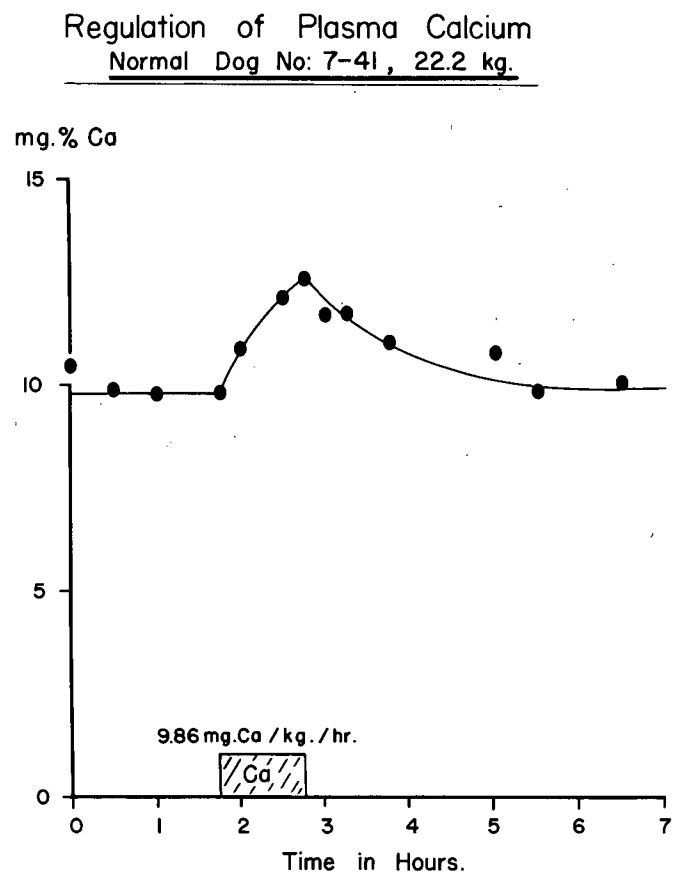

Figure 4. Calcium gluconate infusion in a normal dog, showing a constant bone-blood equilibrium level.

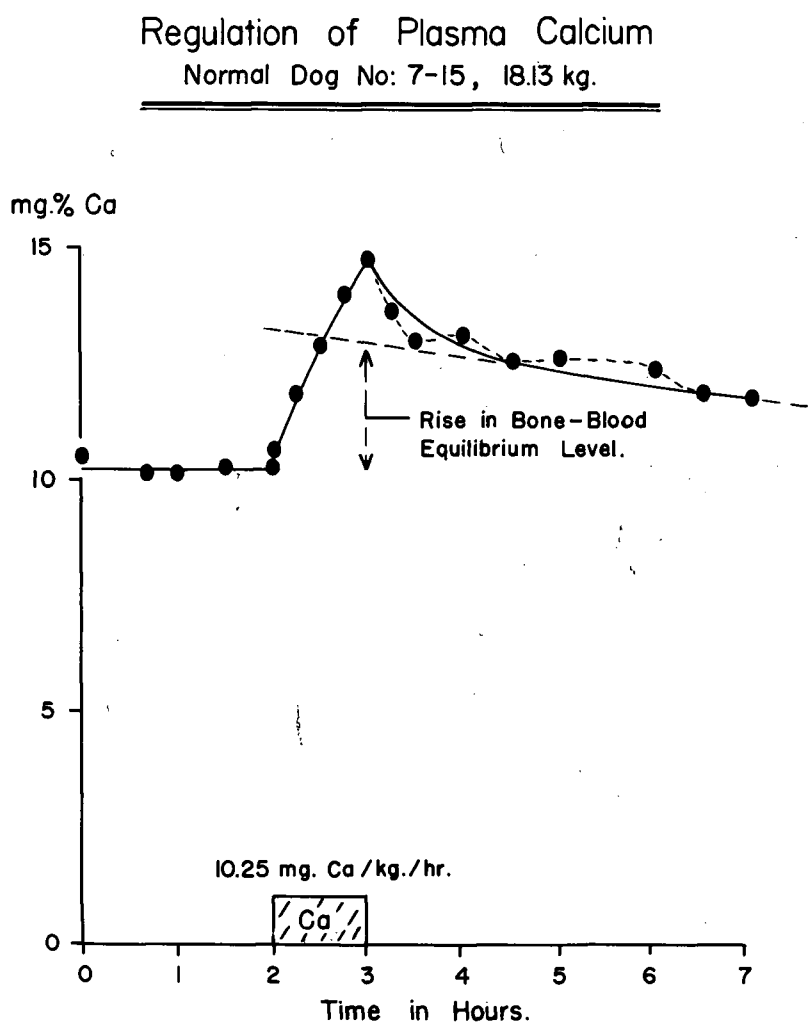

Figure 5. Calcium gluconate infusion in a normal dog, showing a rise in the bone-blood equilibrium level

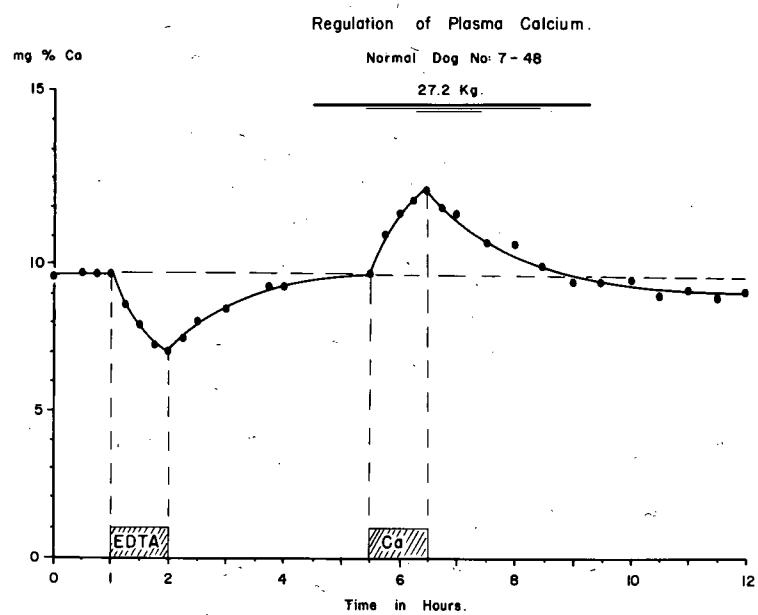

Figure 6. Calcium Regulation. Effects of EDTA and Ca infusion when EDTA is infused prior to Ca.

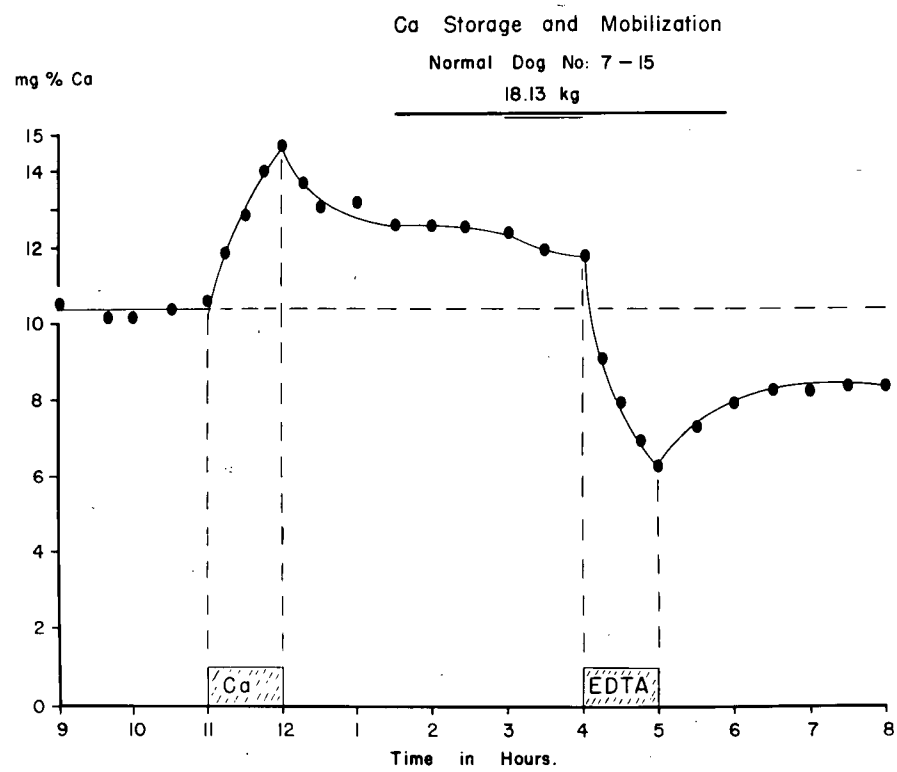

Figure 7. Calcium Regulation. Effects of Ca and EDTA infusion when Ca is infused prior to EDTA.

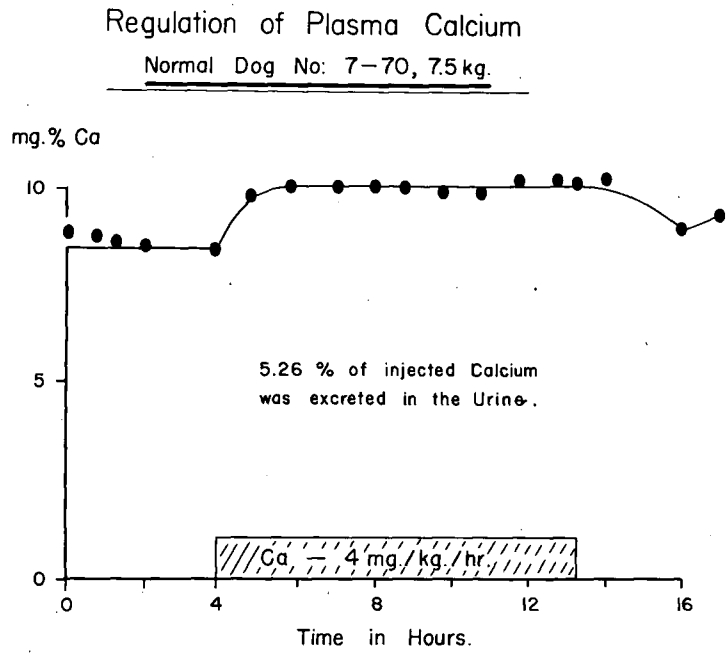

Figure 8. Calcium Regulation. Effects of slow calcium infusion in the normal dog.

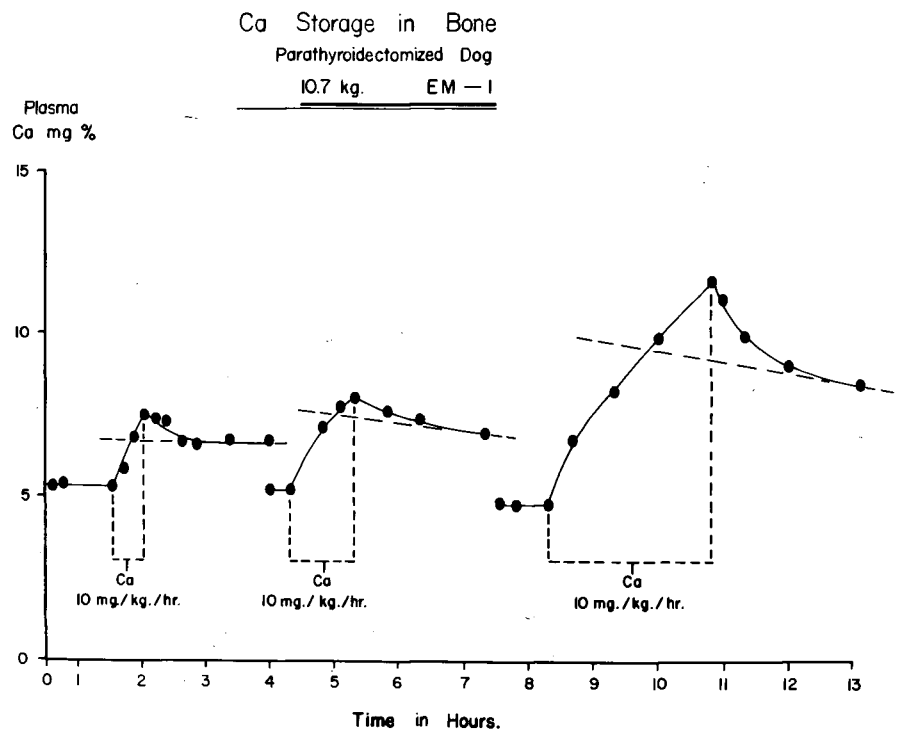

Figure 9. Effect of calcium infusions in parathyroidectomized dog. The bone-blood equilibrium rose in proportion to the total amount of calcium infused.

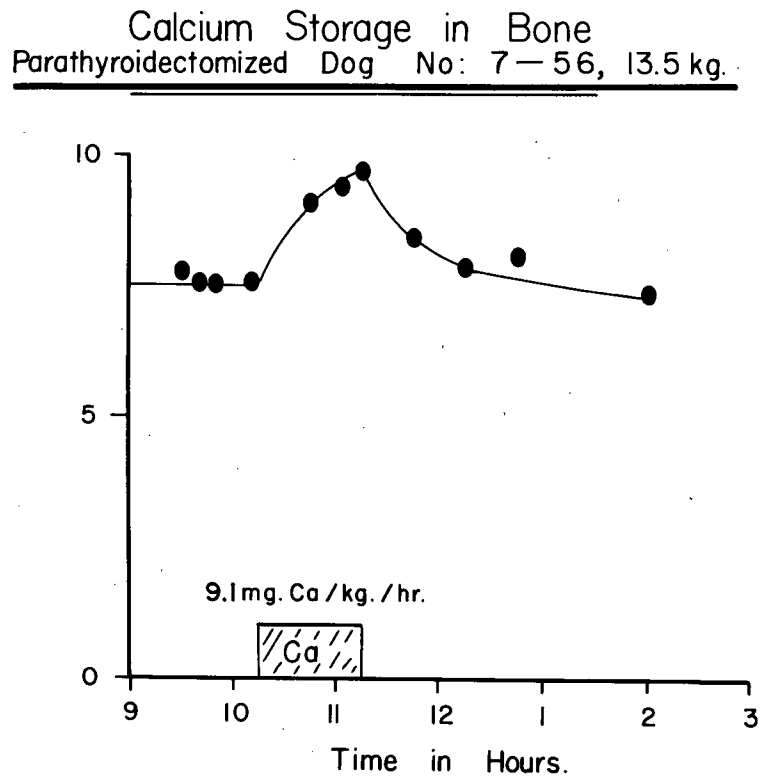

Figure 10. Effect of calcium infusion in parathyroidectomized dog. There is no rise in the bone-blood equilibrium level.

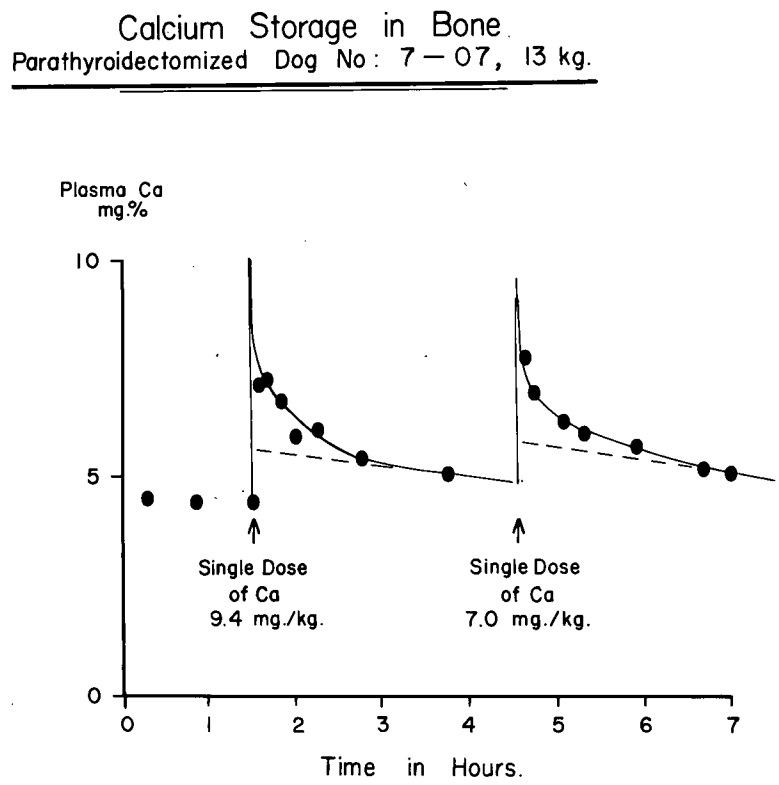

Figure 11. Effect of single injections of calcium in parathyroidectomized dog.

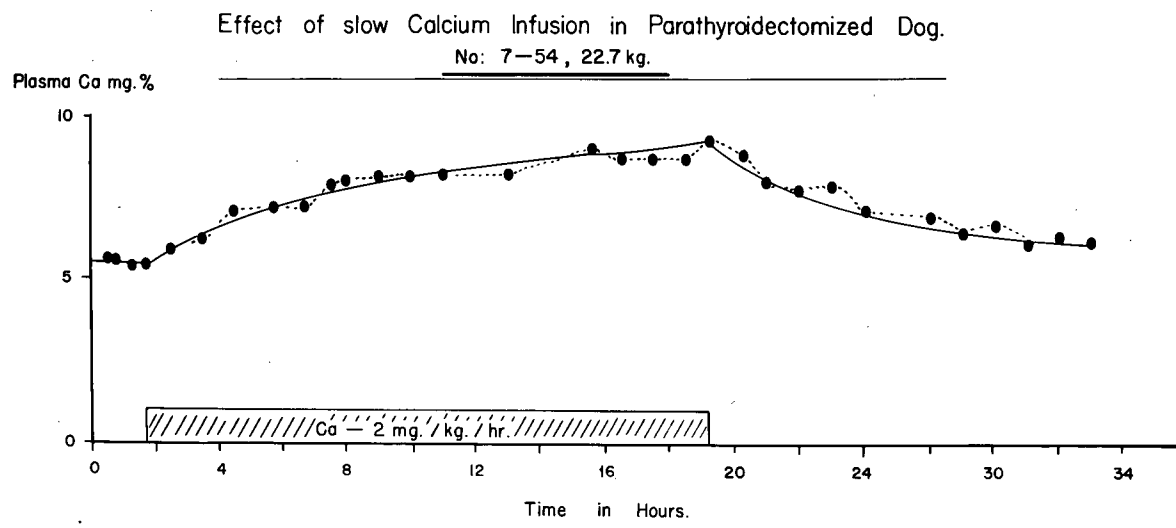

Figure 12. Effect of slow calcium infusion in a parathyroidectomized dog.

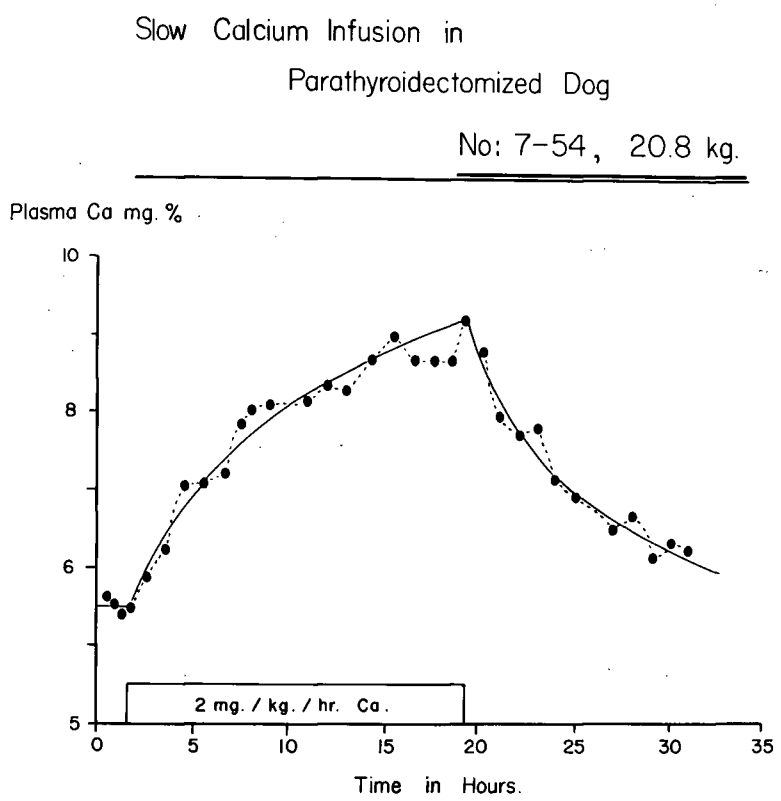

Figure 13. Effect of slow calcium infusion in a parathyroidectomized dog (Figure 12 - same experiment)

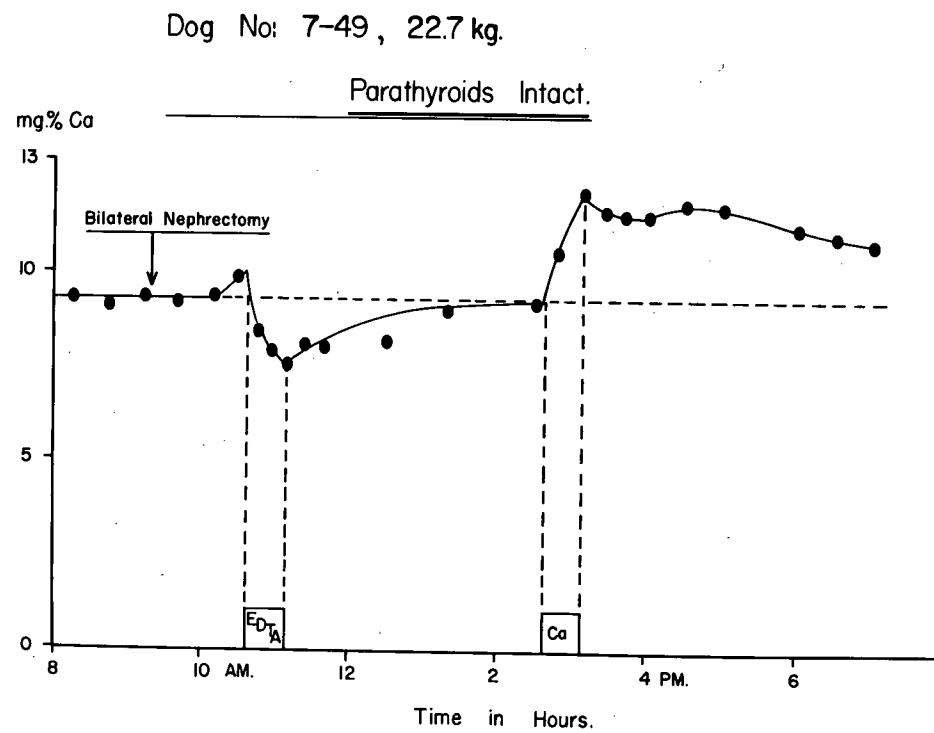

Figure 14. The effect of nephrectomy on calcium mobilization, calcium storage and calcium level.

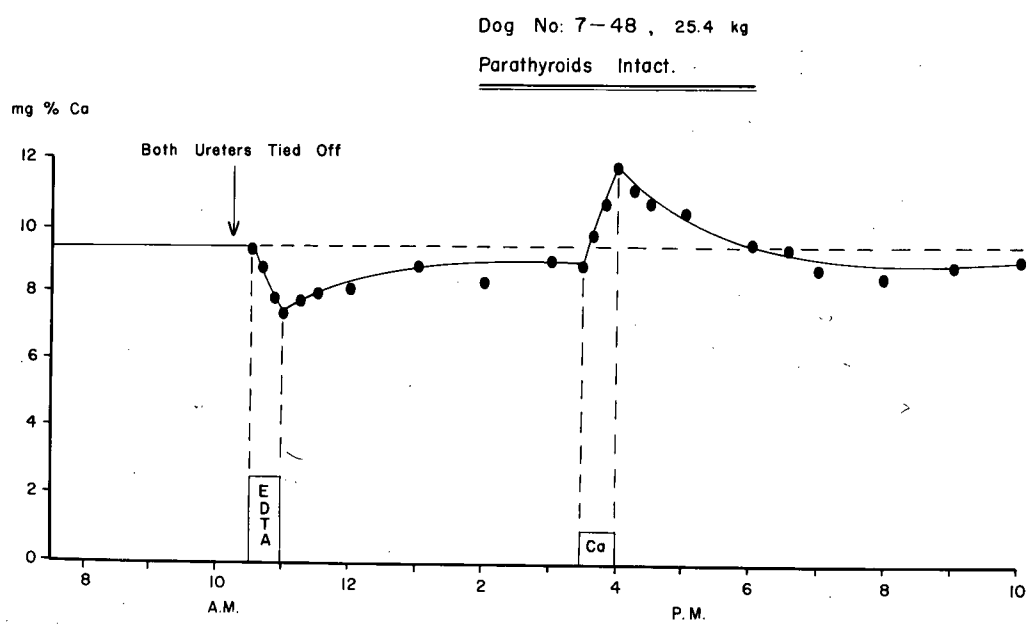

Figure 15. The effect of ureteral ligation on calcium mobilization, calcium storage and calcium level.

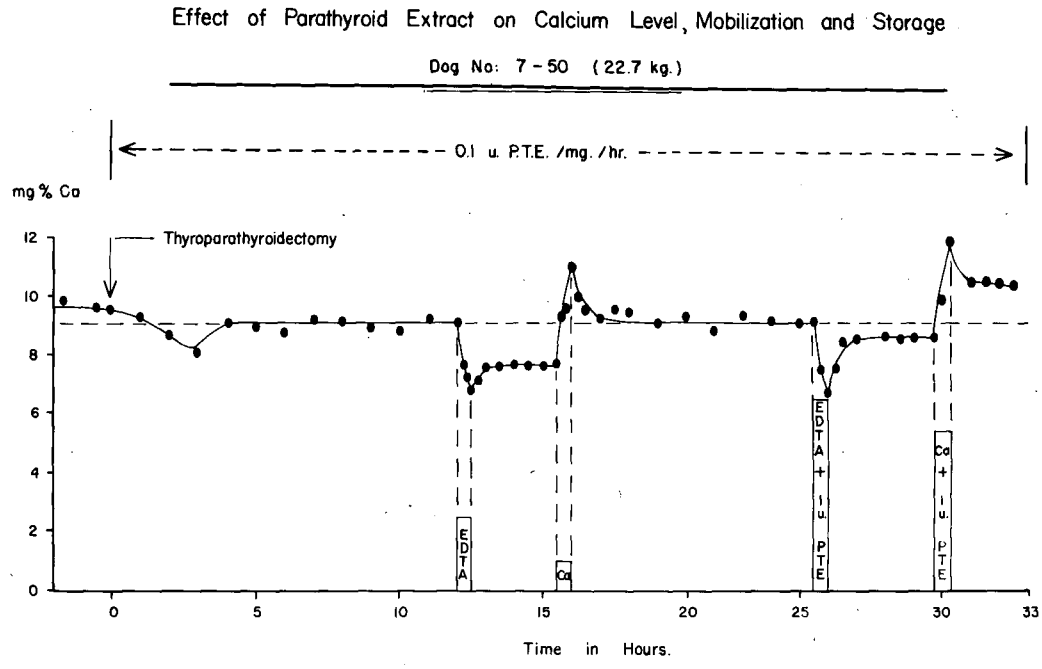

Figure 16. The effect of parathyroid extract on calcium level, mobilization and storage.

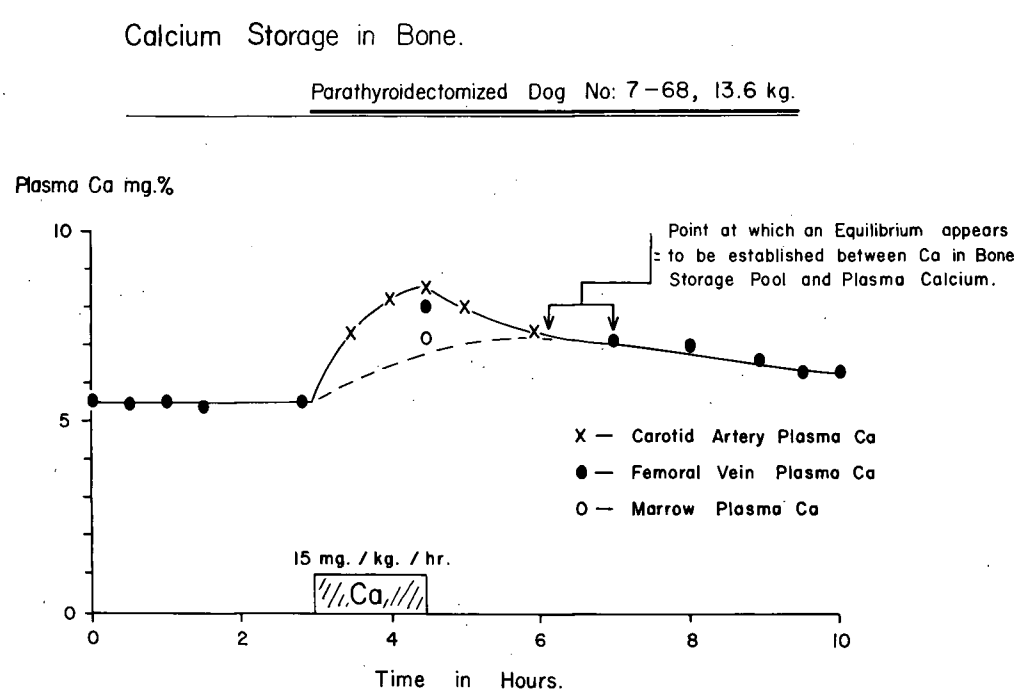

Figure 17. Showing the difference between arterial, venous, and marrow samples during calcium infusion.

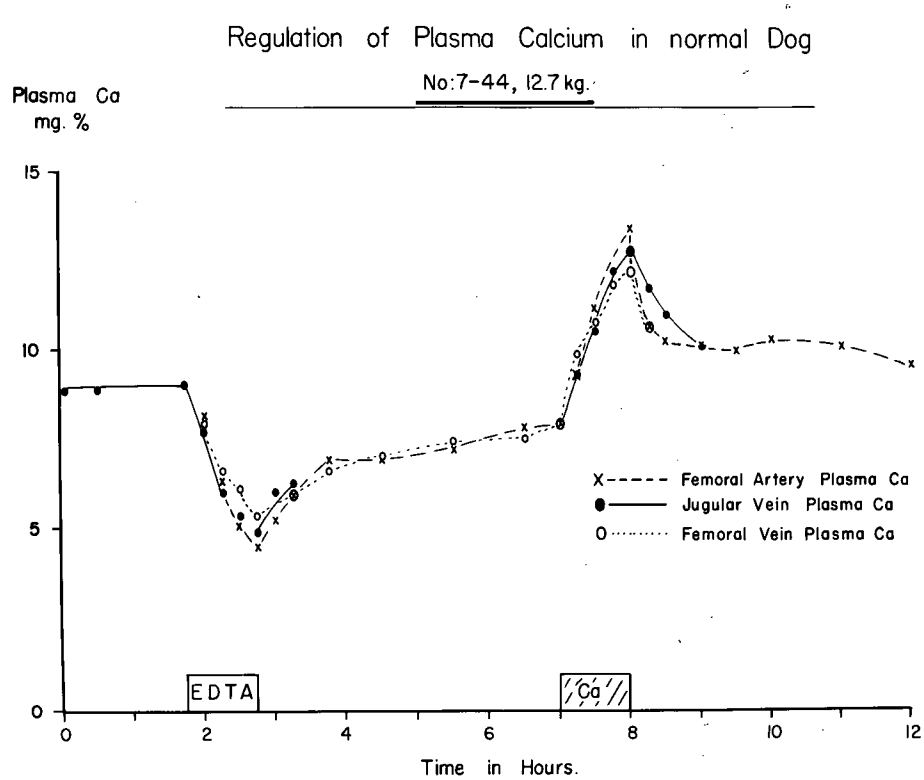

Figure 18. Showing difference between femoral artery, femoral vein, and jugular vein samples during EDTA and calcium infusions.

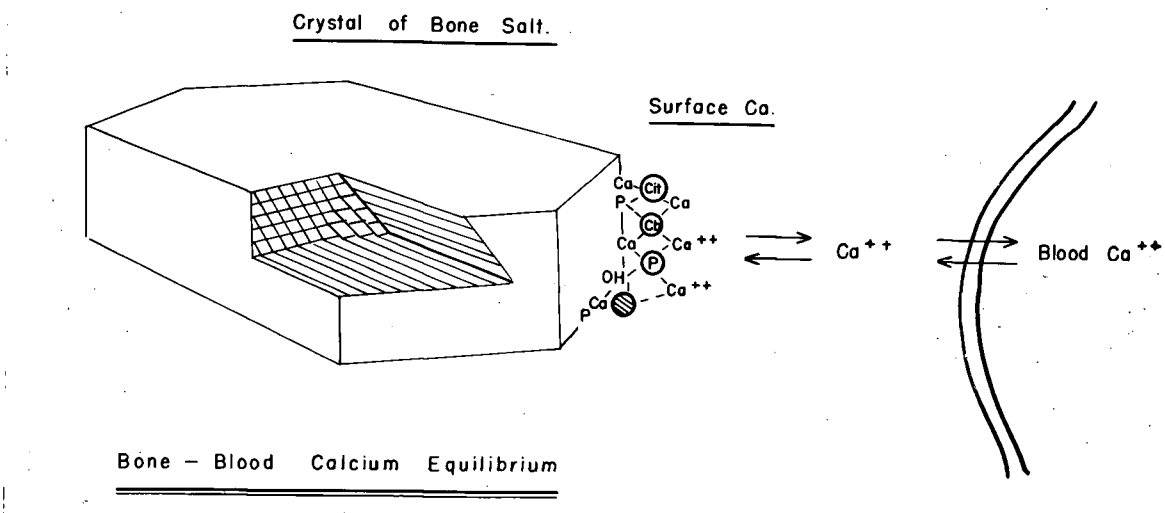

Figure 19. Proposed labile calcium store on surface of bone crystals in equilibrium with the blood calcium.

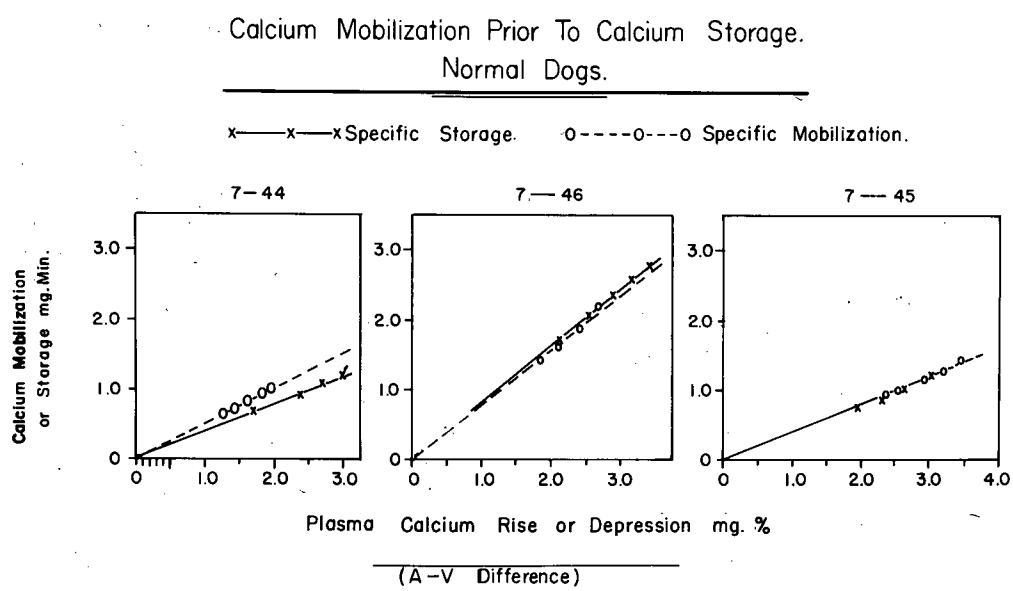

Figure 20. Showing calculated calcium mobilization and calcium storage rates when EDTA infusion was carried out prior to calcium infusion. The slopes of the line represent the effective bone-blood flow.

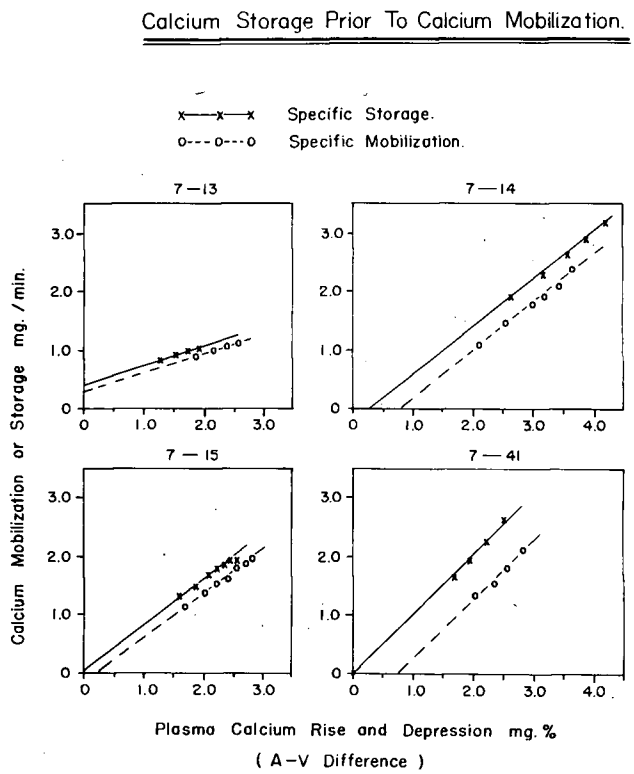

Figure 21. Showing calculated calcium mobilization and calcium storage rates when calcium infusion was carried out prior to EDTA infusion. The slopes of the lines represent the effective bone-blood flow.

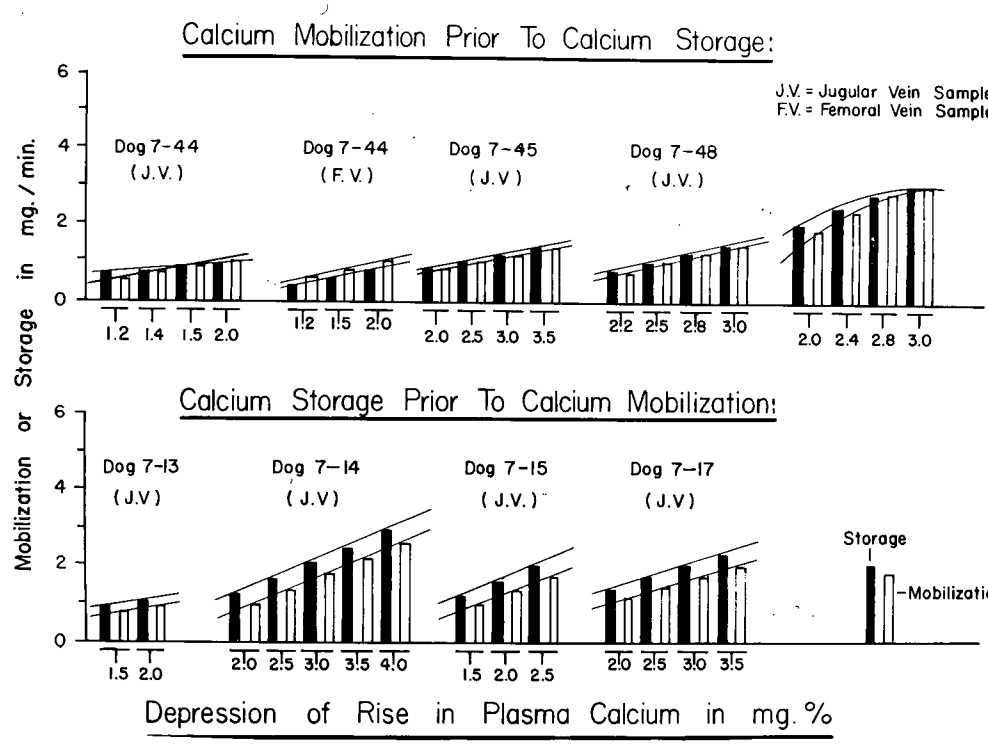

Figure 22. When EDTA was infused prior to calcium infusion, the calculated mobilization and storage rate was almost identical. If calcium was infused prior to EDTA infusion, the mobilization rate and storage rate were similar if expressed as mg/min/mg.% rise or depression in the plasma calcium, (that is the slopes were identical). However, the mobilization rate is less if expressed as mg/min. at a definite depression in the plasma calcium level. This corresponds to the different intercepts of the storage and mobilization rates in Figure 21.

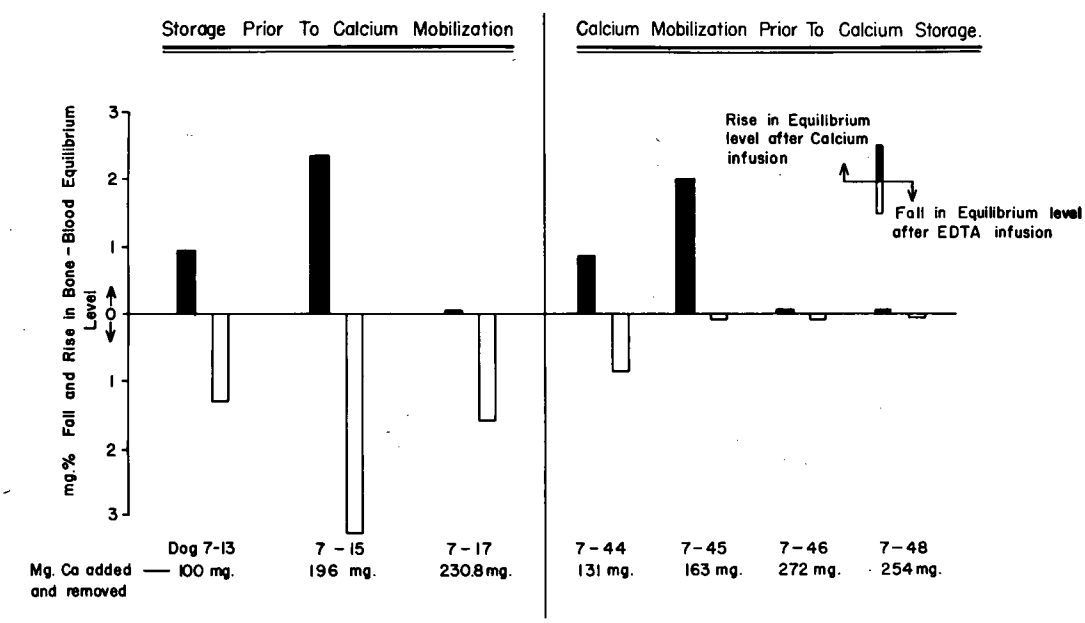

Figure 22A. Showing changes in bone-blood equilibrium level after EDTA and calcium infusions.

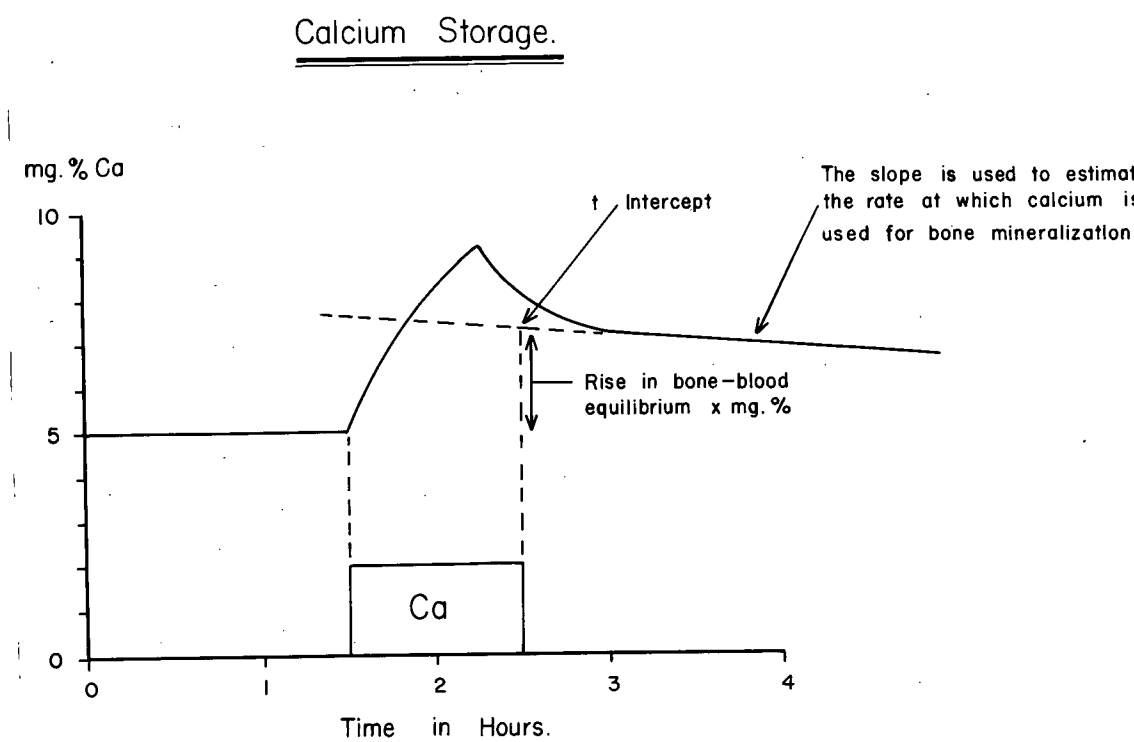

Figure 23. Calcium storage in bone

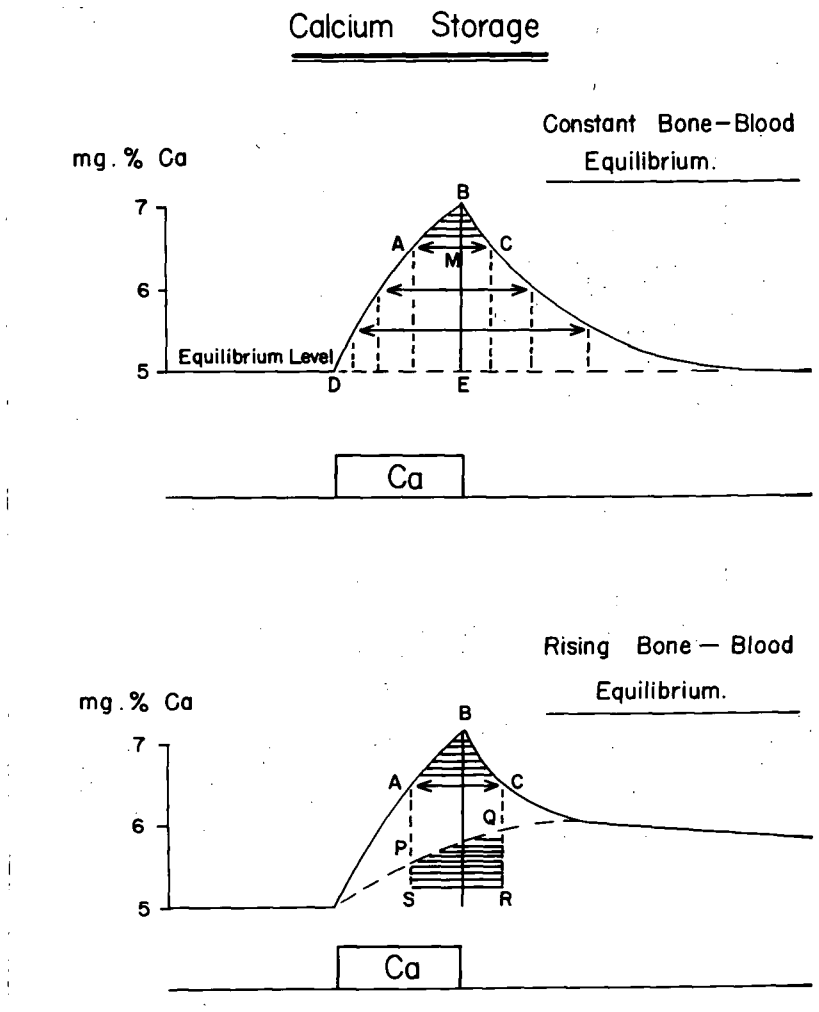

Figure 24 and 25. Method of calculating calcium storage or mobilization.

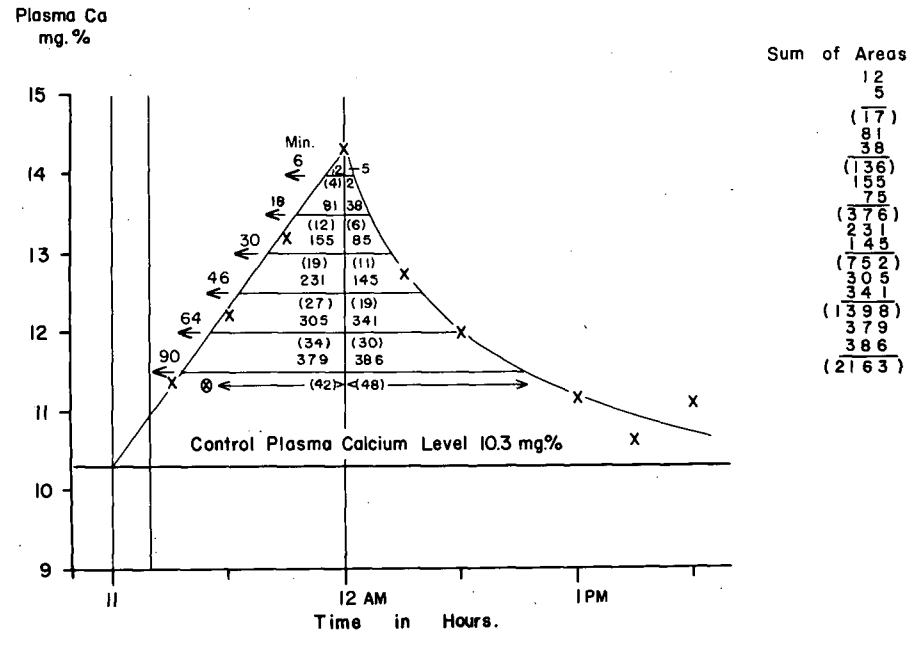

Figure 26. Method of calculating calcium storage.

TABLE I.

Mls. .01 % versene titrated	light absorption
0.0	238
1.0	166
1.2	152
1.4	140
1.6	126
1.8	112
2.0	97
2.2	91
2.4	89
2.6	87

end point = 2.08 ml

Values for titration of calcium standard with 0.01 % Na_2EDTA

TABLE II.

Urine calcium excretion in the normal animal after calcium infusion.

Dog	Rate of calcium infusion mg./kg./hr.	Duration of calcium infusion	Urine calcium expressed as % of calcium injected
7-12	10 mg./kg./hr.	one hour	*3.4% (approximately)
7-13	"	"	*5.0% "
7-14	"	"	*5.0% "
7-15	"	"	*5.0% "
7-17	"	"	*5.0% "
7-19	"	"	*5.0% "
7-70	4.0 mg./kg./hr.	9.5 hours	+5.26% (accurate)

*urine collection by catheterization

+urine collection by canulation of one ureter

TABLE III

Urine calcium excretion in the parathyroidectomized animal.

(accurate Ca analysis urine collection by canulation of one ureter)

Dog	Rate of calcium infusion mg./kg./hr.	Duration of Ca infusion	Urine Ca expressed as % of Ca injected.
EM-1	10 mg./kg./hr.	one hour	0%
7-67	12.95 mg./kg./hr.	1.5 hr.	0.144%
7-68	15.0 mg./kg./hr.	1.5 hr.	3.19%

TABLE IV

Specific calcium storage in normal dogs, with estimated bone blood flow and extracellular calcium.

Dog	Body Wt. Kgms.	Area M^2	Specific Ca Storage mg/min/mgm% rise/M^2	Estim. bone blood flow mls/min.	Bone blood flow as % card. output	I.S.F. calcium mg/kgm	E.C.F. calcium mg/kgm	E.C.F. volume % of body wt.	E.C.F. volume ml/kg
7-11	27.0	0.94	2.11	198	13.20	14.40	19.4	25.4	256
7-12	32.5	1.09	0.81	89	5.06	11.30	16.3	21.2	211
7-13	10.0	0.46	1.26	58	7.42	9.30	14.3	18.3	183
7-14	23.6	0.85	0.89	76	5.23	6.75	11.7	14.7	147
7-15	18.1	0.71	1.13	80	7.06	12.30	17.3	22.6	226
7-16	15.0	0.61	1.02	62	6.35	8.80	13.8	17.5	175
7-17	20.9	0.77	0.87	67	5.43	11.55	16.5	21.4	214
7-21	14.5	0.51	0.91	46	5.70	10.30	15.3	19.7	197
7-39	23.6	0.85	1.32	112	7.56	11.05	16.2	20.9	208
7-41	27.2	0.90	1.13	102	6.62	8.60	13.5	17.2	172
7-44	12.7	0.54	0.77	42	4.80	5.70	10.7	13.2	132
7-45	16.3	0.67	0.61	41	3.82	14.70	19.5	25.8	257
7-46	27.2	0.95	0.86	82	5.37	11.70	16.6	21.6	216
7-48	25.4	0.90	1.16	104	6.80	14.25	19.1	25.3	252
Averages ± S.E.			1.20±0.11*		6.46±0.60*		15.73±0.72*		203.3±10.4*

*Based on the value for cardiac output in the dogs (25)

TABLE V

Specific Calcium Mobilization in Normal Dogs, with Estimated Bone Blood Flow and Extracellular Calcium.

Dog #	Body wt. kg.	Surface area sq.m.	Spec. Ca mobilization mg./min./mg.%/M^2	Estim. bone blood flow ml./min.	Bone blood flow as % cardiac output	Extracellular Ca mg./kg.	Extracellular volume ml./kg.
EM-1	12.2	0.530	1.060	56.18	6.23	14.05	177
7-07	17.2	0.675	1.210	82	7.1	20.80	261
7-39	27.2	0.945	1.28	123	7.6	16.10	209
7-44	12.7	0.540	1.07	58	6.3	-	
7-45	16.3	0.650	0.62	40	3.6	-	
7-46	27.2	0.945	0.83	78	4.8	19.10	253
7-48	25.4	0.895	1.02	92	6.0	23.7	328
7-50	22.7	0.825	1.57	130	9.2	22.7	305
			1.08±0.103		6.37±0.61	19.4±1.7	255±8

TABLE VI

EDTA Infusion Prior to Calcium Infusion. Estimation of Calcium Storage and Calcium Mobilization Rates.

Dog	Calcium storage mg/min/mg%	Calcium mobilization mg/min/mg%	Diff. mg/min/mg%	Specific storage	Specific mobilization	Diff. mg/min/mg%/M^2
7-44	0.3995	0.515	0.1145	0.742	0.954	0.212
7-45	0.4065	0.4096	0.0031	0.612	0.616	0.004
7-46	0.8120	0.778	0.0340	0.812	0.778	0.034
7-48	1.034	0.914	0.1200	1.156	1.022	0.134

TABLE VII

EDTA Infusion Prior to Calcium Infusion. Estimations of Bone Blood Flow, E.C.F. Ca, etc. from both EDTA Curves and Calcium Curves. Dog. No.7-45

	Spec. mob.	Spec. stor.	Bone blood flow % C.O.	Ex-cell. Ca mg./kg.	I.S.F. Ca mg./kg.	I.S.F. volume % body weight	E.C.F. volume % body weight
Calculations made from EDTA curve	0.616		3.62	19.10	14.17	20.2	25.13
Calculations made from calcium curve		0.612	3.82	19.45	14.70	21.0	25.75

TABLE VIII

Calcium infusion prior to EDTA infusion. Estimation of calcium storage and calcium mobilization rates.

Dog	Ca storage: mg/min/mg% rise in plasma Ca.	Ca mobilization: mg/min/mg% depression in plasma Ca.	Differ. mg/min/ mg%	Specific storage: mg/min/ mg%/M^2	Specific mobilization, mg/min/mg%/M^2	Difference: mg/min/mg%/M^2
7-13	0.5815	0.4625	0.1190	1.260	1.005	0.255
7-14	0.7550	0.6020	0.1530	0.890	0.710	0.180
7-15	0.7970	0.6950	0.1020	1.130	0.986	0.144
7-17	0.6660	0.6204	0.0458	0.870	0.812	0.068
			0.1048			0.162

TABLE IX

Calcium infusion prior to EDTA infusion, showing changes in the bone-blood calcium equilibrium.

Dog	Mg.Ca added during Ca infusion.	Mg. Ca removed during EDTA infusion.	Mg.% rise in plasma Ca during Ca infusion.	Mg.% fall in plasma Ca during EDTA infus.	Mg.% rise in equilibrium level after Ca infusion.	Mg.% fall in equilibrium level after EDTA infusion.
7-13	100	100	3.24	4.10	0.973	1.3
7-15	196	196	4.63	5.55	2.37	3.25
7-17	230.8	230.8	4.47	4.40	0	1.60

TABLE X

Showing rise in Bone-Blood Calcium Equilibrium after continuous or single injection of Calcium in the Parathyroidectomized Dog No.EM-1

								Rise in bone-blood equilibrium (mg%)
Continuous infusion		(5 mg.Ca/kg)	- measured from beginning of infusion					1.55
"	"	(10 mg.Ca/kg)	"	"	"	"	"	2.80
"	"	(5 mg.Ca/kg)	"	"	end	"	"	1.45
"	"	(10 mg.Ca/kg)	"	"	"	"	"	2.00
Single	dose	(5 mg.Ca/kg)	-	-	-	-	-	1.40
"	"	(10 mg.Ca/kg)	-	-	-	-	-	2.05

TABLE XI

Dog	Wt. kg.	Area sq.m.	Estim. bone blood flow % card. output	E.C.F. Ca. mg/kg.	Bone storage pool mg.Ca/kg	Ratio of storage pool/E.C.F. calcium	Storage pool as % total bone Ca	Rate at which Ca is used for bone mineralization. mg Ca/kg/hr.	Rate at which Ca is used for bone mineralization as % of total bone Ca/day.
EM-1	12.2	0.53	6.23	14.1	36	2.57	0.1295	1.0	0.087
7-07	17.2	0.675	7.12	20.8	60	3.40	0.4000	1.94	0.310
7-39	23.6	0.85	7.56	16.1	45	3.00	0.3000	0.915	0.100
7-67	14.1	0.58			80.3	4.59	0.5360	1.955	0.314
7-68	15.4	0.62			65.8	3.76	0.4380	2.14	0.343

TABLE XII

Bauer, Carlsson and Lindquist's results in human studies by means of radiocalcium (4)

Age of human years	Weight	Exchange Ca mg/kg.	Accretion rate mg/kg
0.5	4.3	199	2.72
5.12	6.9	155	2.20
1.5	12.9	233	1.93
11	32.0	161	1.02
22	61.0	125	0.54
25	60.0	77	0.32
26	70.0	73	0.28
43	58.0	79	0.26
51	65.0	78	0.23
56	56.0	70	0.35
57	90.0	68	0.20
58	70.0	62	0.29
60	65.0	67	0.36
63	86.0	88	0.21

TABLE XIII

Summary for calculation of all 0.5 mg.% levels.

Mg.% plasma level	t_{Ca} (min)	$t_{storage}$ (min)	Mg/min. Ca added	Mg/min. Ca stored	Mg.% average Ca level	Mg.% average Ca rise (A-V diff.)	Storage mg./min./mg.% rise in plasma Ca. (A-V diff.)
14.0	4	6	14.2	2.365	14.1415	3.8415	0.616
13.5	12	18	42.6	2.365	13.8780	3.5780	0.661
13.0	19	30	67.5	2.250	13.6275	3.3275	0.675
12.5	27	46	96.0	2.085	13.3185	3.0185	0.681
12.0	34	64	120.8	1.890	13.0920	2.7920	0.677
11.5	42	90	149.0	1.660	12.7050	2.4050	0.688

Average = 0.666 mg/min/mg% rise in serum calcium

When the last column is expressed graphically, a straight line through the origin is obtained: (Fig.20)

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