STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM IN ALLOXAN DIABETIC RATS

by

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INTRODUCTION

In considering calcium and phosphorus metabolism one is primarily engaged in a study of bone metabolism. The diagram below from Shohl [1] illustrates why this is so. It shows the relative amounts and concentrations of calcium and phosphorus occurring in the skeleton, in the muscles, and in the rest of the body.

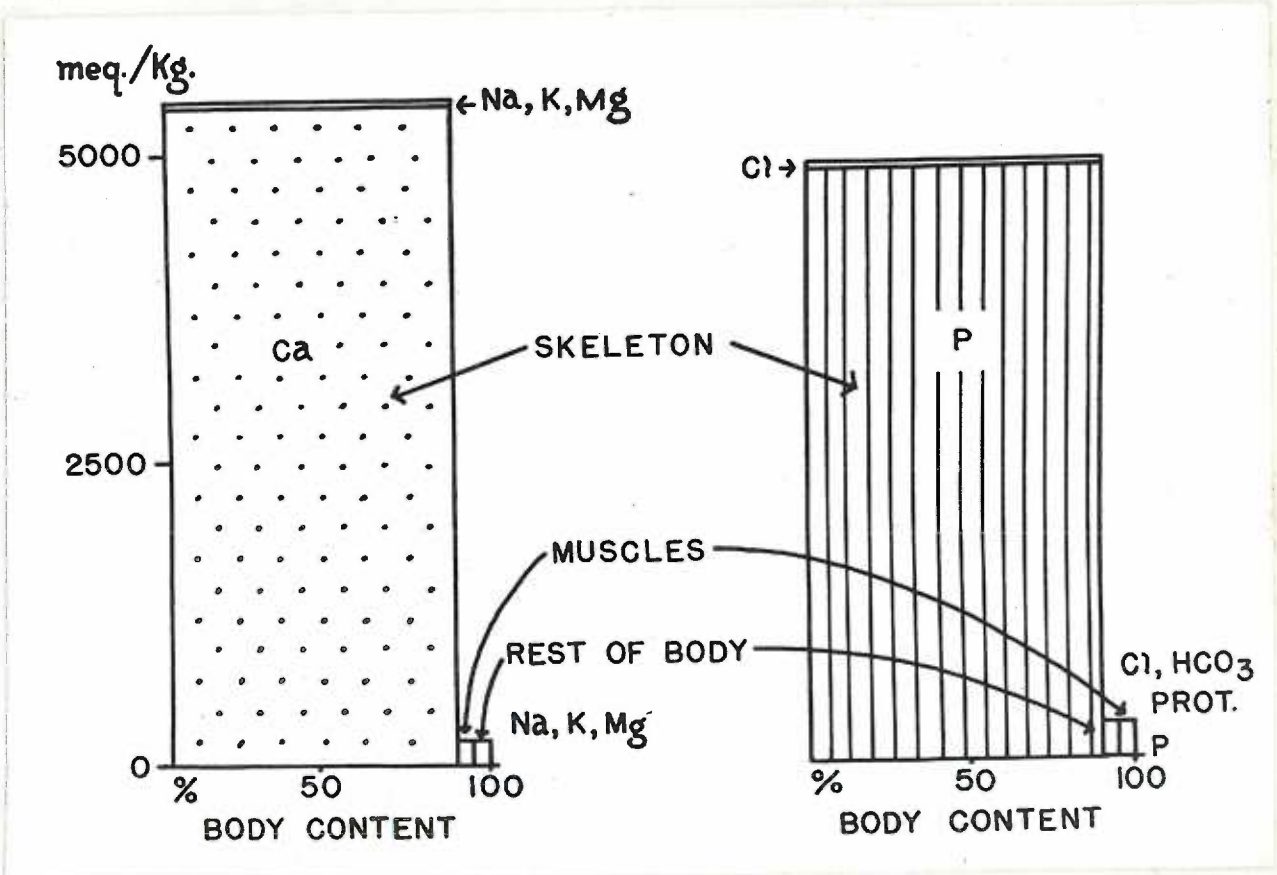


Figure #1.

Distribution of Positive and Negative Equivalents in the Body (Fat-free).

The ordinates represent concentrations in the various organs, and the abscissae the percent of the total positive or negative equivalents of the adult body.

Bone consists of an organic matrix and inorganic matter. The organic matrix develops, by differentiation, from connective tissue, the cells becoming the osteoblasts and the intercellular substance the osteoid matrix. The latter is composed of ossein, which resembles the collagen of other connective tissues, osseoalbuminoid, similar to elastin of elastic fibers, and glycoproteins, which are like the glycoproteins of cartilage. Bone matrix is calcifiable as it is formed and calcification begins as the matrix is developed. (Urist and McLean [2], Bourne [3], and Ascenzi [4]).

The inorganic matter is composed of Ca^{++}, $(PO_4)^{---}$, and some $(OH)^{-}$, $Mg^{}$, $(CO_3)^{--}$, F^{-}, Na^{+}, and Cl^{-} ions arranged in crystals belonging to the apatite system represented by the general formula ---- $3\ Ca_3(PO_4)_2 : CaX$ ---- where X may be $(CO_3)^{--}$, two $(OH)^{-}$, F^{-}, Cl^{-}, and combinations thereof. Since 1862 the apatite structure for bone mineral has been suspected on the basis of its composition (Huggins [5]). X-ray diffraction studies have confirmed the apatite structure for the mineral components of both bone and teeth (Roseberry , Hastings, and Morse [6], Taylor and Sheard [7], and Clark [8]).

The apatite crystal structure of bone mineral suggests formation by precipitation of ions in a manner similar to the formation of other apatite minerals found in nature (Shipley, Kramer and Howland [9]). The process of precipitation of slightly soluble salts can be defined in

terms of the solubility product ($K_{s.p.}$) for the particular salt. For example, the $K_{s.p.}$ for AgCl is 1.56×10^{-10} @ 25 C., which means that whenever the molar concentration of silver ions (Ag^+) times the molar concentration of chloride ions (Cl^-) exceeds 1.56×10^{-10} AgCl will precipitate out of solution.

The apatite crystal structure permits a generous substitution of ions, and because of the complex ionic composition of the plasma, the atomic proportions of the general formula $3\ Ca_3(PO_4)_2 : CaX$, do not occur. Such a proportion as --

$$\left[(Ca_{8.5}\ Mg_{0.25}\ Na_{0.19}\ H_2)\ (PO_4)_{5.07} : Ca_{1.24}\ (OH)_2\right]$$

is characteristic of bone mineral (Hendricks and Hill [10]). A solubility product cannot be assigned to bone mineral of such composition; however, it does approach that for $CaHPO_4$. (Shear and Kramer [11] and Logan [12]).

$K_{s.p.}\ CaHPO_4$ ..... 2.01×10^{-6} in water.

$K_{s.p.}\ CaHPO_4$ ..... 2.5×10^{-6} in serum.

By simultaneous study of blood and bone composition, (Sobel, Rochenmacher and Kramer [13, 14]) have shown that the composition of bone depends upon that of the liquid phase in contact with it, and it is assumed that physicochemical processes are a controlling factor in the phenomemon of calcification (Kruyper [15, 16]). Observations with the aid of radioactive phosphorus <u>etc.</u>

Observations with the aid of radioactive phosphorus support the assumption that bone minerals are in some sort of equilibrium with the corresponding blood minerals (Manly, Hodge and Manly [17]). Neuman and Mulryan [18] have recently demonstrated an ionic exchange and recrystallization of bone mineral occurring in vitro, and from the chemical point of view they emphasize that bone is a labile and sensitive substance. In addition the magnitude of the exchange takes place affirm the dynamic behavior of calcified tissues (Neuman and Riley [19]).

Such material as these authors present is complicated by the diversity of mechanisms by which ions can be fixed in the skeleton .... accretion, ionic exchange, and recrystallization. Considering bone to be in chemical equilibrium with the extracellular fluid, alterations in this medium in turn alter the chemical equilibrium of the bone material.

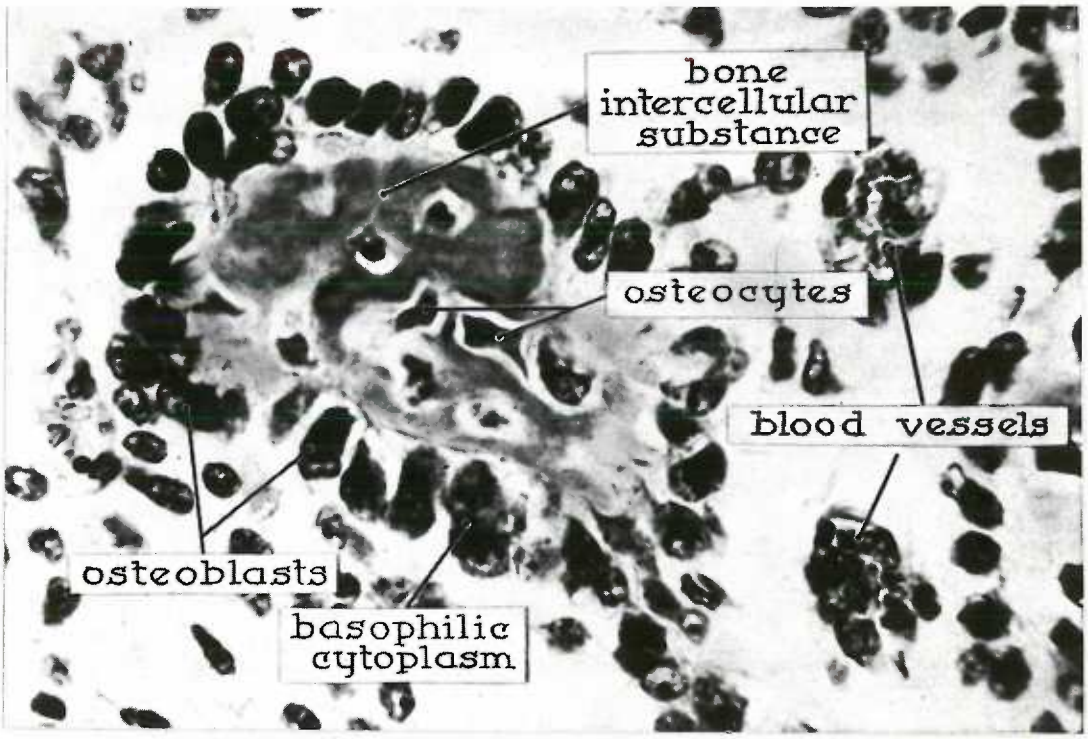
The biochemical details of calcification of hard tissues are not yet fully available; however, it is possible to tentatively sketch a hypothetical sequence of changes leading to calcification. We are limiting our discussion to the skeletal bone of adults and are not considering the growing bone with endochondral calcification. Upon examining a section of bone histologically, one observes surfaces where bone is being deposited, surfaces where it is being resorbed, and others where the

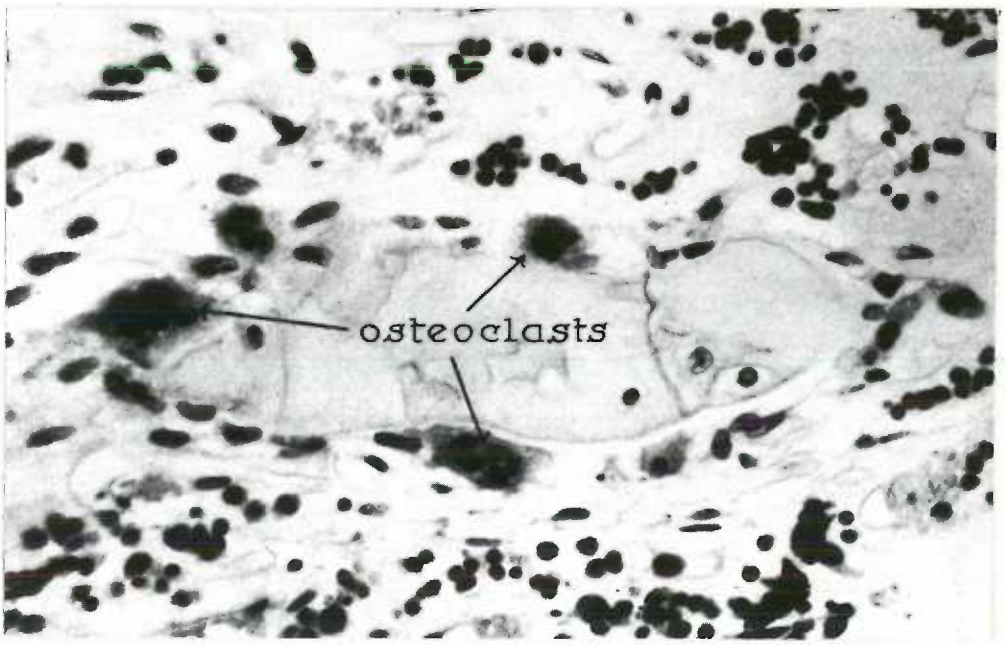
Fig. 2

1. Sketch of a section cut through a newly formed spicule of bone in the developing skull of a pig embryo. Observe that some of the osteoblasts have differentiated into osteocytes and have surrounded themselves with intercellular substance so that they have come to reside in lacunae. Observe that osteoblasts are arranged around the periphery of the spicule, where they are engaged in increasing its extent. (A. W. Ham [19]).

Fig. 3

2. Sketch of a section cut through a trabecula of bone in the marrow cavity of a long bone of a dog. The trabecual occupies the middle of the picture. It is covered with osteogenic cells except in four sites where osteoclasts are present. The dark staining nuclei in the periphery of the picture are those of cells concerned in producing red blood cells in granular leukocytes. (A. W. Ham [20]).


bone
intercellular
substance
osteocytes
blood vessels
osteoblasts
basophilic
cytoplasm


osteoclasts

status quo is being maintained, Fig. 2 and 3.

Initially it should be pointed out that bone deposition occurs by the addition of new bone to free bone surfaces, not interstitial but appositional growth like that of a tree. (Shipley and Macklin [22]).

One may fairly ask what facilitates bone growth on one surface with bone resorption occurring on another, and both processes going on presumably in the same body fluid? The answer to this has come from the findings of Robison [23], Robison and Soames [24]), and (A. B. and E. B. Gutman [25, 26]). Noting the presence of the enzyme, alkaline phosphatase, in tissues actively calcifying, Robison has suggested that it hydrolyzes organic phosphate esters, producing a local increase in the concentration of phosphate ions ($H_2PO_4^-$ and HPO_4^{--}) which causes the solubility product of $CaHPO_4$ to be exceeded, resulting in precipitation. This initial precipitate is gradually altered into the apatite crystal structure and distinctive bone mineral composition.

The serum alkaline phosphatase is practically all produced by the osteoblasts (Gould and Schwachman [27, 28], Fell and Robison [29], Nivens and Robison [30]), and in the absence of liver disease the amount present is considered to be a reflection of the osteoblastic activity of bone. It is always high in conditions in which matrix is being laid down in excess ---- during growth, rickets, osteitis fibrosa cystica, and Paget's disease.

Harris [31] and Glock [32] have demonstrated glycogen in calcifying cartilage and Gutman and Gutman [25] and Gutman, Warrick and Gutman [26] have demonstrated phosphorylase activity, in addition to that of alkaline phosphatase, in calcifying cartilage. Phosphorylase facilitates the phosphorolysis of glycogen to form glucose-1-phosphate, and thereby initiates further enzymatic reactions of anaerobic glycolysis with the net result of producing a mixed substrate of organic phosphate esters for the action of alkaline phosphatase. Phosphorylase is also found in bone tissue, but not in articular cartilage.

In regard to the process of decalcification, Fuller Albright [33-37, 45] suggests that the kidneys play a major role. Under normal conditions the electrolyte composition of the blood, calcium and phosphorus in particular, is maintained at such levels that the interstitial fluid is constantly undersaturated with respect to bone mineral components with the result that a constant dissolution of calcium and phosphorus from bone resorbing surfaces takes place. Any condition that decreases the concentration of calcium and phosphorus ions in the body fluids will increase the rate of bone resorption. An acidosis produced by feeding ammonium chloride will hasten bone resorption. A condition, which occurs in hypoparathyroidism, in which the calcium ion concentration is lowered need not hasten bone mineral resorption because of a compensating rise in phosphate ion concentration.

Any slight change in the ion product of calcium and phosphorus probably influences bone resorption. Apatite deposition on the other hand, is influenced by this ion product only when a marked abnormality in the direction of unsaturation occurs, such as rickets with very low serum calcium or low serum calcium and phosphorus.

Another question is what influences osteoblastic activity? Aside from the influence of nutritional factors such as Vitamin C and hormonal substances such as estrogens and testerone, mechanical stress plays a significant part (Koch [38]). Alterations in bone architecture are in accord with mechanical principles, and bone is laid down in accordance with the stress involved. When a broken bone is set at a new angle the new bone is laid down along lines of stress. Likewise when a limb is put at rest generalized decalcification occurs. (Howard, Parson et al [39, 40, 41], and Deitrick, Whedon, Schorr and Barr [42]).

In regard to the mode of action of the parathyroid hormone there have been two schools of thought. J. B. Collip [43] and his associates believed that the hormone acted directly on the bone tissue causing its dissolution, and that the electrolyte changes of the body fluids were secondary to the bone changes. Recently Neufeld and Collip [44] demonstrated a direct effect of parathyroid hormone on the kidneys.

Fuller Albright [33-37, 45] believes that the

hormone acts on the electrolyte equilibria of the body fluids via the kidneys, and that the bone changes, when they occur, are secondary to the chemical changes. We are following the latter hypothesis because we believe that it offers a better explanation for the known facts.

If one removes the parathroid glands from a normal individual this sequence of events occurs: first, there is an immediate decrease in the phosphorus excretion in the urine; secondly, the serum phosphorus rises; almost simultaneously the serum calcium falls; and finally with the fall in serum calcium, there is a diminished calcium excretion in the urine.

If one administers parathyroid hormone to a normal or hypoparathyroid individual, the same four metabolic functions are altered in the opposite direction but in the same sequence, Fig. 4.

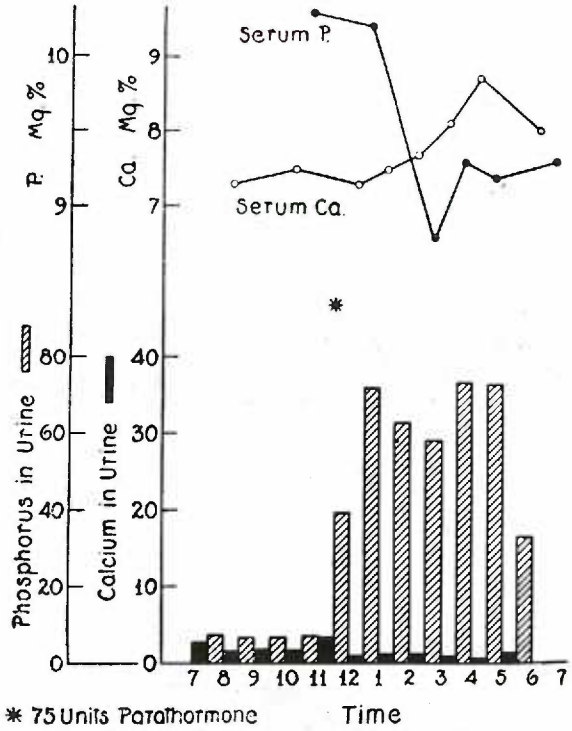


Fig. 4: Effect of Parathyroid Extract on Urinary Calcium and Phosphorus Excretions in a Patient with Idiopathic Hypoparathyroidism. (From Albright and Ellsworth (34)).

From these observations one is naturally led to believe that the serum phosphorus rises as the serum calcium falls or the serum phosphorus falls as the serum calcium rises in keeping with some solubility product relation. As pointed out above, the ionic concentrations of calcium and phosphorus closely approach those for $CaHPO_4$ in serum ($K_{s.p.} = 2.5 \times 10^{-6}$).

Under the influence of the parathyroid hormone the serum calcium rises because the serum phosphorus falls, and serum phosphorus does not fall because the calcium

rises. The stimulus for parathyroid activity is apparently a decreased serum ionic calcium concentration.

In summary, bone, with its organic matrix on which apatite mineral crystals form exists in a state of equilibrium with the body fluids. The calcium and phosphorus ionic concentrations of extracellular fluid are maintained somewhat below the saturation point (the solubility product for apatite), resulting in a continuous dissolution of bone mineral. Bone deposition occurs with the aid of alkaline phosphatase, produced by the osteoblasts, which facilitates the hydrolysis of organic phosphate esters, producing a local increase in the concentration phosphate ions. The product of calcium and phosphorus ion concentrations than exceeds the solubility product for apatite and precipitation, i. e. calcification occurs. Phosphorylase, by its action upon glycogen, provides substrate for the phosphatase enzyme. Mechanical stress is the significant factor in the stimulation of osteoblastic activity. The parathyroid hormone, by increasing the urinary excretion of inorganic phosphorus, causes decalcification and elevated serum calcium. In addition, low serum calcium is a stimulus for enhanced parathyroid activity.

With this group of animals we measured some of the chemical alterations associated with alloxan diabetes. Initially we expected to show a mobilization of bone minerals, both calcium and phosphorus, associated with the excretion of ketone bodies in alloxan diabetic state. This has been

observed in human diabetes by Martin and Westman [46] and Perlzweig, Latham, and Keefer [47].

With increased urinary calcium and phosphorus excretions, representing a loss of bone mineral, we would expect a weakening of the bones, more mechanical stress, and stimulation of osteoblastic activity, which in turn would be reflected by an increase in serum alkaline phosphatase, a sequence occuring in osteomalacia.

Cantor, Tuba, and Capsey [48] have reported an almost four fold increase in the serum alkaline phosphatase of alloxan diabetic rate. Serum alkaline phosphatase is also increased in liver disease; however, the increase as measured by Drabkin and Marsh [49] in alloxan diabetic rats is only about forty per cent above normal. Our measurements of serum alkaline phosphatase were of the same order as those of Cantor, et al, and we believe that the increase in the serum level above forty per cent reflects increased osteoblastic activity.

If the acidosis were the fundamental disturbance in alloxan diabetes with respect to bone metabolism, the addition of base, such as $NaHCO_3$, to the diet should reduce the serum and urinary values toward normal ---- that is, reduce the urinary loss of calcium and phosphorus, lower the serum alkaline phosphatase, raise the serum calcium and lower the serum phosphorus concentrations, respectively. This did not occur, and therefore, we conclude that the

fundamental disturbance in bone metabolism in alloxan diabetes is not due to the acidosis alone. There is an alteration in the mineral metabolism of alloxan diabetic rats which is more profound than that due to acidoisis alone. Treatment of the animals with protamine-zinc insulin relieved the condition.

METHODS

Four groups of adult, male rats, Sprague-Dawley strain, whose weight ranged from 150 to 420 grams and corresponding to ages of 13 to 25 weeks were used. Group I contained 24 control animals, which weighed 300 to 420 grams, corresponding to 18 to 21 weeks' age. Group II contained 25 alloxan diabetic animals, which weighed 150 to 300 grams before the injection of alloxan, corresponding to 15 to 21 weeks' age. The duration of diabetes was 9 days for 1 animal, 10 days for 1 animal, 16 days for 3, 17 days for 5, 19 days for 2, 20 days for 6, 21 days for 2, 22 days for 3, and 34 days for 2.

The animals in Group II whose blood sugar remained above 300 mg. per cent were used for Group III and Group IV. The duration of their diabetes was 30 to 60 days. Group III contained 12 alloxan diabetic rats, which received alkaline drinking water, 75 milli-equivalents of sodium bicarbonate per liter of water, 14 days for 4 animals, 17 days for 4, and 20 days for 4. They weighed 175 to 345 grams. Group IV contained 22 alloxan diabetic rats, which received subcutaneously one unit of Lilly's protamine-zinc insulin for each 100 grams of body weight for 7 days for 3 animals, 8 days for 8 animals, 9 days for 4 animals, 10 days for 4 animals, and 12 days for 3.

At the end of the respective periods blood sugar, serum inorganic phosphorus, serum alkaline phosphatase, and serum calcium determinations were made, and the animals were

placed in metabolism cages for collection of 24-hour urine and feces samples. In the metabolism cages Group I and II animals received water without food, Group III animals alkaline drinking water without food and Group IV animals water without food and insulin. Titratable acidity, calcium, phosphorus and nitrogen determinations were made from the urine samples, calcium and phosphorus from the feces, and calcium and phosphorus from food.

Alloxan monohydrate was prepared according to the method of Speer and Dabovich (50), and a solution containing 60 mg. of pure alloxan per ml. was freshly prepared for subcutaneous injection. Each animal was fasted for 48 hours before receiving alloxan, and 3 mg. of nembutal was given intraperitoneally about ten minutes before the alloxan (Abbot's Veterinarian Nembutal Solution, containing 60 mg. per ml.). The freshly prepared alloxan solution was then given subcutaneously over the flanks, and the overlying skin was gently massaged to aid absorption. 100 to 150 mg. of alloxan per kilogram of body weight resulted in a non-fasting blood sugar of 350 mg. percent or more 48 hours after the injection.

The 48-hour blood sugar determinations were made with tail blood, obtained by warming the tail for one minute in warm water (about 42°C.), drying it thoroughly and hastily severing the tip. Six to eight drops of blood were collected on a spot plate which had been coated with a 5 percent potassium oxalate and 2.5 percent sodium fluoride solution and

dried over-night at room temperature (Somogyi [51]). The blood and anti-coagulant were mixed; 0.1 ml. of blood was immediately transferred to a 15-ml. centrifuge tube containing 7.5 ml. distilled water. A blood filtrate was made from the laked blood solution by the Somogyi [52] procedure, using 0.5 ml. of 0.3 N $Ba(OH)_2$ and 0.5 ml. of 5 percent $ZnSO_4$ solution. Five ml. of blood filtrate were added to 5 ml. of Somogyi [53] reagent, according to the Somogyi [52] procedure.

The reagent is an alkaline solution of cupric ions with phosphate buffer. The amount of glucose, oxidized by the cupric ions is reflected in the quantity of cuprous ions produced. The cuprous ions are, in turn, oxidized by free iodine. There is a known concentration of free iodine, which is in excess, and the iodine that is not reduced by the cuprous ions is reduced by titration with 0.005 N thiosulfate solution. Blank determinations are run simultaneously. The glucose content of the blood filtrate is equivalent to the quantity of sodium thiosulfate obtained by the difference of the blood filtrate titration and that of the blank.

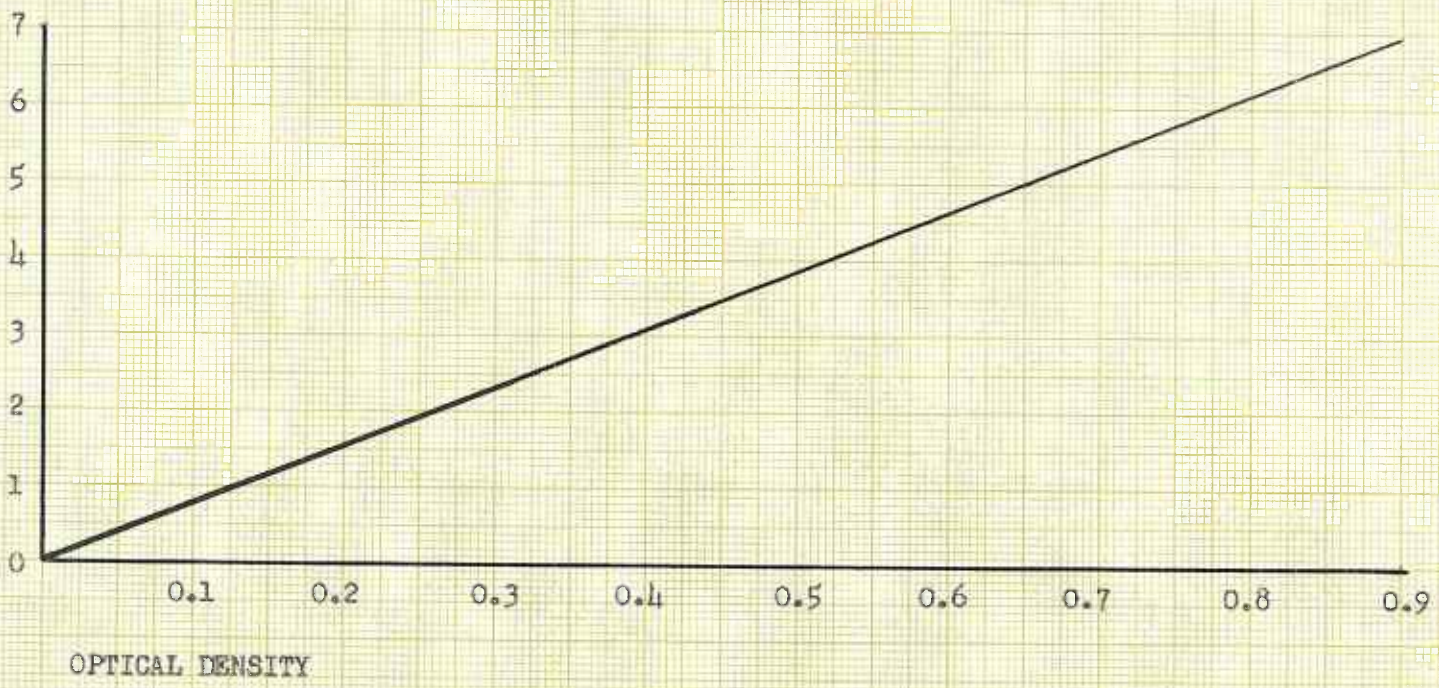
To obtain heart blood each animal was anesthetized by intraperitoneal injection of 3 mg. Nembutal for each 100 grams of body weight (Abbott's Veterinarian Nembutal solution 60 mg. per ml.) and a heart puncture was made through the chest wall. One ml. of blood was slowly withdrawn into a tuberculin syringe and 0.2 ml. was quickly

transferred to a spot plate coated with anti-coagulant for blood sugar determination *vide supra*. The remaining 0.8 ml. was transferred to a clean, dry 15 ml. centrifuge tube, which was immediately stoppered and placed in a water bath kept at 37°C. to promote clotting. After forty to sixty minutes, when clotting was complete, the stoppered tubes were centrifuged at 1500 r. p. m. for twenty minutes. Almost 0.4 ml. of serum was withdrawn and transferred to a small serum tube.

The serum inorganic phosphorus and alkaline phosphatase were determined by the micro-technique of Shinowra, Jones, and Reinhart [54], adapted to 0.05 ml. of serum. The substrate for alkaline phosphatase was a buffered beta-glycerol phosphate solution maintained at pH 10.4±0.1 in order to have a final pH of 9.3±0.1 upon the addition of serum. The proteins of the serum and serum with substrate were precipitated with trichloracetic acid. The protein-free filtrate was treated with an acid molybdate solution, which formed phosphomolybdic acid from any phosphorus present. The phosphomolybdic acid was reduced by adding stannous chloride solution, producing a blue color whose intensity was proportional to the amount of phosphate present. Transmissions were read at 600 mμ and their values in gamma of phosphorus were obtained from a previously standardized chart (Chart #1). The Coleman junior spectrophotometer, model 6a, was used for all photometric measurements.

The serum calcium was determined by the micro-metod

CALIBRATION CURVE FOR SERUM INORGANIC PHOSPHORUS AND ALKALINE PHOSPHATASE @ 600 mu.



of Sendroy [55], using 0.1 ml. of serum. The calcium was precipitated as calcium oxalate, which, in turn, was converted into oxalic acid by the addition of sulfuric acid. A known concentration of cerric sulfate solution was added to the oxalic acid, and the cerric sulfate was in excess of that required to oxidize the acid. After the oxidation of the oxalic acid was complete, potassium iodide was added, and a part of the iodide was oxidized to iodine by the remaining cerric sulfate. Transmissions were read at 400 mu, and the concentrations of free iodine were measured from a previously standardized chart (Chart #2). A standard sodium oxalate solution and a blank were determined simultaneously with the serum samples.

Because the relationship of iodine concentration to the intensity of transmitted light is not a linear one, the formula used for calculating the oxalate concentration is developed as follows:

$$2\ Ce(SO_4)_2 + H_2C_2O_4 \longrightarrow Ce_2(SO_4)_3 + 2\ CO_2 + 2\ H_2SO_4 \quad (1)$$

$$2\ Ce(SO_4)_2 + 2\ KI \longrightarrow Ce_2(SO_4)_3 + I_2 + K_2SO_4 \quad (2)$$

S_1 = Standard Sodium Oxalate

S_2 = Standard Blank

U = Unknown Sample

$$\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{S_1} = \left[I_2\right]_{S_1} \quad (3)$$

$$\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{S_2} = \left[I_2\right]_{S_2} \quad (4)$$

$$\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{U} = \left[I_2\right]_{U} \quad (5)$$

$$\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{S_1} = \left[I_2\right]_{S_1} \quad (3)$$

$$-\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{U} = \left[I_2\right]_{U} \quad (5)$$

$$\left[Ox\right]_U - \left[Ox\right]_{S_1} = \left[I_2\right]_{S_1} - \left[I_2\right]_U \quad (6)$$

$$\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{S_2} = \left[I_2\right]_{S_2} \quad (4)$$

$$-\left[Ce^{++++}\right] - \left[\text{oxalate}\right]_{U} = \left[I_2\right]_{U} \quad (5)$$

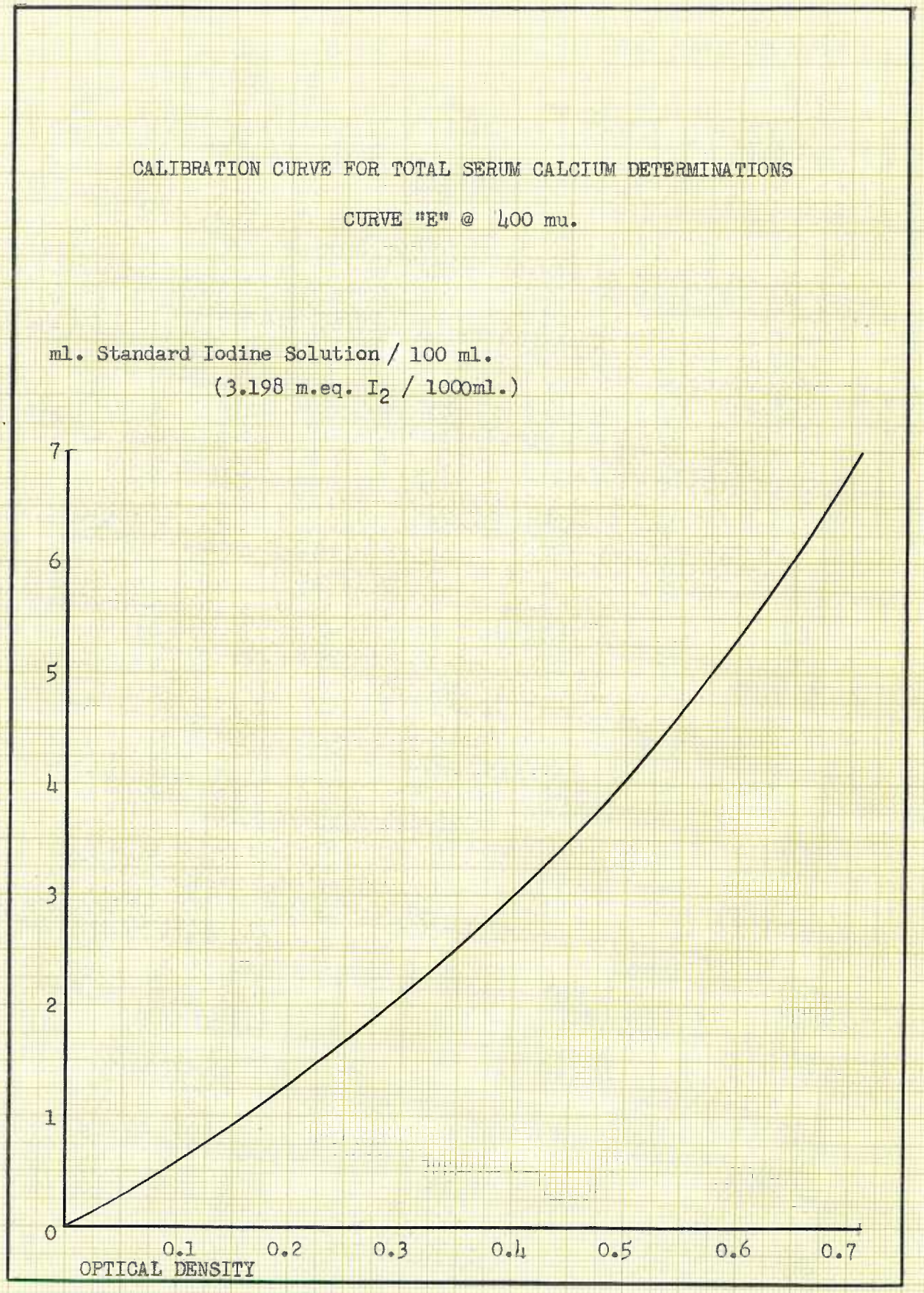
$$\left[Ox\right]_U - \left[Ox\right]_{S_2} = \left[I_2\right]_{S_2} - \left[I_2\right]_U \quad (7)$$

$$2\left[Ox\right]_U - \left[Ox\right]_{S_1} - \left[Ox\right]_{S_2} = \left[I_2\right]_{S_1} + \left[I_2\right]_{S_2} - 2\left[I_2\right]_U \quad (8)$$

$$\left[Ox\right]_U = \frac{\left[Ox\right]_{S_1} + \left[Ox\right]_{S_2} + \left[I_2\right]_{S_1} + \left[I_2\right]_{S_2}}{2} - \left[I_2\right]_U \quad (9)$$

CALIBRATION CURVE FOR TOTAL SERUM CALCIUM DETERMINATIONS

CURVE "E" @ 400 mu.



The quantity of oxalate, which is inversely proportional to the concentration of iodine, is equavalent to the calcium, which is initially precipitated as calcium oxalate.

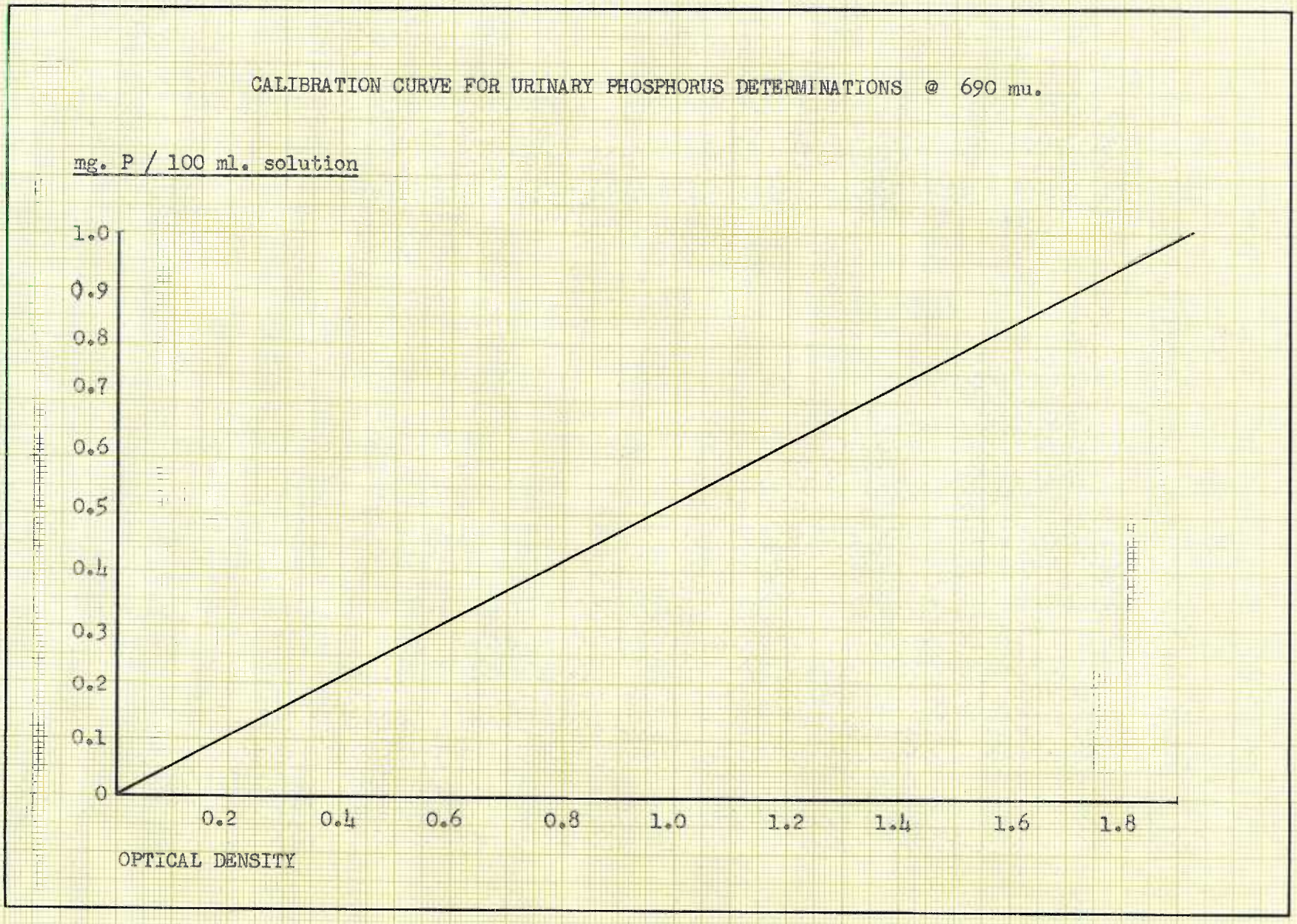
In addition each group of serum determinations was checked with 0.1 ml. of a standard calcium solution containing 10.1 mg. percent of calcium. The stock calcium solution was prepared with 0.5045 grams of calcite dissolved in 25 ml. of 0.1 NHCl and diluted with distilled water to 100 ml. 5 ml. of the stock solution was then diluted to 100 ml. with distilled water for the standard calcium solution.

After recovery from the anesthetic, given to obtain heart blood, the animals were put in metabolism cages for collection of 24-hour urine and fece samples. The urine was funnelled into a 125 ml. Erlenmeyer flask containing 5 ml. of toluene, and the feces were collected on a small copper-wire screen seated in the funnel.

The volume of each 24-hour urine specimen was measured, and the urine filtered. The titratable acidity was determined by the Folin (56, 57) method, using 5 ml. of the filtered urine with 0.1 N NaOH solution in the presence of 2 ml. of 30 percent neutral potassium oxalate solution.

The urinary calcium was determined by the method of McCrudden (58, 59) as given by Hawk, Oser and Summerson (62) with the following exceptions: (1) 2 ml. of filtered urine were transferred to a 15 ml. centrifuge tube, (2) the calcium oxalate precipitate was obtained by centrifuging at 2600 r.p.m.

CALIBRATION CURVE FOR URINARY PHOSPHORUS DETERMINATIONS @ 690 mu.



for 5 minutes; (3) the supernatant was withdrawn by suction applied to a small pipette made from a glass tube drawn out to a fine point and curved at the bottom; (4) the sides of the tube were washed with 3 ml. of 2 percent NH_4OH solution, the tubes centrifuged and the supernatant again withdrawn. (5) The precipitate was forcefully washed with a fine stream of a mixture containing equal parts of ethyl alcohol, ethyl ether, and distilled water (about 1 ml.). The sides of the tube were rinsed with 3 ml. of the solution and the tubes were centrifuged for 5 minutes at 2600 r.p.m. and the supernatant again withdrawn. (6) The washing was repeated and after centrifuging again the supernatant withdrawn. (7) Two ml. of 1.0 N H_2SO_4 were added and the tubes heated in a boiling water bath for a few minutes and titrated while hot with 0.14 N potassium permanganate solution.

Five ml. of filtered urine were diluted to 100 ml. with distilled water for total nitrogen and inorganic phosphorus. Total nitrogen was determined by a micro-Kjeldahl method, digesting 5 ml. of the diluted urine with 3 ml. of selenium digestion mixture.* The nitrogen was converted into ammonia in the presence of solid NaOH pellets and distilled over into N/70 H_2SO_4. The excess acid was titrated with N/70 NaOH. A blank determination with digestion mixture and 5 ml. of distilled water was run simultaneously.

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*Selenium digestion mixture: 1 liter of concentrated sulfuric acid, 1 liter of distilled water, 150 grams of potassium sulfate, and 5 ml. of selenium oxychloride.

The urinary inorganic phosphorus was determined by the method of Fiske and SubbaRow [61] as given by Hawk, Oser, and Summerson [62], adapted to 10 ml. of the diluted urine. An acid molybdate solution added to the dilute urine formed phosphomolybdic acid from any phosphorus present. The phosphomolybdic acid was reduced by adding 1,2,4-amino-naphtholsulfonic acid reagent, producing a blue color the intensity of which was proportional to the amount of phosphate present. Transmissions were read at 690 mμ and their values in mg. phosphorus were read from a previously standardized chart (Chart #3). The Coleman Jr. Spectrophotometer, Model 6a, was used for photometric measurements.

Feces samples were dried in the oven at 110 degrees C. for one week and measured for dry weight. Those samples of more than 0.5 grams dry weight were powdered in a mortar and a sample approximately 0.5 grams was ashed in a muffle furnace for four hours at 600 degrees C. For a feces sample below 0.5 grams, the entire sample was ashed. After cooling 10 ml. of equal parts concentrated HCl and water were added to each sample. The acid solution with some undissolved ash was transferred to a 100 ml. Kjeldahl flask and digested with 3 ml. of the selenium digestion mixture. A blank determination with digestion mixture and water was run simultaneously. The digested sample was transferred to a 100 ml. volumetric flask and made up to volume with distilled water.

Fecal calcium was determined on a 2 ml. aliquot of

the diluted solution, which was transferred to a 15 ml. centrifuge tube and neutralized with a few drops of concentrated NH_4OH. Thereafter the procedure was identical to the McCrudden procedure [58, 59] outlined above for urinary calcium.

Fecal phosphorus was determined on a 5 ml. aliquot of the diluted solution by the Fiske and SubbaRow [60] method as given by Hawk, Oser, and Summerson [62].

A triplicate analysis of Purina Chow Checkers for calcium and phosphorus was made. Parts of several checkers were ground together in a mortar and about 0.5 grams of the powder was digested in a Kjeldahl flask with 6 ml. of the selenium digestion mixture. From this point, the procedure for calcium and phosphorus determinations followed that outlined above for fecal calcium and phosphorus respectively.

RESULTS AND DISCUSSION

The analyses for the animals in Groups I, II, III, and IV are listed in Tables I to IV respectively, and the average values for the determinations made from each group are given in Table V. The blood sugar and the serum inorganic phosphorus, alkaline phosphatase, and total calcium determinations are non-fasting results. The urine, feces, and food figures represent values per twenty-four hours per hundred grams of body weight.

The twenty-four normal control animals of Group I had an average blood sugar of 117.6 mg. percent and an average serum inorganic phosphorus of 7.6 mg. percent, an alkaline phosphatase of 46.1 Bodansky units, and a total calcium of 10.0 mg. percent. The urine determinations averaged 4.06 mg. of phosphorus, 1.24 mg. of calcium, 62.0 mg. of nitrogen and 3.78 ml. of 0.1 N NaOH for the titratable acidity. The fecal phosphorus averaged 8.2 mg. and fecal calcium 15.3 mg. During the 24-hour period the animals consumed an average of 5.8 grams of food containing 40.6 mg. of phosphorus and 65 mg. of calcium.

The twenty-five diabetic animals of Group II showed an average blood sugar of 436 mg. percent and a serum inorganic phosphorus of 8.0 mg. percent, an alkaline phosphatase of 164 Bodansky units, and a total calcium of 9.56 mg. percent. The urinalyses averaged 7.7 mg. of phosphorus, 3.0 mg. of

calcium, 105.5 mg. of nitrogen, and 4.66 ml. of 0.1 N NaOH for the titratable acidity. The fecal phosphorus was 15.7 mg. and the calcium 34.3 mg. For the 24-hour periods food consumption averaged 11.6 grams which contained 81 mg. of phosphorus and 125 mg. of calcium.

The alloxan diabetic animals of Group II showed an increase in urinary phosphorus and calcium about twice the normal excretion and urinary nitrogen was seventy per-cent above the normal amount. The serum alkaline phosphatase was elevated to almost four times the normal level, the same quantitative increase as that found by Cantor et al (48).

It was initially believed that the alterations in mineral metabolism associated with alloxan diabetes might be due to the prolonged state of acidosis. In an effort to neutralize the acidosis sodium bicarbonate was added to the drinking water with the surmise that by relieving the state of acidosis the disturbances in mineral metabolism might be corrected. A concentration of 75 milli-equivalents $NaHCO_3$ per liter of water was found sufficient to reduce the titratable acidity of the urine to the normal range. In addition, it seemed adequate since this amount represents a concentration of the sodium ion, Na^+, about one-half that of the normal extracellular fluid and a concentration of the bicarbonate ion, $(HCO_3)^-$, about three times the normal extracellular fluid concentration. The Group III animals, 12 alloxan-diabetic rats, were given the alkaline drinking water for 14 to 20 days, and, at the end of that time their average

urinary acidity was somewhat below the normal average -- 2.62 ml. as compared to the normal of 3.78 ml. of 0.1 N NaOH.

The twelve diabetic animals maintained on sodium bicarbonate solution in Group III had an average blood sugar of 470 mg. percent and a serum inorganic phosphorus of 7.67 mg. percent, an alkaline phosphatase of 220 Bodansky units and a total calcium of 10.1 mg. percent. The urine determinations averaged 8.5 mg. of phosphorus, 3.24 mg. of calcium, 112.0 mg of nitrogen, and 2.6 ml. of 0.1 N NaOH for the titratable acidity. The fecal phosphorus was 9.3 mg. and the calcium 20.6 mg. The average 24-hour food consumption was 9.6 grams, containing 67 mg. of phosphorus and 117.6 mg. of calcium.

Though the alkaline therapy reduced the urinary acidity, it had no influence on the urinary loss of phosphorus, calcium, and nitrogen, and there was no significant change in the respective blood and serum values compared to those of the diabetic animals in Group II. With the discovery that alkaline therapy did not correct the disorder in mineral metabolism associated with alloxan diabetes, a more profound derangement than urinary acidosis appeared to be involved.

Because there was no fundamental change in the metabolism of the diabetic rats maintained on alkaline drinking water, a program of insulin therapy was instituted. It was found that one unit of Lilly protamine-zinc insulin per hundred grams of body weight given subcutaneously each day reduced the average blood sugar level from 436 mg. percent to

190 mg. percent. The serum and urine determinations made at the end of the respective periods of insulin therapy (7 to 12 days) showed a significant return toward normal values.

The twenty-two diabetic animals maintained on insulin, Group IV, showed an average blood sugar of 190 mg. percent and a serum inorganic phosphorus of 7.77 mg. percent, an alkaline phosphatase of 76.0 Bodansky units and a total calcium of 9.96 mg. percent. The urinalyses averaged 7.3 mg. of phosphorus, 1.76 mg. of calcium, 74.8 mg. of nitrogen, and 2.1 ml. of 0.1 N NaOH for the titratable acidity. The fecal phosphorus was 112.2 mg. and the calcium 27.8 mg. The food consumed in 24 hours averaged 9.3 grams, containing 65 mg. of phosphorus and 104.5 mg. of calcium.

The alloxan diabetic animals of Groups II and III showed an increase in urinary and phosphorus and calcium about twice the normal excretion, which could be attributed to the increase in food consumption, which was about double the normal, and to skeletal decalcification. The diabetic animals maintained with insulin consumed approximately the same quantity of food with only a slight increase in urinary phosphorus and calcium; so, skeletal decalcification was thought to be the principal source for the additional phosphorus and calcium excreted in the urine by alloxan diabetic rats.

The seventy percent increase in urinary nitrogen occurring in the diabetic animals was not reduced by alkali

therapy and was reduced by insulin, matching the urinary excretion of phosphorus and calcium. Recently Mackler and Guest [63], by analysis of amino acid excretion in poorly controlled alloxan diabetic rats, showed that this increased catabolism is simply a quantitative increase of normal protein breakdown.

Analyses of freshly dried, fat-free bone shows a protein content of approximately thirty percent (Baker, Butterworth and Langley [64] and Strobine and Farr [65]), and one group of collagen-nitrogen determinations on guinea pig bone shows 15 to 18 percent calculated as protein (Lightfoot and Coolidge [66]), which indicates a significant protein content of bone matrix.

The facts that alkaline therapy of diabetic rats failed to decrease the urinary calcium and phosphorus while insulin therapy was effective in decreasing the excretion of these substances under the same food intakes suggest that much of the calcium and phosphorus loss was due to skeletal decalcification primarily associated with catabolism of the protein of the bone matrix.

TABLE I

	BLOOD SUGAR	SERUM ALKALINE PHOSPHATASE	SERUM INORGANIC PHOSPHORUS	TOTAL SERUM CALCIUM	TOTAL URINARY NITROGEN	TITRATABLE ACIDITY OF URINE	URINARY PHOSPHORUS	URINARY CALCIUM	FECAL PHOSPHORUS	FECAL CALCIUM	FOOD PHOSPHORUS	FOOD CALCIUM	BODY WT.	
	mg. %	units	mg. %	mg. %	mg.	ml. 0.1 N NaOH	mg.	mg.	mg.	mg.	mg.	mg.	gm.	
	130	36.1	9.7		74.1	6.2	3.0	0.7			40.6*	65.0*	335	1
	120	48.3	8.7		53.7	5.6	3.2	0.6					334	2
	120	39.6	8.2		72.7	7.8	4.7	0.7					341	3
	130	45.6	8.2		41.2	6.0	4.2	0.75					294	4
	110	22.6	6.3	9.2		3.6	3.9	2.0					397	5
	120	38.1	6.6			4.3	3.1	1.7					345	6
	100	51.9	7.0		52.4	5.8	4.1	3.2					393	7
	110	38.1	7.0			5.0	3.8	3.8					397	8
	160	41.9	8.9	7.4	62.3	1.7	4.1	1.4	8.0	16.2			329	9
	130	30.6	6.4		61.6	1.0	2.1	1.7					302	10
	120	61.8	8.2			6.7	5.6	1.8	6.7	13.9			392	11
	110	26.1	7.7			6.4	4.2	2.0	9.9	15.9			364	12
	120	53.7	9.2	10.7		1.3	3.3	0.2					344	13
	115	49.5	7.1	10.4		4.0	7.0	0.7					347	14
	121	59.5	7.8	9.0		2.0	5.1	0.3					376	15
	146	63.8	8.2	10.7		1.9	4.3	1.1					420	16
	98	64.6	9.4	9.0		2.5	4.7	0.5					342	17
	98	49.6	9.2	9.9		1.9	4.0	0.7					321	18
	103	42.5	6.7	11.8		1.5	2.5	0.3					318	19
	107	39.4	5.8	10.9		2.3	5.0	0.8					386	20
	124	38.4	5.8	10.8		1.8	4.7	1.0					379	21
	125	36.1	6.8	11.2									371	22
	94	46.9	7.4	9.3									370	23
	112	81.2	7.6	10.2									361	24

*Average food consumption by 4 normal animals during eleven 24-hour periods.

TABLE II

DURATION OF DIABETES	BLOOD SUGAR	SERUM ALKALINE PHOSPHATASE	SERUM INORGANIC PHOSPHORUS	TOTAL SERUM CALCIUM	TOTAL URINARY NITROGEN	TITRATABLE ACIDITY OF URINE	URINARY PHOSPHORUS	URINARY CALCIUM	FECAL PHOSPHORUS	FECAL CALCIUM	FOOD PHOSPHORUS	FOOD CALCIUM	BODY WT.
days	mg. %	units	mg. %	mg. %	mg.	ml.0.1N NaOH	mg.	mg.	mg.	mg.	mg.	mg.	gm.	
9	490	103.5	6.5		124.3	6.4	5.5	3.6					290	1
10	400	118.1	8.1	9.5	98.0	3.5	7.1	2.2			81.0*	130.0*	264	2
16	490	199.6	7.8	11.3		9.1	7.7	3.4					150	3
16	330	107.8	9.4	8.9		11.3	7.3	2.2					246	4
16	510	146.4	8.0	8.9		12.3	8.6	3.4					184	5
17	490	206.2	6.9		84.9	2.5	3.4	2.7					290	6
17	500	188.6	6.6	9.2	82.1	2.5	3.2	1.5					275	7
17	470	256.7	7.1	11.0	173.5	4.0	6.5	3.1					190	8
17	300	105.2	10.0	8.9		6.8	6.6	2.7	15.1	37.1			247	9
17	390	148.3	10.0	8.3		1.8	6.4	1.6	9.1	17.9			257	10
19	520	180.5	9.3	11.6	114.4	3.6	8.8	2.2					196	11
19	520	161.5	7.8	9.2	86.4	2.0	3.8	2.4					200	12
20	380	106.4	10.0	9.7	125.1	4.0	11.5	2.0					227	13
20	360	114.8	6.8	9.5		3.4	11.4	3.3					192	14
20	540	201.3	7.7	7.8		5.1	14.4	5.5	10.3	19.9			186	15
20	460	189.7	9.1	6.1	68.1	4.5	11.6	4.5					235	16
20	430	179.9	8.1	8.3		9.7	8.8	2.6					194	17
20	340	171.6	7.2	10.6		7.0	11.3	5.7					187	18
21	380	143.1	8.7	9.3	80.8	2.5	5.3	1.7	7.7	16.0			300	19
21	240	104.9	8.7		74.5	2.1	10.3	2.8					275	20
22	420	195.7	7.1	10.3	110.7	1.0	4.2	1.6					258	21
22	390	209.0	6.6	11.6	106.4	4.3	6.3	3.2	34.3	74.7			270	22
22	590	194.4	6.6	11.6	80.4	1.1	4.8	1.7	28.5	65.0			280	23
34	470	195.6	8.8	11.2	161.1	3.6	11.8	6.2	4.3	9.2			175	24
34	490	186.0	6.6	9.6	116.8	2.4	5.6	2.7					230	25

*Average food consumption by 14 alloxan diabetic animals during three 24-hour periods.

TABLE III

DURATION OF $NaHCO_3$ THERAPY	BLOOD SUGAR	SERUM ALKALINE PHOSPHATASE	SERUM INORGANIC PHOSPHORUS	TOTAL SERUM CALCIUM	TOTAL URINARY NITROGEN	TITRATABLE ACIDITY OF URINE	URINARY PHOSPHORUS	URINARY CALCIUM	FECAL PHOSPHORUS	FECAL CALCIUM	FOOD PHOSPHORUS	FOOD CALCIUM	BODY WT.	
days	mg. %	units	mg. %	mg. %	mg.	ml.0.1N NaOH	mg.	mg.	mg.	mg.	mg.	mg.	gm.	
14	260	224.7	6.1	10.8	99.6	1.3	7.8	1.1			67.0*	117.6*	245	1
14	440	253.3		10.1	119.1	1.0	6.2	2.6					220	2
14	450	221.6	6.9	10.1	120.8	-1.1	7.5	4.1					215	3
14	410	209.4		10.1	104.0	1.5	8.1	3.0					210	4
17	440	234.2	8.0	9.4	68.1	2.0	9.8	4.3	20.6	46.1			177	5
17	480	208.0	7.4	9.9	102.5	1.3	7.5	4.2					191	6
17	500	231.3	6.9		164.6	0.0	10.7	8.1					138	7
17	440	240.7	8.5		119.1	1.9	8.2	1.6					206	8
20	650	209.1	8.7		154.0	9.7	14.3	6.0					142	9
20	540	203.6	7.6		105.4	5.8	6.4	0.95					202	10
20	490	226.8	8.2		102.8	4.0	6.5	2.2					208	11
20	540	177.5	8.4		84.0	4.1	9.0	0.7					345	12

*Average food consumption by 6 diabetic animals maintained on $NaHCO_3$ for five 24-hour periods.

TABLE IV

DURATION OF INSULIN THERAPY	BLOOD SUGAR	SERUM ALKALINE PHOSPHATASE	SERUM INORGANIC PHOSPHORUS	TOTAL SERUM CALCIUM	TOTAL URINARY NITROGEN	TITRATABLE ACIDITY OF URINE	URINARY PHOSPHORUS	URINARY CALCIUM	FECAL PHOSPHORUS	FECAL CALCIUM	FOOD PHOSPHORUS	FOOD CALCIUM	BODY WT.	
days	mg. %	units	mg. %	mg. %	mg.	ml. 0.1N NaOH	mg.	mg.	mg.	mg.	mg.	mg.	gm.	
7	220	93.3	6.4	10.2	77.9	2.9	8.7	2.3	19.3	42.7	65.0*	104.5*	345	1
7	230	94.1	7.1	9.8	72.2	3.2	8.7	2.0					320	2
7	120	102.2	8.2	9.8	73.6	3.7	9.3	2.5	14.2	40.5			300	3
8	330	60.1	7.9	10.2	101.0	2.2	8.7	2.1	5.3	9.9			245	4
8	180	31.5	7.9	10.5	105.9	2.1	8.4	1.1	9.6	26.0			290	5
8	30	94.5	9.1	9.7	60.7	0.4	3.0	0.6	4.1	11.2			278	6
8	160	67.8	8.6	10.5	53.9	1.8	7.1	0.6	9.8	27.5			298	7
8	60	89.6	11.0	11.1	92.7	2.3	8.4	1.3	3.4	14.4			292	8
8	290	78.0	6.6	6.6	65.5	2.3	6.8	1.4	5.5	9.3			320	9
8	100	68.3	7.1	8.2	70.5	1.7	6.8	1.6	10.4	23.0			355	10
8	100	90.6	6.4	9.4	63.0	2.0	6.7	1.6					320	11
9	280	73.1	8.3	11.0	101.6	3.2	10.0	2.7	7.3	12.9			222	12
9	320	68.1	7.1	10.8	98.8	2.5	9.4	1.7					282	13
9	250	49.9	6.9	10.5	85.3	2.3	8.2	1.7					286	14
9	200	39.9	7.1	10.5	48.3	1.8	7.2	1.4					342	15
10	70	86.9	8.9	9.8	89.4	1.4	6.7	2.2					280	16
10	70	67.4	7.6	9.5	63.5	2.4	5.3	1.4					295	17
10	200	88.4	7.4	9.5	85.7	2.2	8.1	3.2	18.7	38.4			330	18
10	170	84.7	7.9	10.2	73.6	1.3	6.4	1.3					325	19
12	180	87.4	8.2	10.8	58.6	1.8	6.2	2.3	20.7	48.3			265	20
12	240	75.4	7.4	10.5	47.3	1.0	4.5	1.6					290	21
12	380	80.1	7.9	9.8	56.1	1.8	6.3	2.2	17.4	47.0			325	22

*Average food consumption by 8 diabetic animals maintained on insulin for six 24-hour periods.

TABLE V

GROUP	BLOOD SUGAR	SERUM ALKALINE PHOSPHATASE	SERUM INORGANIC PHOSPHORUS	TOTAL SERUM CALCIUM	TOTAL URINARY NITROGEN	TITRATABLE ACIDITY OF URINE	URINARY PHOSPHORUS	URINARY CALCIUM	FECAL PHOSPHORUS	FECAL CALCIUM	FOOD PHOSPHORUS	FOOD CALCIUM	
	mg. %	units	mg. %	mg. %	mg.	ml.0.1 N NaOH	mg.	mg.	mg.	mg.	mg.	mg.	
I	117.6 *(24)	7.66 (24)	46.1 (24)	10.0 (24)	3.78 (22)	62.0 (8)	4.06 (22)	1.24 (22)	8.2 (3)	15.3 (3)	40.6 (44)	65.0 (44)	
II	436.0 (25)	8.00 (25)	164.6 (25)	9.56 (21)	4.66 (25)	105.5 (16)	7.70 (25)	3.00 (25)	15.7 (7)	34.3 (7)	81.0 (42)	130 (42)	
III	470.0 (12)	7.67 (10)	220.0 (12)	10.1 (6)	2.62 (12)	112.0 (12)	8.50 (12)	3.24 (12)	9.3 (3)	20.8 (3)	67.0 (30)	117.6 (30)	
IV	190.0 (22)	7.77 (22)	76.0 (22)	9.96 (22)	2.10 (22)	74.8 (22)	7.3 (22)	1.76 (22)	11.2 (13)	27.8 (13)	65.0 (48)	104.5 (48)	

*Figures in parenthesis indicate the number of determinations, respectively.

AVERAGE BLOOD, SERUM AND URINE VALUES FOR GROUPS I, II, III AND IV

Blood Sugar
(mg. glucose per 100 ml.)

Serum Alkaline Phosphatase
(Bodansky Units per 100 ml.)

Serum Inorganic Phosphorus
(mg. per 100 ml.)

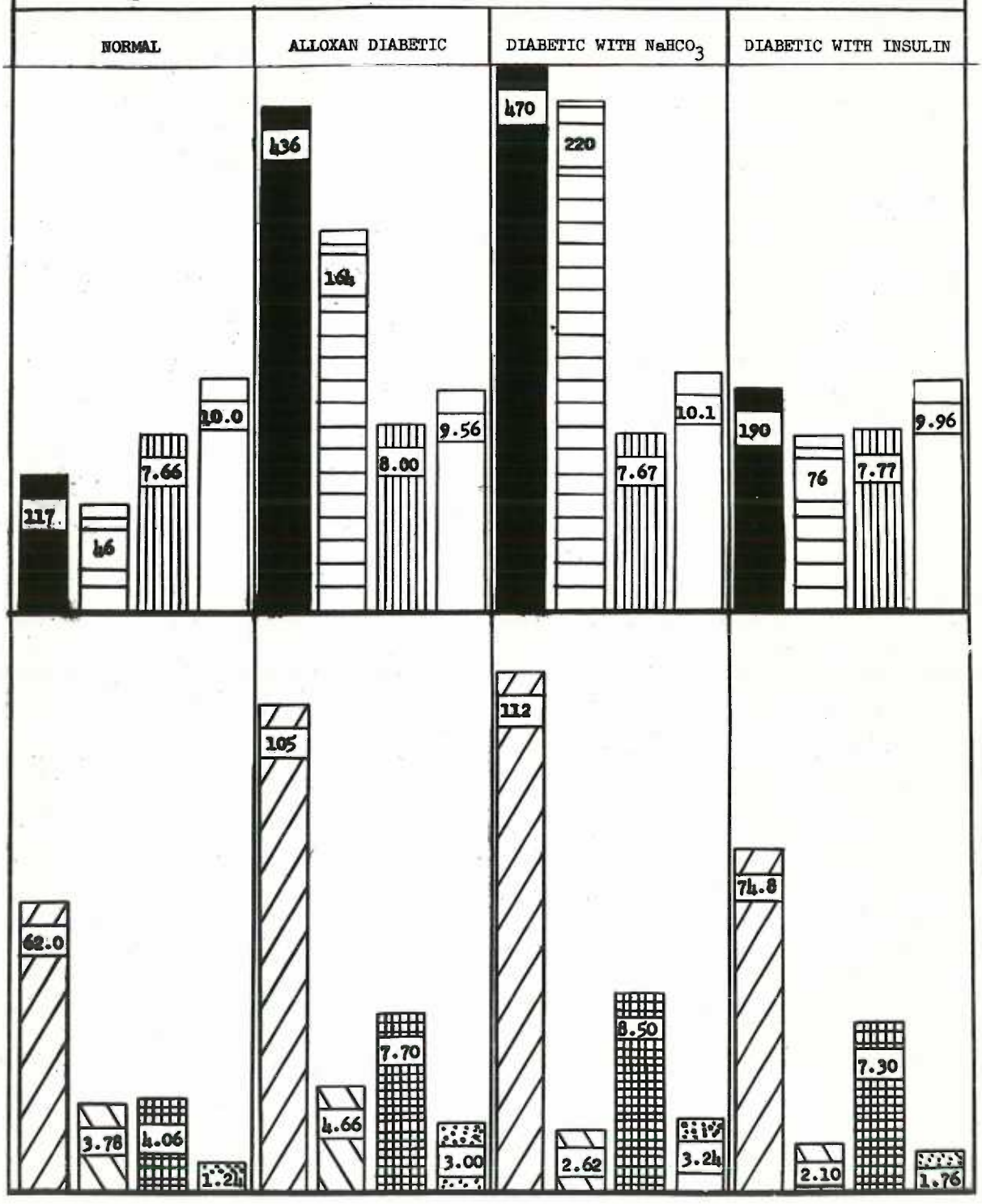
Total Serum Calcium
(mg. per 100 ml.)

Total Urinary Nitrogen
(mg. per 100 gm. body weight.)

Titratable Acidity of Urine
(ml. of 0.1 N NaOH per 100 gm. body weight.)

Urinary Phosphorus
(mg. per 100 gm. body weight.)

Urinary Calcium
(mg. per 100 gm. body weight.)



SUMMARY

1. A study of calcium and phosphorus metabolism is primarily a study of bone metabolism.

2. Bone with its organic matrix on which crystals of apatite mineral form exists in a state of equilibrium with the body fluids.

3. A hypothetical sequence of events leading to calcification has been outlined.

4. Alloxan diabetic rats showed an elevated serum alkaline phosphatase concentration and an increased excretion of calcium, phosphorus, nitrogen and titratable acidity of the urine.

5. Alkaline drinking water (75 milliequivalents of sodium bicarbonate per liter) which neutralized the urinary acidosis was fed to diabetic rats for 14 to 20 days with no significant alterations in urinary loss of calcium, phosphorus and nitrogen, nor in the blood sugar, serum calcium, phosphorus and alkaline phosphatase concentrations.

6. The blood, serum, and urinary values returned toward normal in diabetic rats treated with protamine zinc insulin.

7. The alloxan diabetic animals, the diabetic animals fed alkaline drinking water, and the diabetic animals given insulin consumed approximately equal quantities of food.

8. The calcium and phosphorus content of the feces was roughly proportional to the food intake.

9. Bone matrix contains 15 to 30 percent protein, and catabolism of tissue proteins occurs in alloxan diabetic animals.

10. The facts that alkaline therapy of diabetic rats failed to decrease the urinary phosphorus and calcium while insulin therapy was effective in decreasing the excretion of these substances under the same food intake suggest that much of the calcium and phosphorus loss was due to skeletal decalcification primarily associated with catabolism of the protein of bone matrix.

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