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PARATHYROID HORMONE EFFECTS ON INTERMEDIARY METABOLISM

by

JOHN C. COLLA

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June

1962

STRITCH SCHOOL LOYOLA UNIVERSITY OF MEDICINE

LIFE

John C. Colla was born in West Allis, Wisconsin on December 11, 1930.

He was graduated from Central High School, West Allis, Wisconsin in February, 1949. In June, 1953, he was graduated from the University of Wisconsin, Madison, Wisconsin, with the Degree of Bachelor of Science.

From September, 1953, to September, 1955, he served on active duty with the United States Army. From January, 1956, to August, 1957, he held the position of Junior Research Biochemist with the Rahr Malting Company, Manitowoc, Wisconsin.

In September, 1957, he began his graduate work in the Department of Biochemistry of Loyola University at the Stritch School of Medicine. In February, 1960, he received the Degree of Master of Science in Biochemistry.

From September, 1957 to June, 1959, he held the position of Teaching Assistant in the Department of Biochemistry. From July, 1959, to July, 1960, he was a Standard Oil Foundation Fellow. Since November, 1960, he has held a Research Fellowship sponsored by the Atomic Energy Commission.

He is co-author of the following publications:

1. "The Use of Carbon-14 Labeled Material in Malting and Brewing Research", Amer. Soc. Brew. Chemists Proceedings., 26, 1 (1957).

2. "In Vitro Effects of Parathyroid Extract", 60C, Abstracts of papers presented at Chicago, Illinois, September 3-8, 1961, Division of Biological Chemistry of the American Chemical Society, 140th National Meeting.

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The Author wishes to express his gratitude to Dr. Maurice V. L'Heureux for his guidance and constructive criticisms throughout this work, to the other members of the staff for their support and to my fellow graduate students for their companionship and friendly banter without which graduate study would have been less exciting.

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CHAPTER I

INTRODUCTION

Hanson in 1924 (80) and Collip in 1925 (33,34) independently extracted the biologically active principle of parathyroid glands which consistently prevented or relieved tetany in thyroparathyroidectomized dogs. For many years the best hormone preparations were of low potency and it was not until 35 years later that Rasmussen and Craig (170,171) purified the active principle and determined that the protein hormone had a molecular weight of approximately 9500 with a potency of 2000 units per milligram.

Early in the development of parathyroid hormone research it was established that the hormone monitored calcium and phosphate homeostasis. However, the general mechanism by which it accomplished this was a subject of controversy. On the one hand it was thought that the calcium-mobilizing activity was the main function of the hormone and that the phosphaturic activity of the hormone was an artifact. On the other hand there were those who maintained that the phosphaturic activity was the primary function. The demonstration that the highly purified parathyroid hormone has both calcium-mobilizing and phosphaturic activities resolved this issue (9,170) and it is accepted today that the hormone affects both the kidney and bone with respect to calcium and phosphate metabolism.

Since both calcium and phosphate are intimately involved in many areas of metabolism, the elucidation of the specific mechanism of action of parathyroid hormone on these two substances is complex. For example, calcium participates in the formation of bones and teeth, in the maintenance of blood coagulation, in membrane permeability, in cardiac rhythmicity, in excitability of nerves and muscles, in muscular contraction, in renal tubular concentrating mechanisms and in enzyme activities (172). Phosphate is involved in the formation of teeth and bones, in the formation of organic phosphates for structure or energy-transferring functions and in maintaining the body fluid hydrogen ion concentration through the phosphate buffering system (172). Thus slight changes in calcium or phosphate levels can lead to gross biochemical changes which can easily be misinterpreted to be directly involved in the mechanism of action of the parathyroid hormone. Furthermore, there are other biological substances that affect calcium and phosphate metabolism and these may become involved directly or indirectly by the parathyroid hormone. Two of these substances are vitamin D and insulin.

In the case of vitamin D, the relationship between it and the parathyroid hormone is not yet clear. Both vitamin D and parathyroid hormone at times appear to have the same effects on the organism (172). Harrison and Harrison (83) observed that the levels of serum citrate progressively increased

following the intramuscular injection of vitamin D. Along with the increase in citrate there occurred increases in serum phosphate and serum calcium levels. However, the injection of citric acid was not effective in the treatment of rachitic rats (147) and vitamin D deficient rats did not respond to injected parathyroid hormone (125). Vitamin D, simultaneously administered with parathyroid extracts to albino rats, prolongs the activity of parathyroid hormone while the hormone probably increases the inhibitory effect of vitamin D on tubular reabsorption of phosphate (95). It has been suggested that vitamin D is necessary for the normal action of parathyroid hormone (98, 124,148). Parathyroidectomy in rats, however, did not affect either the absorption of calcium or response of calcium absorption to administration of vitamin D (72). Selye et al (188) called attention to the fact that the same sensitizing agents that are effective in increasing the action of parathyroid hormone also proved to be potent sensitizers for the induction of cardiovascular lesions by certain vitamin D derivatives and corticoids.

With reference to insulin, there have been numerous reports in the literature concerning the hypoglycemic action of parathyroid hormone (21,151,154,157,168,179,185,229). Subsequent to parathyroid hormone injection there occurs a fall in the blood glucose of normal and diabetic men (152,153,154) and in normal and diabetic dogs (157,168,185,229) while para-

thyroidectomy in rats raises the blood sugar level (129). Various experiments and histological investigations in rats have shown that the hormone stimulates insulin secretion (179, 129) by stimulating the islands of Langerhans (152) and that it increases the glucose-tolerance and sensitivity to insulin (179). No effect of parathyroid hormone is obtained in severe cases of diabetes nor is there a local hypoglycemic effect by the hormone (153).

Insulin administration to normal and parathyroidectomized dogs not in tetany will increase calcemia and decrease phosphatemia (174); while in tetany, insulin will relieve the symptoms, usually completely, and will produce hypophosphatemia (174,175). (In every case of tetany, the net result of insulin injection is an increase of the calcium/phosphate ratio in the serum).

Thus, bearing in mind the many areas of metabolism in which calcium and phosphate are involved and the various agents other than parathyroid hormone that affect calcium and phosphate, it is worthwhile to elaborate on the general aspects of parathyroid hormone regulation of calcium and phosphate.

The parathyroid glands have been demonstrated to be under direct humoral control. Copp (38) and others (62,76, 138,159,210) perfused isolated thyroid-parathyroid glands with high or low calcium blood. The low calcium blood appeared to

release parathyroid hormone or a substance with identical action. Patt and Luckhardt (159) showed that when the perfusate was injected intravenously into normal dogs, there was an increase in serum calcium within one and one-half to three hours and also in most instances an increase in serum inorganic phosphate of almost the same magnitude. Crawford et al (40) postulated that hyperphosphatemia per se stimulates increased parathyroid activity. Chang (23) found that transplants of parathyroid glands could maintain the biological requirements for the hormone which suggested that humoral control rather than the nervous system was responsible for stimulating parathyroid hormone secretion.

The principal sites of regulation of calcium and phosphate in the blood are (1) the intestine, which is the portal of entry, (2) bone, which is a storehouse of calcium and phosphate and (3) the kidney, which is the portal of exit (146). In addition to these three sites, the mammary glands and the salivary glands have been implicated as areas of parathyroid hormone regulation of calcium although again to a much lesser degree (105). In both of these glands parathyroid hormone causes an increase in the calcium content of their respective secretions. The stimulatory effect of parathyroid hormone on the intestinal absorption of calcium is not very striking although it has been shown to occur (36,39,169,180).

The direct action of parathyroid hormone on bone

independent of its action on the kidney has been established (11,12,23,24,35,44,49,66,68,75,93,186). Collip (35) found histological evidence of parathyroid-induced resorption in nephrectomized animals. Barnicot (11,12) and Chang (23,24) transplanted parathyroid tissue into direct contact with bone and demonstrated histologically-active bone resorption in the area immediately adjacent to the transplant. Chang (23) also observed that a small amount of new bone formation occurred laterally to the parathyroid graft. Ellsworth (49), Selye (186), Ingalls (93), Stewart (199) and Grollman (75) also reported the hypercalcemic action of parathyroid hormone in nephrectomized animals. In a classical series of experiments, Gaillard (66-70) demonstrated that parathyroid hormone induced bone resorption in tissue cultures and that the effects were proportional to the amount of hormone used.

The effect of parathyroid hormone on the kidney has been a subject of many studies (1,2,40,48,77,85,90,96,114,132, 144,148,212, 217,218). As early as 1925, Greenwald (74) demonstrated a reduced urinary excretion of phosphate following parathyroidectomy. This observation has since been confirmed by other investigators (3,14,20,176,201,216). Albright and Ellsworth (1) first demonstrated the phosphaturic action of parathyroid hormone on a patient with hypoparathyroidism. Hogben and Bollman (91) using intravenous injections of parathyroid extract found no inhibition of phosphate reabsorption

but did obtain a phosphate diuresis within two hours in intact dogs. A phosphate diuresis was also obtained in intact mice (20).

Ito et al. (94) found a 70 per cent increase in tubular reabsorption following parathyroidectomy. Samiy and coworkers (178) injected parathyroid hormone into parathyroidectomized dogs and found increased phosphate excretion but decreased tubular reabsorption. The effects appeared to be directly on the reabsorption in both proximal and distal segments of the nephron (178). A biological assay of the phosphaturic activity of parathyroid hormone is based on the increased urinary excretion of phosphate by two month old male rats during the first hour after parathyroidectomy (102).

With the advent of very pure preparations of parathyroid hormone, the controversy of whether the parathyroid hormone affects the kidney directly has been resolved. Pullman and Lavender (164) and Lavender et al. (109) on adminstration of very dilute solutions of the purified hormone by slow infusion into one renal artery in dogs demonstrated a unilateral phosphaturia which was a consequence of a depressed tubular reabsorption. Davies (41) and Fujita et al. (65) have isolated parathyroid hormone from human urine in both normal and hyperparathyroid subjects.

The current belief is that the action of parathyroid hormone on bone and kidney involves some effect on intermediary

metabolism which in turn regulates calcium and phosphate. The primary evidence for this is that plasma is normally supersaturated with bone salts (139,202). As a consequence, (1) some cellular mechanism must be postulated by which a supersaturated condition can be maintained *in vivo*, (2) the driving force of the mineralization process in calcification is present in plasma itself and (3) some active mechanism must be present in the intestinal wall to allow the absorption of calcium against a concentration gradient (202). The function of the parathyroid hormone is related to the first of these consequences while vitamin D is related to the last.

Hansard *et al* (79) observed that in young cattle calcium disappeared from the blood at a rapid rate but despite this the animals were able to maintain the concentration of calcium ion in the blood plasma at approximately the same level. The demonstration of the rapidity of the turnover of calcium in the blood illustrates that the relatively slow acting parathyroid control, while responsible for hour to hour or day to day adjustment, is inadequate for the minute to minute interaction between blood and bone. Thus, in spite of the establishment of a parathyroid hormone regulation of serum calcium by some action on bone resorption, it is necessary to postulate a dual mechanism for the homeostatic control of calcium. One part acts rapidly, requires ion transfer or ion exchange between blood and bone, is independ-

ent of the function of the parathyroid glands and in the absence of these glands maintains the plasma calcium level at approximately 7 mg per cent (37). Talmage and Elliott (205) applying the technique of peritoneal lavage demonstrated that parathyroidectomized rats could mobilize calcium from bone at the same rate as normal rats. These same authors using the same technique in nephrectomized rats on a calcium-free diet and parathyroidectomized during lavage found that changes in radio-calcium were the same as calcium if the tracer was injected 2-3 weeks before the experiment, but injections just prior to the experiment created a situation in which parathyroidectomy caused a drop in total calcium lost to the wash but no change in radio-calcium lost to the wash.

The second part of the dual mechanism for control of plasma calcium acts by a feedback through the parathyroids and is responsible for the maintenance of the normal serum calcium level. This part has access to the stable fraction of the bone mineral by control of osteoclastic resorption (238). The humoral control of parathyroid function discussed previously is implicated in this mechanism.

Additional evidence in support of the hypothesis that parathyroid hormone affects some area of intermediary metabolism is the fact that one of the first effects of hormone administration is the elevation of the serum citrate concentration (115).

Changes in the content of citrate and calcium tend to parallel each other in epidermal carcinogenesis (133) and in blood (25). Shorr et al, (196) found parallel changes in the calcium and citrate content of the urine of males and post-menopausal women following variations in the diet and also in one subject with hypoparathyroidism subsequent to injection with parathyroid extract. Dickens (42) demonstrated that bone has a high concentration of citrates, approximately 70-90 per cent of that found in the body. This observation has been confirmed by Class (29) and Kenney et al, (101). Dickens (42) also demonstrated that the variation of citrate content greatly exceeds that of the other bone constituents, which suggested that the citrate is in a relatively available form in vivo.

Bone mineral has a high affinity for citrate ion (6,118,142). Mandl et al, (118) injected white rats with radioactive citrate and reported five per cent of the activity in the skeleton in one hour and very little radioactivity in the expired air four hours after injection.

The ability of parathyroid hormone to alter citrate metabolism was first suggested by Greenwald(73). L'Heureux and Roth (115) showed that subcutaneous injection of parathyroid extract into normal and thyroparathyroidectomized rats resulted in a definite increase in the serum citric acid level. Sjostrom (197) showed the influence of parathyroid hormone level on citric acid in the plasma when he found an

elevated level of citric acid in patients with hyperparathyroidism. Elevation of the plasma citrate level was noted to accompany bone resorption in dogs (5). Dixon and Perkins (44) studying the disintegration of bone in response to parathyroid extract postulated some direct action of the hormone on bone which was partly due to an influence on citrate metabolism. Freeman (57) linked the mechanism of bone resorption and maintenance of normal level of calcium in the circulating fluids to citrate and its metabolism. Elliott and Talmage (47) showed that tracer calcium was lost to the fluid used for peritoneal lavage in nephrectomized and parathyroidectomized-nephrectomized rats and this amount of calcium lost by the rats was increased when citrate was added to the fluid. Control rats lost more calcium than parathyroidectomized rats. The demonstration that the addition of citrate to the fluid did not affect the loss of tracer calcium administered less than twenty-four hours previously supported the suggestion that citrate mobilized calcium from deep areas of bone (47).

One mechanism of action of parathyroid hormone is related to the ability of citrate to complex with calcium. Sendroy in 1927 (189,190) and Thunberg in 1929 (214) postulated that citrate in the tissues and body fluids formed a soluble diffusible complex with calcium ion. This postulation has been supported by other investigators (17,86,133,140,141,156). The first direct evidence for the complex formation between

citrate and calcium was obtained by Shear and Kramer (191).

Many of the di-and tricarboxylic acid intermediates of the Krebs cycle can react with calcium forming relatively unionized soluble complexes (99,121,122,184). The tendency for complex formation is greatest for the tricarboxylic acids with citrate being the most active chelating compound of these (99,101).

Clark and Geoffroy (28) elicited a rapidly increased urinary excretion of radioactive calcium in rats with subcutaneous injections of sodium citrate regardless of whether the isotope was given two or sixty days before the injection of citrate. In the case of animals treated sixty days before with the isotope, the increased urinary radio-calcium resulting from sodium citrate, amounting to a 6 to 10 fold increase, must be derived from a storehouse of older bones and not from recently deposited calcium.

Kenney et al, (101) analyzed media from resorbing mouse calvaria in tissue cultures for citric acid, calcium and phosphate while studying the resorption of bone seen morphologically. He found parallel increases in citric acid, calcium and phosphate; the cumulative increase in citric acid was 8-19 times greater than the total citric acid content of the original calvaria. He concluded that resorbing bone was capable of producing citric acid metabolically. Other workers have demonstrated an increased release or production of citric

acid from bone after injection of parathyroid extract (42,52, 130, 145,186). Harrison (81) presented evidence that parathyroid hormone increased the activity of bone cells particularly with respect to the production of citrate.

Elliott and Freeman (45) found that nephrectomy produced a transient hypercitricemia and a hypercalcemia in rats and rabbits while parathyroidectomy four days prior to nephrectomy abolished these effects. It seemed that parathyroid hormone had some direct effect on citric acid metabolism because the hormone restored the calcium and citrate responses of parathyroidectomized rats to nephrectomy.

Because of the high initial content of citrate in bone, it is difficult to distinguish the relatively inert extracellular citrate from more labile intracellular citrate. Lekan (111,112) incubated bone with pyruvate-2-C^{14} and obtained approximately twice as much incorporation of isotope in the citrate produced by bone from parathyroid hormone-treated animals as that of control bone. Ranney(167) studied the incorporation of acetate-1-C^{14} into bone citrate as influenced by parathyroid hormone and estrone and found that estrone increased both the citrate synthesis and concentration while parathyroid extract enhanced labeled citrate formation with little change in bone citrate concentration.

Bone as a tissue is difficult to work with and therefore an extensive study of the enzymes in it is lacking. However,

the Krebs cycle has been shown to be present and functioning in bone (44,107,108,110,221,222,223). The presence of considerable isocitric dehydrogenase activity has been demonstrated in femurs (104,221,222,223) in contradiction to Dixon and Perkins (44) who found low values for this enzyme. Hekkelman (87) reported that parathyroid hormone caused a significant decrease of isocitric dehydrogenase in rabbit bone with no significant change in aconitase activity. Van Reen and Michalakis (223) however, have not been able to confirm these results. Normal bone has an extremely low rate of carbohydrate metabolism (108). Raisz et al (165) reported that glycolysis was active in bone and that it appeared necessary for calcium mobilization. Laskin and Engel (107,108) demonstrated that after administration of parathyroid extract, anaerobic glycolysis was unaffected in bone but the oxygen consumption was markedly depressed. Further investigations by these authors revealed the decreased respiration was related to diminished succinic dehydrogenase activity. Schartum and Nichols (181) found that the defects of glucose metabolism were dependent upon steps beyond phosphate dehydrogenase, the rate of lactate production seemed only indirectly involved. Cohn and Forscher (30,31) indicated that parathyroid hormone produced modification in the respiratory pathways of metabolism in bone although no evidence for alteration of citrate metabolism in the demineralization of bone were obtained. Raisz et al (165) also were unable to find any alteration of

citrate metabolism in the demineralization. They did indicate, however, that the mineral equilibrium between surviving bone and its bathing fluid was dependent on the metabolic activity and could be modified by prior changes in parathyroid function.

The kidney is the organ which removes circulating citrate from the serum (25,43,59,60,89,127,128,131). The liver takes only a small part in the metabolism of circulating citrate (127,128,155). Martensson (126) first reported the active part taken by the renal parenchyma in the oxidation of citrate and indicated that the amount of this compound to be found in the urine is dependent on both renal oxidation and the serum citric acid level. In nephrectomized rabbits the serum concentration of citric acid rises 4 to 5 times its normal value. Freeman and Elliott (61) reported that the elevation of plasma citrate produced by fluoroacetate (an inhibitor of the further degradation of citric acid in the Krebs cycle) into rats was augmented by parathyroidectomy and nephrectomy and that parathyroid extract in sufficient amounts to restore the plasma calcium to normal, reduced the response of parathyroidectomized rats to sodium fluoroacetate. These authors also reported a maximal hypercalcemia of approximately 15 milligrams per cent occurs in rats with elevation of the plasma citrate level and that endogenous citrate can mobilize calcium from the skeleton in the absence of parathyroid glands.

Lindenbaum (116) showed that within one hour after the

injection of sodium fluoroacetate, a fifty-fold increase in the concentration of citric acid in the kidney occurred. By far, the largest part of citric acid supplied to the kidney is converted, only a small part being excreted in the urine (127). Citrate is quickly eliminated from the blood following intravenous injection: 60-120 mg per kg body weight disappears practically completely in 1 to 1.5 hours (128). Herndon and Freeman (89) studying the simultaneous renal blood flow and citrate uptake by the kidneys of dogs, determined that several grams of citrate may be removed daily from the circulation by this organ, an amount far in excess of that excreted in the urine. The capacity of the kidney to metabolize circulating citrate is much greater than the actual performance under ordinary conditions. Meintzer and Freeman (131) demonstrated the rapid turnover of circulating citrate by following the radioactive carbon dioxide in the expired air following the injection of C^{14}-labeled citrate. The half-life was determined to be 47 minutes; nephrectomy increased this to 80 minutes and parathyroidectomy increased it to 65 minutes. Combined nephrectomy and parathyroidectomy lead to a half-life of circulating citrate equal to 148 minutes. The injection of vitamin D or parathyroid extract could restore the citrate half-life of parathyroidectomized animals to near normal.

On administration of malic acid, citric acid is not formed in the kidneys (127). L-malic acid suppresses or arrests

the normal breakdown of citric acid in the kidneys and at the same time brings about a large increase in the urinary excretion of citric acid (127,198).

Komarkova et al (103) demonstrated that parathyroid extract raises sharply the concentration of citric acid in the kidney after latent periods of 60 minutes and reaches a maximum in 2-3 hours after injection. The concentration of citric acid remains at approximately the same level up to 10 hours after injection and returns to about the starting concentration after 24 hours.

Statement of the Problem

The early studies of the effect of parathyroid hormone on the kidney were concerned with the dynamics of phosphate metabolism. Only recently has the effect of parathyroid hormone on the intermediary metabolism of the kidney been investigated.

The observation that parathyroid hormone injection leads to a rise in the citrate content of the kidney, in spite of the fact that the kidney has a great capacity for removing citrate from the circulation, poses the problem of whether this increased citrate arises from the increased citrate concentration in the serum or whether the metabolism of citrate in the kidney is affected by the parathyroid hormone.

Most measurements of the influence of parathyroid hormone on tissues in vitro have been concerned with the effect of the hormone on one or two enzyme levels or on the utilization

or production of one or two metabolites. In this study an effort was made to measure the influence of parathyroid hormone on the metabolism of several intermediates of the Krebs cycle in metabolizing kidney tissue.

Homogenates of kidneys from parathyroid hormone-treated rats and control rats were incubated with various C^{14}-labeled substrates in sealed Warburg vessels. The substrates included glucose-1-C^{14}, acetate-1-C^{14}, succinate-2,3-C^{14} and citrate-1,5-C^{14}. The metabolism of the homogenates was stopped and the radioactive carbon dioxide evolved was collected and its activity determined. The radioactive organic acids of the Krebs cycle, arising from the metabolism of the C^{14}-labeled substrates, were separated by column chromatography and the activity associated with them determined. A comparison of the radioactive carbon dioxide evolved and the organic acid profiles of the control kidneys to that of the parathyroid hormone-treated kidneys indicated whether the intermediary metabolism of the kidneys was affected by the parathyroid hormone.

In addition, in the early stages of study of the effect of parathyroid hormone on intermediary metabolism, an attempt was made to obtain a preparation of bone suitable for incubation with labeled substrates.

CHAPTER II

MATERIALS AND METHODS

A. Standard Analytical Procedures.

The standard procedures for the determination of glucose (54), phosphorus (53), deoxyribonucleic acid (183) and citric acid (13) are presented in the Appendix. The standard curves for these analyses are given in Figures 1 to 4. Two methods for the determination of calcium were used. One was the method of Ashley and Roberts (8) and the other that of Roe and Kahn (177). The standardization procedures for these methods are presented in the Appendix.

B. Paper Chromatography of Organic Acids.

The organic acids were chromatographed on paper by two different methods. The method of Jones et al (97) employed an alkaline developing solution consisting of 95% ethyl alcohol, ammonium hydroxide and water (8:1:1) while the method of Loeffler and Reichel (117) employed an acid developing solution consisting of ethyl acetate, acetic acid and water (3:1:1). Small quantities of the organic acids (usually between 2 and 10 gamma) were spotted on Whatman #1 chromatography paper. Ascending chromatography was employed at room temperature for 22 hours in 6 by 18 inch chromatography jars. When the alkaline system was used, the movements of the individual acids were compared

to that of malic acid. The chromatograms developed with the alkaline system were dried at room temperature for one hour and then sprayed with a 0.4% solution of chlorophenol red in ethanol made slightly alkaline with 0.1N sodium hydroxide. The acids appeared as yellow spots on a purple background.

When the acid system was used it was necessary to dry the chromatograms at room temperature for at least two days to insure that all the acetic acid in the developing solvent was removed. These chromatograms were sprayed with Hargreaves indicator (7). The organic acids appeared as yellow spots on a green background. The results of a number of determinations employing both systems are given in Table I.

TABLE I

PAPER CHROMATOGRAPHY OF ORGANIC ACIDS

Acid	Alkaline System R_f (% Malic)*	Acid System R_f*
Citric	0.30	0.47
Aconitic	0.50	0.55
Malic	1.00	0.78
Succinic	1.16	0.84
Fumaric	1.41	0.91

* Average of a minimum of five determinations.

C. Column Chromatography of Organic Acids.

The modified method of Swim and Utter (203) using Celite as the stationary phase and chloroform and butanol as developing solvents was employed to separate and characterize the organic acids.

Celite 535 was prepared for chromatography by percolating 1 liter of peroxide-free ether per 100 grams of material packed in a large diameter column. The rate of percolation did not exceed 25 ml/minute. The Celite was spread in a thin layer on aluminum foil and allowed to dry at room temperature overnight. It was then heated in an oven at 100° C for 6 hours and finally stored in a closed container.

The preparation of the column and the addition of the sample was as follows: A 10 gram sample of the prepared Celite 535 was thoroughly mixed with 5.0 ml of 0.5N sulfuric acid in a 250 ml beaker. After mixing, a 100 ml aliquot of chloroform (previously washed with 0.5N sulfuric acid) was added and the fine slurry was transferred to a 1.6 cm column containing a cotton plug at the bottom. The transfer was best accomplished in small portions with each addition being accompanied with gentle tapping of the sides of the column. This insured that trapped air bubbles rose to the surface. When addition was complete, the column was packed under nitrogen pressure (3.5 lbm/sq in.). A 1.0 ml aliquot of the sample to be chromatographed was mixed with 2.0 grams of Celite in a small beaker.

A small amount of chloroform was added and the mixture was transferred to the column with the aid of a small spatula. The beaker was rinsed with small portions of chloroform and the washings were added to the column. The last traces of Celite were removed from the beaker by wiping it with a glass wool plug and the plug was inserted into the column. The plug serves to hold the Celite in place and should be large enough to sweep down particles adhering to the top and sides of the column. A small aliquot of chloroform was added to the column and the column was repacked with nitrogen.

Chloroform, and chloroform containing 5,10,15,20,25, 30,35,40 and 50 per cent (v/v) n-butanol were used as the developing solvents. Each solvent was previously equilibrated with an equal volume of 0.5N sulfuric acid. 100 ml of each developing solution, except the 50 per cent mixture, where 200 ml was used, was allowed to run though the column at a rate not exceeding 4 ml/minute. The effluent was collected in 10 ml fractions with the aid of a Gilson Medical Automatic Fraction Collector.

One drop of phenol red indicator solution and 1 ml of distilled water was added to each fraction and the acid in the fraction was titrated with 0.0075N sodium hydroxide. During the titration, intimate contact between the organic and aqueous phases was obtained by mixing with a Vortex mixer.

Various standards were chromatographed individually

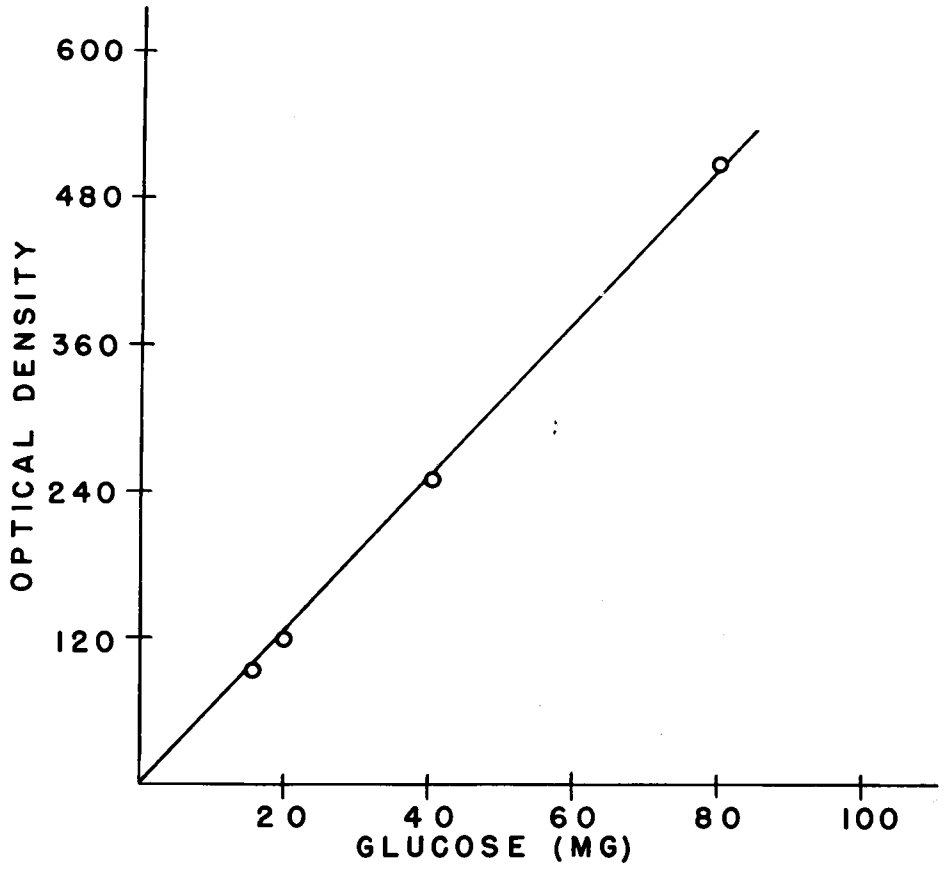

FIGURE 1.

GLUCOSE STANDARD CURVE

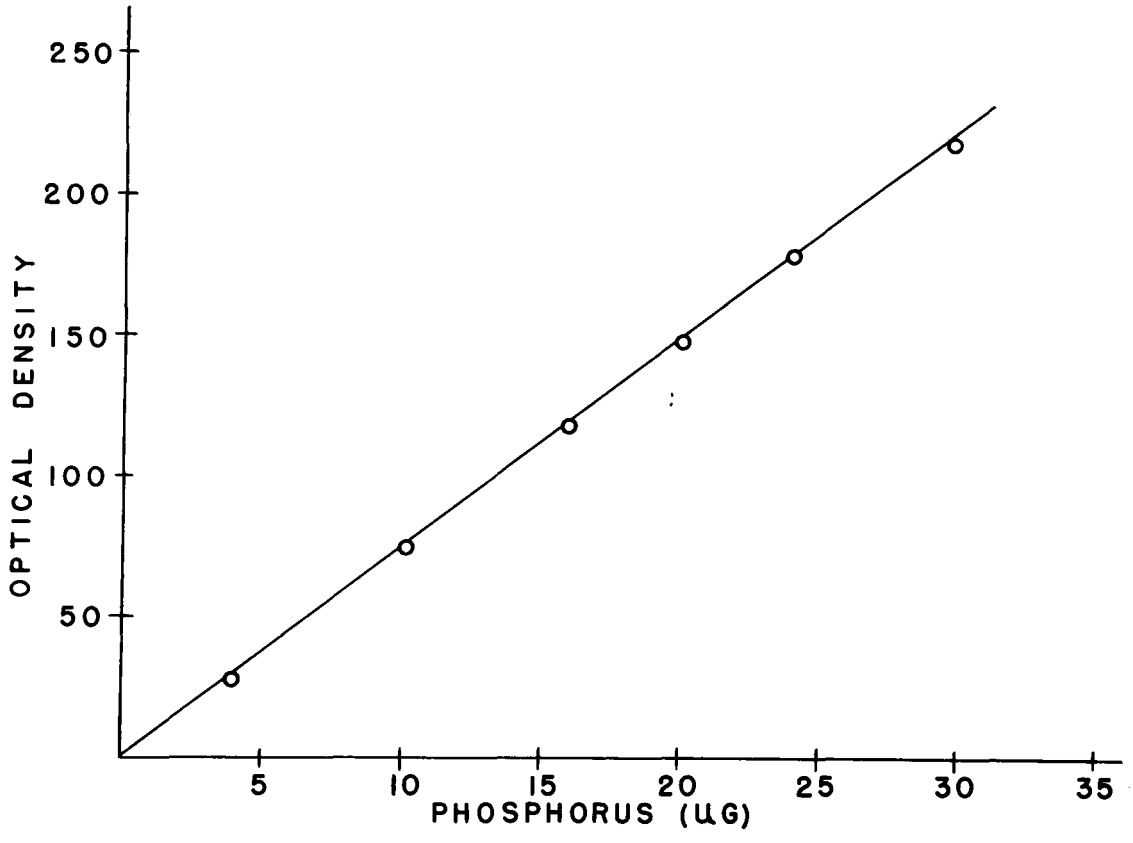

FIGURE 2.

PHOSPHORUS STANDARD CURVE

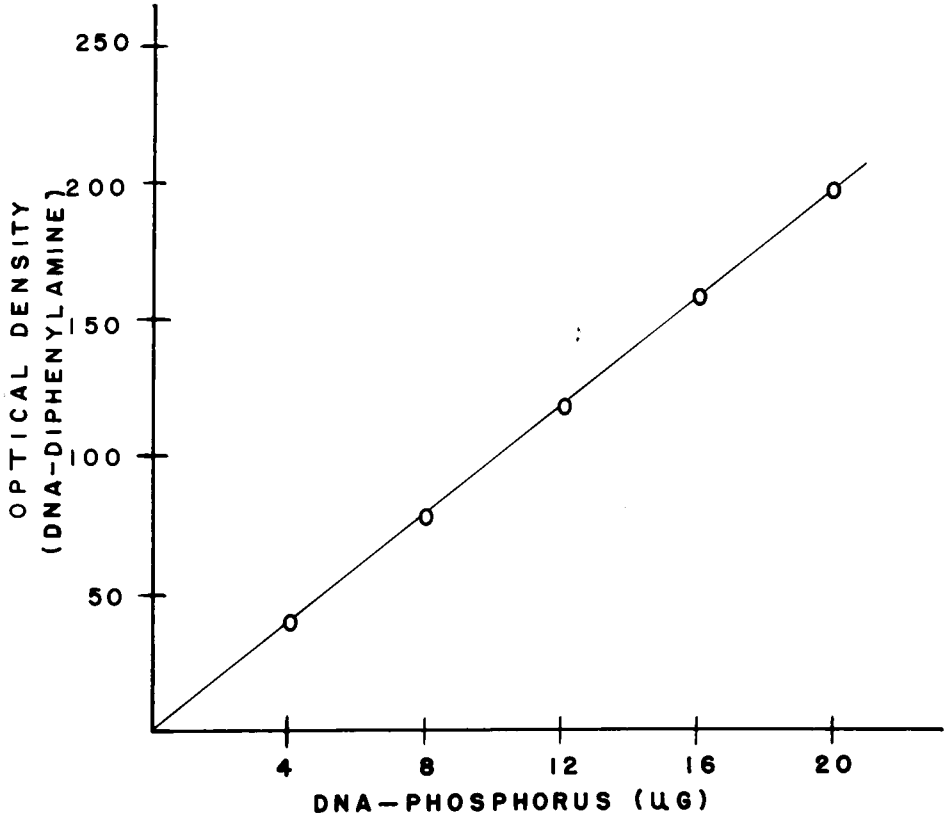

FIGURE 3.

DEOXYRIBONUCLEIC ACID STANDARD CURVE

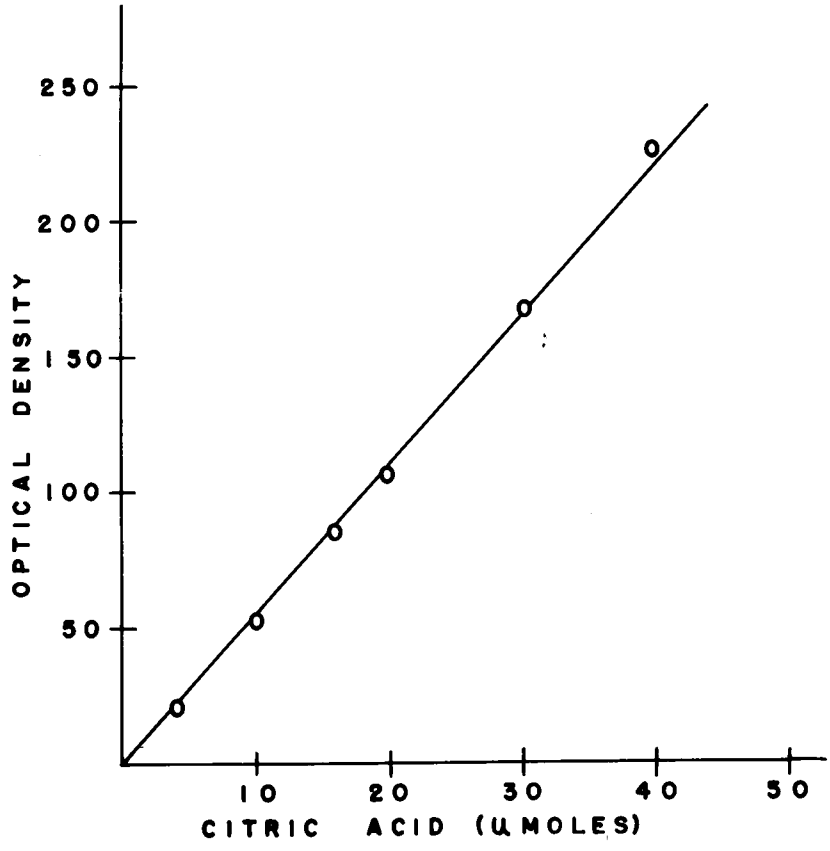

FIGURE 4.

CITRIC ACID STANDARD CURVE

and in combinations with each other. A typical separation of a mixture of organic acids is presented in Figure 5. A summary of the results obtained with acids chromatographed individually and in combination is given in Table II. The effluent peak volume is the volume at which the maximum concentration of the acid emerged from the column. The acids were confirmed by paper chromatography; citrate was also determined by chemical analysis.

D. Radioisotope Techniques.

Carbon dioxide evolved by the kidney homogenate incubations in Warbug vessels was trapped in potassium hydroxide in the center wells of the vessels by the method of Umbreit et al (219). The expired carbon dioxide of animals receiving carbon-14 labeled substrates was collected in alkali scrubbers placed at the exits of the respiration chambers. The carbon dioxide was plated as barium carbonate and the radioactivity was determined in a Tracerlab windowless gas-flow counter assembly. The measured activities were corrected for the self-absorption of barium carbonate. The procedure followed is given in the Appendix and the self-absorption curve appears in Figure 6.

The radioactivity associated with the organic acids in the fractions collected by column chromatography was determined as follows: a 1.0 ml sample of water plus 1 drop of phenol red indicator was added to each fraction and the acid

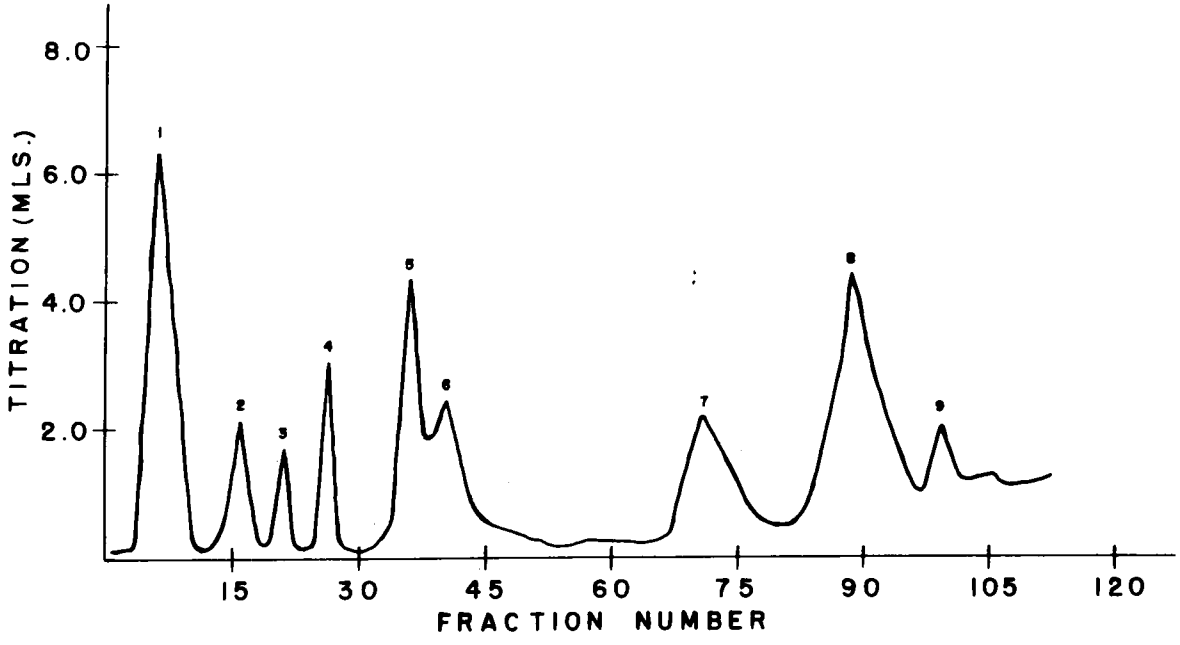

FIGURE 5.

SEPARATION OF ORGANIC ACIDS BY COLUMN CHROMATOGRAPHY

The numbers over the peaks correspond to the numbers of the acids listed in Table II.

TABLE II

COLUMN CHROMATOGRAPHY OF ORGANIC ACIDS

Organic Acid	Number of Determinations	Peak Effluent Volume (ml)
1. Trichloroacetic	18	70
2. Acetic	18	170
3. Pyruvic	8	200
4. Fumaric	17	260
5. Succinic	12	370
6. alpha-Ketoglutaric	4	410
7. Malic	13	720
8. Citric	13	890
9. Isocitric	13	980

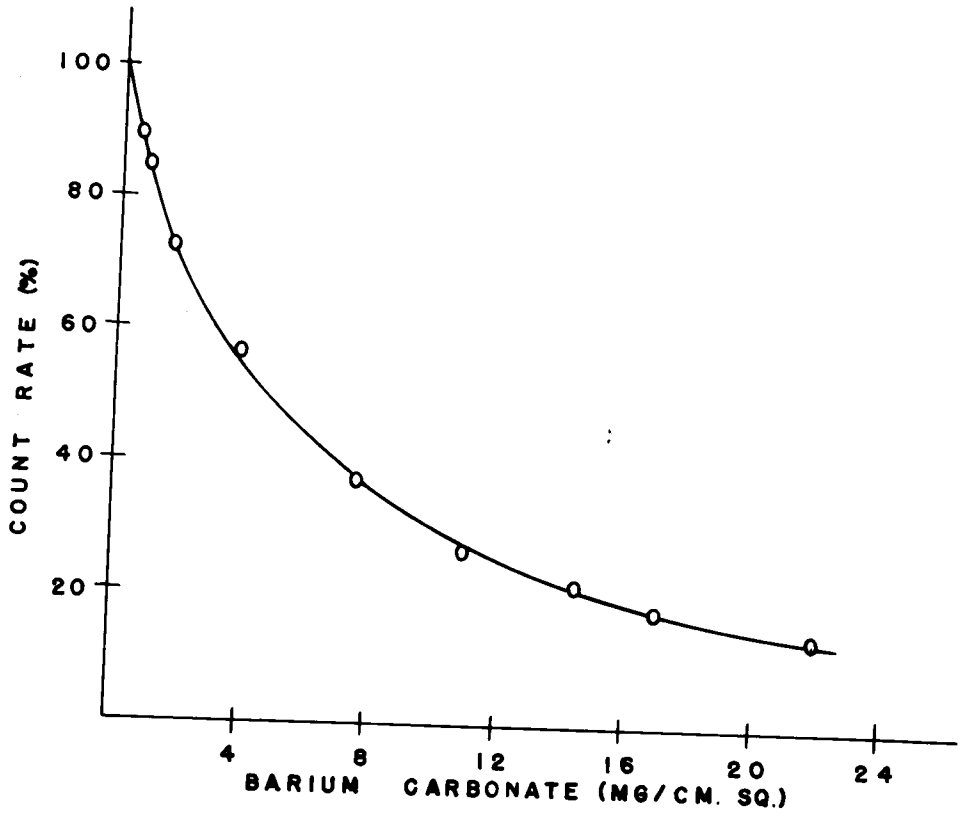

FIGURE 6.

SELF-ABSORPTION OF BARIUM CARBONATE

was titrated with 0.0075N sodium hydroxide. Thorough mixing was obtained with a Vortex mixer. Titration was continued until the aqueous layer remained a definite red. When the aqueous layer separated from the organic layer, it was siphoned off and transferred to a stainless steel planchet. The solution was then evaporated to dryness in an oven. The organic phase was washed two more times with 0.0075N sodium hydroxide. Each wash was transferred to the planchet that contained the original aqueous layer and it was re-evaporated to dryness. The radioactivity was then measured with a Tracerlab windowless gas-flow counter assembly.

CHAPTER III

EXPERIMENTAL AND RESULTS

A. Embryo Experiments

As was previously mentioned, there has been very little work done with bone homogenates. The study of the bone tissue presents many technical problems. It is not easily dissected from the animal. It is not susceptible to homogenization by the ordinary mild techniques and it is difficult to obtain uniform and reproducible preparations from experiment to experiment. It was hoped that a tissue not yet calcified to any great degree would circumvent some of these difficulties.

The most likely prospect appeared to be embryonic bone. However, before embryonic bone was used, it was necessary to determine whether the growing embryo was affected by parathyroid hormone since it was essential to obtain experimental bone which was under the influence of parathyroid hormone.

It was decided to determine the calcium and phosphorus contents of the embryo and the fluids bathing the embryo after the embryo was injected with parathyroid hormone. If the hormone affected the embryo, it was hoped that this would be reflected in the levels of calcium and phosphorus in the embryo or the fluids bathing the embryo.

In the first experiment one dozen fertile white

Leghorn eggs were incubated at 37° C for 15 days. Each day the eggs were rolled and then cooled to room temperature for fifteen minutes. Candling of the eggs revealed that eight embryos were apparently alive at the end of fifteen days.

Four of the eggs were injected with 0.15 ml of physiological saline and four were injected with 0.15 ml of lyophilized Injection Parathyroid (Lilly) reconstituted with physiological saline. The reconstituted hormone preparation contained 50 USP units/0.15 ml. The solutions were injected into the allantoic area of the eggs. The eggs were then incubated at 37° C for four additional hours. At the end of this time the eggs were quickly processed as follows: Each was broken open into an evaporating dish and the embryo was separated from the yolk sac and allantoic sac. The embryo was washed with distilled water, blotted on filter paper, weighed on a triple beam balance and then homogenized with 25 ml of distilled water in a Waring Blender. The yolk sac, allantoic sac, and washing remaining in the evaporating dish were homogenized in a Potter-Elvehjem homogenizer. Each homogenate was then treated with an equal volume of cold 20 per cent trichloroacetic acid. The precipitated material was removed by centrifugation. The supernatant was poured off and saved. The precipitate was washed with 5 per cent trichloracetic acid and recentrifuged. The wash was combined with the original supernatant. The washing and the recentri-

fugation of the precipitate was repeated a second time. The supernatant and the washings were combined and after dilution to volume, calcium and inorganic phosphate were determined. The calcium was determined by the method of Roe and Kahn (177). The washed precipitate was extracted five times with 20 ml portions of ethanol. After each extraction, the precipitate was centrifuged and the ethanol was discarded. The precipitate was then extracted twice with 10 ml of ether and the centrifuged extracts were also discarded. The final precipitate was extracted four times with 25 ml portions of hot 5 per cent trichloracetic acid and the deoxyribonucleic acid content of the extract was determined. The weights of the embryos and the analytical results for calcium, phosphate and DNA determinations are given in Tables III and IV. In the surviving egg embryos there are no striking differences in the calcium and phosphate content between controls and parathyroid treated embryos or the fluids bathing them.

The experiment was repeated with more eggs. All the conditions were identical with those in the first experiment except that additional aliquots in duplicate were removed from the embryo homogenates and fluid homogenates for glucose determination. Glucose was determined on the protein-free filtrates of both the embryo and fluid homogenates. Calcium was determined by the modified method of Ashley and Roberts (9). The results of the various analyses are summarized in

TABLE III

ANALYSIS OF CONTROL CHICK EMBRYOS

		Calcium (mg)		Phosphorus (mg)		DNA (mg)	
Embryo	Weight (gm)	Embryo	Fluid	Embryo	Fluid	Embryo	Fluid
1	14	25	38	9	3	23	14
2	15	23	28	11	3	23	16
3	14	30	46	11	3	21	12

The values represent the average of duplicate determinations.

TABLE IV

ANALYSIS OF PARATHYROID HORMONE-TREATED CHICK EMBRYOS

		Calcium (mg)		Phosphorus (mg)		DNA (mg)	
Embryo	Weight (gm)	Embryo	Fluid	Embryo	Fluid	Embryo	Fluid
1	13	25	30	8	3	22	16
2	11	31	31	9	2	17	11
3	13	27	34	9	2	17	12

The values represent the average of duplicate determinations.

Tables V through VIII. The slight differences between controls and parathyroid-treated embryos and their fluids are not significant.

B. Incubation of Bone in vitro with C^{14}-Labeled Glucose.

Because the attempt to demonstrate an effect of parathyroid hormone on chick embryos was unsuccessful, experimentation was begun to study the effect of parathyroid hormone on adult bone. Two female white rats of the Sprague-Dawley strain were used for the experiment. One rat was injected intraperitoneally with two 1.0 ml doses of Injection Parathyroid (Lilly) four hours apart. The other rat received 2.0 ml of isotonic saline in the same manner. The animals were given food and water ad libitum for 24 hours. They were then sacrificed by exsanguination.

A femur was dissected from each rat, it was cleaned of tissue and marrow, weighed and broken into fragments. The fragments were transferred to a Warburg vessel containing 3.0 ml of Krebs-Ringer bicarbonate (219). A 0.2 ml aliquot of a solution of glucose-1-C^{14} in water (1×10^6 cpm) was added to the sidearm. The flasks were connected to manometers, inserted into a 30° C bath, gassed for five minutes with 95 per cent oxygen and 5 per cent carbon dioxide and then equilibrated for five minutes before the labeled glucose was tripped into the main compartment. The flasks were shaken for four hours at the rate of 100 oscillations per minute. At the end of the incubation

TABLE V

ANALYSIS OF CONTROL CHICK EMBRYOS

Egg #	Weight (gm)	Calcium (mg)	Phosphorus (mg)	Glucose (mg)	DNA (mg)
1	12	13	8	21	15
2	13	18	11	23	17
3	10	12	7	13	16
4	13	13	8	21	17
5	13	18	11	24	14
6	10	10	5	30	11
7	--	14	9	23	15
Mean*	12 ± 1	14 ± 3	8 ± 2	22 ± 5	15 ± 2

* Mean value with the Standard Deviation.

TABLE VI

ANALYSIS OF PARATHYROID HORMONE-TREATED CHICK EMBRYOS

Egg #	Weight (gm)	Calcium (mg)	Phosphorus (mg)	Glucose (mg)	DNA (mg)
1	13	11	7	21	15
2	14	24	15	23	17
3	13	21	10	13	16
4	14	22	14	21	17
5	12	15	10	24	14
6	11	9	6	30	11
7	12	10	5	23	15
Mean*	13 ± 1	16 ± 6	10 ± 4	22 ± 5	15 ± 2

* Mean value with the Standard Deviation.

TABLE VII

ANALYSIS OF FLUIDS FROM CONTROL CHICK EMBRYOS

Egg #	Weight (gm)	Calcium (mg)	Phosphorus (mg)	Glucose (mg)
1	12	46	5	29
2	13	37	3	23
3	10	43	5	23
4	13	43	5	23
5	13	38	5	28
6	10	41	5	38
7	--	39	3	35
Mean*	12 ± 2	41 ± 3	4 ± 1	29 ± 6

* Mean value with the Standard Deviation.

TABLE VIII

ANALYSIS OF FLUIDS FROM PARATHYROID HORMONE-TREATED CHICK EMBRYOS

Egg #	Weight (gm)	Calcium (mg)	Phosphorus (mg)	Glucose (mg)
1	13	40	4	26
2	14	37	5	26
3	13	35	4	28
4	14	38	4	27
5	12	40	4	28
6	11	40	5	27
7	12	35	5	26
Mean*	13 $\pm$ 1	38 $\pm$ 2	4 $\pm$ 1	27 $\pm$ 1

* Mean value with the Standard Deviation.

0.17 ml of 35 per cent perchloric acid was added to the side-arm and 0.3 ml of 2N potassium hydroxide and a piece of folded filter paper were placed in the center well. The flasks were reconnected to the manometers and the perchloric acid was tripped into the main compartment. The flasks were shaken for an additional hour and then allowed to stand in the refrigerator overnight.

The paper in the center well was removed and transferred to a graduated tube. The potassium hydroxide was removed with a capillary pipet and transferred to the tube containing the paper. The well was then washed four times with 0.2 ml aliquots of distilled water; each wash was added to the original material. The total volume was adjusted to 10.0 ml and the paper was eluted for 2 days. The results of the radioactive carbon dioxide evolved by the bone preparations appear in Table IX. The radioactive carbon dioxide evolved by incubation of bone with glucose-1-C^{14} was less for bone obtained from the parathyroid hormone-treated animal than that obtained from the control animal.

TABLE IX

RADIOACTIVE CARBON DIOXIDE EVOLVED BY INCUBATION OF BONE WITH GLUCOSE-1-C^{14}

Condition	Total cpm	cpm per gm Bone	cpm per mg DNA
Control	87,000	73,000	1,500
PTH-treated	57,000	57,000	970

C. Injection of Glucose-1-C^{14} into Rats.

Because the bone from the parathyroid hormone-treated rat appeared to evolve less radioactivity than did control bone when both were incubated with glucose-1-C^{14}, it was decided to determine whether normal rats utilize glucose-1-C^{14} differently than do rats injected with parathyroid hormone.

Blood samples were obtained by cardiac puncture from two fasted male white rats of the Sprague-Dawley strain. One animal was injected intraperitoneally with 1.0 ml of physiological saline followed by 1.0 ml of glucose-1-C^{14} solution (2.5×10^6 cpm). This animal served as a control. The experimental animal received 1.0 ml of Injection Parathyroid (Lilly) plus 1.0 ml of glucose-1-C^{14} solution in the same manner. The animals were placed in separate desiccators and air was passed through the desiccators with the aid of a water aspirator. Each desiccator was connected with scrubbers containing 10% NaOH to remove the carbon dioxide entering the desiccator and to trap that leaving. Every half-hour, for three hours, an aliquot of the solution which trapped the expired carbon dioxide was removed for radioactivity measurements. At the end of three hours, blood samples were quickly taken. The animals were sacrificed and a femur was dissected from each. Glucose was determined on the protein-free filtrates of the final blood samples. The femurs were dissolved in 5 ml of 4N HCl per gram of bone.

The radioactive carbon dioxide expired by the animals is shown in Table X. It appears that the control animal is expiring more $C^{14}O_2$ than the experimental animal and that the expired carbon dioxide reaches a higher maximum specific activity.

TABLE X

RADIOACTIVE CARBON DIOXIDE EXPIRED BY RATS INJECTED WITH GLUCOSE-1-C^{14}

Time Interval (Hr)	Total Activity for Interval (cpm)		Specific Activity for Interval (cpm/mg $BaCO_3$)	
	Control	PTH-Treated	Control	PTH-Treated
0-0.5	300	400	2	2
0.5-1.0	14,000	4,000	39	27
1.0-1.5	27,000	20,000	220	35
1.5-2.0	64,000	26,000	67	130
2.0-2.5	98,000	71,000	80	73
2.5-3.0	99,000	66,000	85	70

The radioactivity of the blood and bone was divided into two parts. One part was that which exchanged with IRA-400 anion exchange resin (referred to as the organic acid fraction) and the other was that which passed through the column (referred to as glucose). The IRA-400 resin was prepared for exchange by the method of Bryant and Overell (19) as fol-

lows: Coloring matter was removed from the resin, successively washing with 1.0N sodium carbonate, water and then 1.0N hydrochloric acid, in that order, for several cycles over the resin packed in the form of a large column. All fine particles were removed by suction. The resin was then exchanged with 1N sodium carbonate overnight, at a ratio of 5 grams of resin to 500 ml of carbonate. Five grams of resin were then packed into 1.0 cm columns. After passage of another 500 ml of 1N sodium carbonate, water was passes to wash out all excess alkali. Neutralized samples of the protein-free filtrate of the blood and of the bone digest were percolated through the resins at a rate of 1.0 ml/minute. The material which passed through the columns was collected and saved. The columns were washed with distilled water and the original effluent and washings were combined and evaporated to dryness in an oven. The residue was taken up in a small amount of water, transferred to a stainless steel planchet, dried and then counted in the windowless gas-flow counter. The anions on the colums were eluted with 500 ml of 1N ammonium carbonate. The eluates were evaporated to dryness in the oven. These residues were taken up with small volumes of water, transferred to planchets, evaporated to dryness and counted in the windowless gas-flow counter. The radioactivities of the final blood and bone samples with respect to the "organic acids" and "glucose" are given in Tables XI and XII. There is more radioactivity present in both the

blood and bone of the experimental animal than in the control. In addition, the radioactivity associated with the "organic acid" fraction of the blood and bone and that associated with "glucose" is greater for the parathyroid hormone-treated animal than for the control.

The influence of the parathyroid hormone on glucose metabolism, as reflected by the expired $C^{14}O_2$, was repeated with two more control and two more parathyroid hormone-treated rats. Each control animal was injected intraperitoneally with 1.0 ml of physiological saline followed by 0.2 ml of glucose-1-C^{14} solution (5.0×10^5 cpm). The experimental animals received 1.0 ml of parathyroid extract (Lilly) plus 0.2 ml of glucose-1-C^{14} solution in the same manner. Expired $C^{14}O_2$ was collected for half-hour intervals for six and one-half hours. The collection of $C^{14}O_2$ was continued for a total period of twenty-four hours. The results are shown in Table XIII. There does not appear to be any significant difference between the amount of $C^{14}O_2$ evolved or the specific activity of evolved $C^{14}O_2$ by controls and that evolved by the experimentals.

D. Incubation of Kidney Homogenates with C^{14}-Labeled Substrates.

The preliminary experiments with bone and whole animals indicated that some measure of success was possible in using labeled substrates to study the effect of parathyroid hormone on intermediary metabolism. The study of the effect of

TABLE XI

RADIOACTIVITY IN BLOOD THREE HOURS AFTER INTRAPERITONEAL INJECTION OF GLUCOSE-1-C^{14}

Animal	Total Activity (cpm/ml)	"Organic Acids" (cpm/ml)	"Glucose" (cpm/ml)	Specific Activity "Glucose" (cpm/mg)
Control	1,600	980	620	670
PTH-Treated	5,600	3,900	1,800	1,600

TABLE XII

RADIOACTIVITY IN BONE THREE HOURS AFTER INTRAPERITONEAL INJECTION OF GLUCOSE-1-C^{14}

Animal	Total Activity (cpm/gm)	"Organic Acids" (cpm/gm)	"Glucose" (cpm/gm)
Control	830	480	360
PTH-Treated	1,800	590	1,200

TABLE XIII

RADIOACTIVE CARBON DIOXIDE EXPIRED BY RATS INJECTED INTRAPERITONEALLY WITH GLUCOSE-1-C^{14}

Time Interval (Hr)	Total Activity for Interval (cpm)		Specific Activity for Interval (cpm/mg $BaCO_3$)	
	Control	PTH-Treated	Control	PTH-Treated
0-0.5	100	100	2	2
0.5-1.0	2,300	1,300	14	10
1.0-1.5	5,000	3,000	24	21
1.5-2.0	6,000	8,000	28	29
2.0-2.5	10,000	11,000	32	36
2.5-3.0	11,000	12,000	34	36
3.0-3.5	13,000	11,000	30	23
3.5-4.0	16,000	11,000	28	25
4.0-4.5	12,000	13,000	18	22
4.5-5.0	11,000	14,000	21	18
5.0-5.5	12,000	10,000	20	16
5.5-6.0	10,000	12,000	24	19
6.0-6.5	10,000	13,000	19	20
6.5-24	110,000	130,000	6	7

Each value represents the mean of the values obtained with two animals.

parathyroid hormone on the utilization of labeled substrates by the kidney was begun.

1. The Effect of Parathyroid Hormone on the Utilization of Glucose-1-C^{14} and Acetate-1-C^{14} by Kidney Homogenates.

Blood samples were obtained by cardiac puncture from four adult male Sprague-Dawley rats. Two of the rats were injected intraperitoneally with 3.0 ml of isotonic saline and the other two were injected with 3.0 ml of parathyroid extract (Lilly) in 1.0 ml doses for three successive days. The animals received small portions of food each day and were allowed water ad libitum. On the fourth day a final blood sample was taken and the animals were sacrificed. The kidneys were removed, weighed and then homogenized in a Potter-Elvehjem homogenizer with ten volumes of cold 0.25M sucrose solution. The reaction system for the incubation is given in Table XIV.

All the components of the reaction system, except the kidney homogenate, were placed in the main compartment of the Warburg flask in the form of a pre-mix. The labeled substrate was either glucose-1-C^{14} (2.4×10^6 cpm) or acetate-1-C^{14} (2.2×10^6 cpm) and separate pre-mixes were prepared for each. A 0.4 ml aliquot of the ten per cent kidney homogenate was placed in the sidearm of the Warburg vessel. Duplicate determinations were made for each kidney homogenate incubation with each labeled substrate. Before the reaction was initiated, 0.2 ml of 10% KOH and a piece of folded filter paper were placed in

TABLE XIV

REACTION SYSTEM FOR KREBS CYCLE OXIDATION IN RAT KIDNEY HOMOGENATES

Material	Volume (ml)	Micromoles
0.08M Phosphate buffer, pH 7.4	0.4	32
0.1M Fumarate	0.1	10
0.1M Pyruvate	0.1	10
0.15M Acetate	0.1	15
0.60M Glucose	0.1	60
0.01M ATP	0.3	3
0.12M $MgCl_2$	0.1	12
0.50M Sucrose	1.0	500
C^{14}-Labeled substrate	0.4	---
10% Kidney homogenate in 0.25M sucrose	0.4	100
Total volume	3.0	

the center well of each Warburg flask. The vessels were sealed with greased glass stoppers and after five minutes equilibration, the homogenate was tripped into the main compartment of the vessel. The flasks were shaken at the rate of 100 oscillations per minute for one hour in a Dubnoff-type water bath at a temperature of 37° C. The reaction was stopped by quickly opening the sidearm vent, introducing 0.5 ml of 50% trichloroacetic acid, closing the vent and spilling the trichloroacetic acid into the main compartment. The flasks were shaken for one hour and then stored in the refrigerator overnight. The potassium hydroxide in the center well was quantitatively collected and the trapped radioactive carbon dioxide was determined as the barium carbonate. The results of the experiment are given in Table XV. The kidneys from control animals evolved approximately four times the amount of radioactive carbon dioxide than did those from the parathyroid hormone-treated animals when incubated with acetate-1-C^{14}. There was no observed effect of parathyroid hormone on the utilization of glucose-1-C^{14} under the conditions of this experiment.

2. <u>The Effect of Parathyroid Hormone on the Utilization of Glucose-1-C^{14} and Acetate-1-C^{14} of Higher Specific Activity and of Succinate-2,3-C^{14} by Kidney Homogenates</u>.

The experiment was repeated. However, the specific activities of the initial reactants were increased by reducing

TABLE XV

CARBON DIOXIDE EVOLVED BY KIDNEY HOMOGENATES INCUBATED WITH ACETATE-1-C^{14} AND GLUCOSE-1-C^{14}

Substrate	Experimental Condition	$C^{14}O_2$ (cpm)	Specific Activity (cpm/mg $BaCO_3$)
Acetate-1-C^{14}	Control	10,000	200
Acetate-1-C^{14}	Control	9,000	250
Acetate-1-C^{14}	PTH-Treated	2,200	70
Acetate-1-C^{14}	PTH-Treated	2,400	62
Glucose-1-C^{14}	Control	800	19
Glucose-1-C^{14}	Control	700	24
Glucose-1-C^{14}	PTH-Treated	700	26
Glucose-1-C^{14}	PTH-Treated	700	30

the amount of carrier substrates added to the reaction mixture. In addition, succinate-2,3-C^{14} was used as a substrate.

A 0.5 ml aliquot of 10 per cent kidney homogenate was added to the reaction mixture of 1.5 ml which contained 30 uM phosphate buffer, pH 7.4, 1 uM each of fumarate, pyruvate and acetate, 8 uM $MgCl_2$, 2 uM ATP, 5 uM glucose, 300 uM sucrose and either acetate-1-C^{14} (2.0×10^6 cpm), glucose-1-C^{14} (2.8×10^6 cpm) or succinate-2,3-C^{14} (1.9×10^6 cpm). The total volume of the incubation mixture was 2.0 ml. Incubation was for two hours at 37° C. The reaction was stopped with 0.3 ml of cold 50% trichloroacetic acid. The radioactive carbon dioxide was quantitatively collected and determined as previously described. The incubation mixtures were centrifuged and the supernatants were saved for determination of their radioactive organic acid profiles.

The data for the evolved $C^{14}O_2$ appear in Table XVI. There is a significantly greater amount of $C^{14}O_2$ evolved by the control kidneys than by the kidneys obtained from PTH-treated animals and the specific activity of evolved $C^{14}O_2$ is significantly higher in the controls than in the experimentals when incubated with acetate-1-C^{14}. There appears to be relatively little difference in either the total amount of $C^{14}O_2$ evolved or the specific activities of evolved $C^{14}O_2$ with succinate-2,3-C^{14} or glucose-1-C^{14} incubations between kidneys from control and parathyroid hormone treated rats.

TABLE XVI

CARBON DIOXIDE EVOLVED BY KIDNEY HOMOGENATES INCUBATED WITH ACETATE-1-C^{14}, SUCCINATE-2,3-C^{14} AND GLUCOSE-1-C^{14}

Substrate	Experimental Condition	$C^{14}O_2$ (cpm)	Specific Activity (cpm/mg $BaCO_3$)
Acetate-1-C^{14}	Control	820,000	120,000
Acetate-1-C^{14}	Control	950,000	120,000
Acetate-1-C^{14}	PTH-Treated	330,000	66,000
Acetate-1-C^{14}	PTH-Treated	310,000	78,000
Succinate-2,3-C^{14}	Control	730,000	29,000
Succinate-2,3-C^{14}	Control	810,000	32,000
Succinate-2,3-C^{14}	PTH-Treated	710,000	36,000
Succinate-2,3-C^{14}	PTH-Treated	700,000	35,000
Glucose-1-C^{14}	Control	27,000	900
Glucose-1-C^{14}	Control	29,000	1,200
Glucose-1-C^{14}	PTH-Treated	22,000	1,100
Glucose-1-C^{14}	PTH-Treated	23,000	1,600

Each value represents the average of duplicate determinations.

The organic acid profiles of the supernatants of the centrifuged incubation mixtures were determined by column chromatography and the activities associated with the acids are presented in Tables XVII, XVIII and XIX.

Only with the acetate-1-C^{14} incubations are there any differences in the organic acid profiles between the kidneys of the control and parathyroid hormone-treated rats. More activity is incorporated in the organic acids of the latter than in the controls and the observed differences are most pronounced in the case of fumaric, malic, citric and isocitric acids. Further, it is noted that the utilization of acetate-1-C^{14} is markedly greater for the control kidney homogenates.

3. <u>The Effect of Parathyroid Hormone on the Utilization of Acetate-1-C^{14} and Citrate-1,5-C^{14} by Kidney Homogenates</u>.

The incubation of kidney homogenates with acetate-1-C^{14} was repeated. Included in this experiment was the incubation of kidney homogenates with citrate-1,5-C^{14}. All conditions of the experiment were identical with the previous experiment with the exception of the total activity used, which was 1.2×10^6 cpm acetate-1-C^{14} and 1.2×10^6 cpm citrate-1,5-C^{14}.

The $C^{14}O_2$ evolved by the incubations are given in Tables XX and XXI. As in the previous experiments, the results of the incubations with acetate-1-C^{14} show that the total $C^{14}O_2$ evolved and the specific activity of $C^{14}O_2$ evolved are greater

TABLE XVII

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING KIDNEY HOMOGENATES WITH ACETATE-1-C^{14}

	Activity (cpm)	
Acid	Control	PTH-Treated
Acetic	13,000	330,000
Pyruvic	2,000	2,500
Fumaric	11,000	31,000
Succinic	18,000	15,000
Malic	61,000	110,000
Citric	33,000	50,000
Isocitric	6,000	13,000

TABLE XVIII

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING KIDNEY HOMOGENATES WITH SUCCINATE-2,3-C^{14}

	Activity (cpm)	
Acid	Control	PTH-Treated
Acetic	11,000 (20,000)	14,000 (20,000)
Pyruvic	52,000	50,000
Fumaric	23,000 (22,000)	26,000 (30,000)
Succinic	57,000 (55,000)	54,000 (50,000)
Malic	56,000	60,000
Citric	31,000	30,000
Isocitric	10,000	9,000

Values in parentheses represent the activity found in a duplicate kidney homogenate.

TABLE XIX

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING KIDNEY HOMOGENATES WITH GLUCOSE-1-C^{14}

Acid	Activity (cpm)	
	Control	PTH-Treated
Acetic	600	900
Pyruvic	3,900	6,400
Fumaric	5,700	3,900
Succinic	4,900	5,300
Malic	14,000	12,000
Citric	5,900	6,000
Isocitric	1,800	1,500

TABLE XX

CARBON DIOXIDE EVOLVED BY KIDNEY HOMOGENATES INCUBATED WITH ACETATE-1-C^{14}

Substrate	Experimental Condition	Activity (cpm)	Specific Activity (cpm/mg $BaCO_3$)
Acetate-1-C^{14}	Control	400,000	40,000
Acetate-1-C^{14}	Control	430,000	31,000
Acetate-1-C^{14}	Control	400,000	35,000
Acetate-1-C^{14}	Control	350,000	31,000
Acetate-1-C^{14}	PTH-Treated	270,000	17,000
Acetate-1-C^{14}	PTH-Treated	250,000	16,000
Acetate-1-C^{14}	PTH-Treated	16,000	2,000
Acetate-1-C^{14}	PTH-Treated	13,000	2,000

TABLE XXI

CARBON DIOXIDE EVOLVED BY KIDNEY HOMOGENATES INCUBATED WITH CITRATE-1,5-C^{14}

Substrate	Experimental Condition	Activity (cpm)	Specific Activity (cpm/mg $BaCO_3$)
Citrate-1,5-C^{14}	Control	340,000	43,000
Citrate-1,5-C^{14}	Control	370,000	32,000
Citrate-1,5-C^{14}	Control	360,000	41,000
Citrate-1,5-C^{14}	Control	360,000	38,000
Citrate-1,5-C^{14}	PTH-Treated	220,000	23,000
Citrate-1,5-C^{14}	PTH-Treated	190,000	19,000
Citrate-1,5-C^{14}	PTH-Treated	110,000	12,000
Citrate-1,5-C^{14}	PTH-Treated	100,000	11,000

for the kidneys of control animals than for the kidneys of parathyroid hormone-treated animals. In addition, the incubation of the kidney homogenates with citrate-1,5-C^{14} led to similar results as those observed with the incubation of acetate-1-C^{14}.

The organic acid profiles of the incubation mixtures are shown in Tables XXII, XXIII, XXIV and XXV. The observed differences between control and experimental animals in the radioactive organic acid profiles of the kidneys incubated with acetate-1-C^{14} are in agreement with the previous experiment. The organic acid profiles of homogenates of kidneys obtained from parathyroid hormone-treated animals and incubated with citrate-1,5-C^{14} indicate a decreased utilization of this substrate. Also, the incorporation of the label into fumaric, malic and isocitric acids is greater in the kidneys of the hormone-treated animals.

TABLE XXII

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING HOMOGENATES OF KIDNEYS FROM CONTROL RATS WITH ACETATE-1-C^{14}

	Activity (cpm)	
Acid	Control Rat #1	Control Rat #2
Acetic	66,000 (60,000)	33,000 (31,000)
Pyruvic	2,100	800
Fumaric	12,000 (10,000)	12,000 (11,000)
Succinic	7,000	5,000
Malic	29,000	24,000
Citric	6,300	8,800
Isocitric	1,400	2,300

The values in parentheses represent the activity found in a duplicate kidney incubation.

TABLE XXIII

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING HOMOGENATES OF KIDNEYS FROM PTH-TREATED RATS WITH ACETATE-1-C^{14}

	Activity (cpm)	
Acid	Experimental Rat #1	Experimental Rat #2
Acetic	280,000 (260,000)	820,000 (800,000)
Pyruvic	1,000	300
Fumaric	16,000 (15,000)	3,000 (3,500)
Succinic	5,000	1,000
Malic	41,000	10,000
Citric	23,000	11,000
Isocitric	13,000	4,000

The values in parentheses represent the activity found in a duplicate kidney incubation.

TABLE XXIV

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING HOMOGENATES OF KIDNEYS FROM CONTROL RATS WITH CITRATE-1,5-C^{14}

	Activity (cpm)	
Acid	Control Rat #1	Control Rat #2
Pyruvic	1,600	1,400
Fumaric	4,300	4,600
Succinic	5,200	5,100
Malic	23,000	26,000
Citric	17,000	37,000
Isocitric	6,800	7,200

TABLE XXV

C^{14}-ACTIVITY IN THE ORGANIC ACIDS OBTAINED BY INCUBATING HOMOGENATES OF KIDNEYS FROM PTH-TREATED RATS WITH CITRATE-1,5-C^{14}

	Activity (cpm)	
Acid	Experimental Rat #1	Experimental Rat #2
Pyruvic	2,100	1,800
Fumaric	13,000	14,000
Succinic	6,400	7,800
Malic	65,000	91,000
Citric	120,000	440,000
Isocitric	56,000	63,000

CHAPTER IV

DISCUSSION

The attempt to obtain a preparation of embryonic bone which was under the influence of parathyroid hormone and which was suitable for incubation with labeled substrates was not successful. However, the conditions for the experiments were arbitrarily set with respect to age of embryo, dose of parathyroid hormone, length of time allowed for the hormone to elicit an effect, analysis to be made and the division of whole embryo into the actual embryo and the fluids bathing it. Different experimental conditions may have been more successful.

The radioactive carbon dioxide produced by bone obtained from parathyroid hormone-treated animals was less than that of the controls when both types of bone were incubated with glucose-1-C^{14}. This indicated that parathyroid hormone-treated bone oxidized less glucose-1-C^{14} than did control bone. This finding was supported by the results obtained from studying the fate of injected glucose-1-C^{14} into control and parathyroid hormone-treated rats. It was indicated that the parathyroid hormone affected the utilization of glucose because, at the end of the experiment more "glucose" activity was remaining in both the blood and bone of the experimental animal than in the control. The "organic acid" content of both blood and bone was also higher in the parathyroid hormone-treated animal than in

the control. The decreased amount of $C^{14}O_2$ expired by the parathyroid hormone-treated animal was additional evidence that less glucose was used by this animal than by the control. However, further studies with rats injected intraperitoneally with glucose-1-C^{14} failed to support the initial findings that the amount of $C^{14}O_2$ expired by controls and PTH-treated rats was basically different.

The distinct differences in the total amount of $C^{14}O_2$ and the specific activity of $C^{14}O_2$ evolved between control and parathyroid hormone-treated kidney homogenates incubated with acetate-1-C^{14} is evidence that the utilization of acetate in the kidney is affected by the parathyroid hormone. The results of incubations with acetate-1-C^{14} of higher specific activity substantiate the findings.

Only slight differences are exhibited between control and parathyroid hormone-treated animals in both the total activity and the specific activity of evolved $C^{14}O_2$ when the kidneys are incubated with glucose-1-C^{14}. The parathyroid hormone either exhibits no effect on the kidney metabolism of glucose or the rate limiting conditions to produce an effect have not been obtained. Furthermore, the metabolism of glucose-1-C^{14} via the Embden-Meyerhof glycolytic pathway will lead to pyruvate-3-C^{14} which enters the Krebs cycle as acetyl-CoA labeled in the methyl position of acetate. Since the 1- and 2-carbons of acetate are not expired as CO_2 at the same position in the Krebs cycle, the

possibility arises that the differences in evolved $C^{14}O_2$ obtained between acetate-1-C^{14} and glucose-1-C^{14} in the homogenates is an indication that the effect of parathyroid hormone occurs in the area in which the carboxy-labeled carbon of acetate is eliminated as carbon dioxide. Under normal conditions, 50% of the label of acetate-1-C^{14} is eliminated as $C^{14}O_2$ on the second turn of the Krebs cycle in the step oxalsuccinate to alpha-ketoglutarate.

The incubation of kidney homogenates with succinate-2,3-C^{14} reveals that the activity of $C^{14}O_2$ evolved is higher for the controls while the specific activity of evolved $C^{14}O_2$ is greater for the experimentals. The significance of this is not known. However, the differences may represent the effect of parathyroid hormone on the utilization of succinate or one of its metabolites by different routes (Figures 7 and 8). Fumarate, malate and oxalacetate are products of succinate metabolism. Both exalacetate and malate, by different decarboxylation reactions, can yield pyruvate which via another decarboxylation reaction can enter the Krebs cycle as acetyl-CoA. In addition, oxalacetate can be decarboxylated, in a nucleotide-dependent reaction, to phosphoenol-pyruvate which after conversion to pyruvate can again be decarboxylated and yield acetyl-CoA.

The marked differences in evolved $C^{14}O_2$ by kidneys incubated with citrate-1,5-C^{14} reflect a decreased utilization

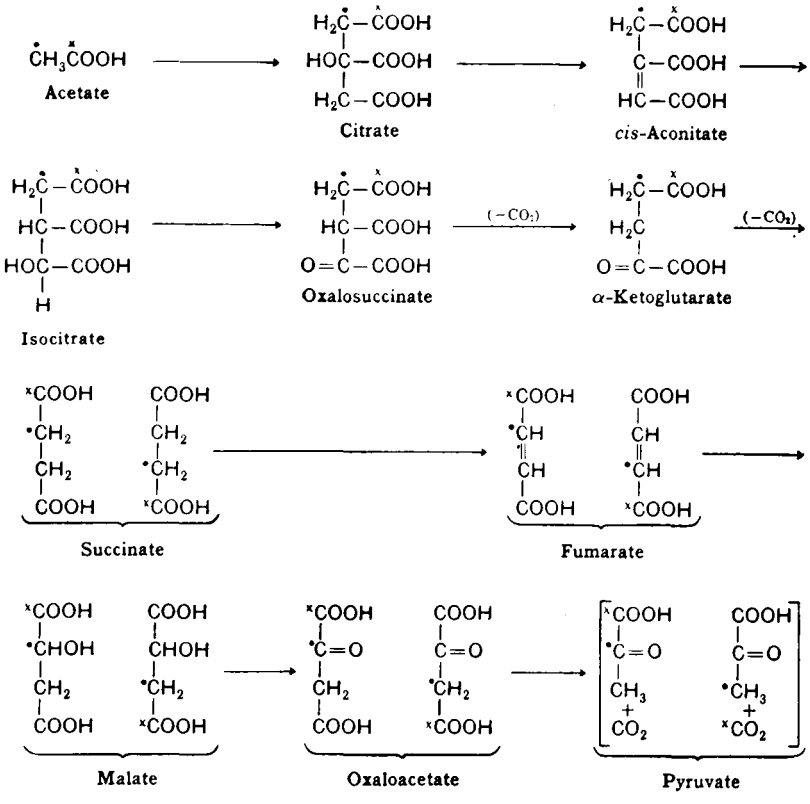

FIGURE 7.

DISPOSITION OF ISOTOPICALLY LABELED CARBON ATOMS OF ACETIC ACID IN THE CITRIC ACID CYCLE.
White et al (226)

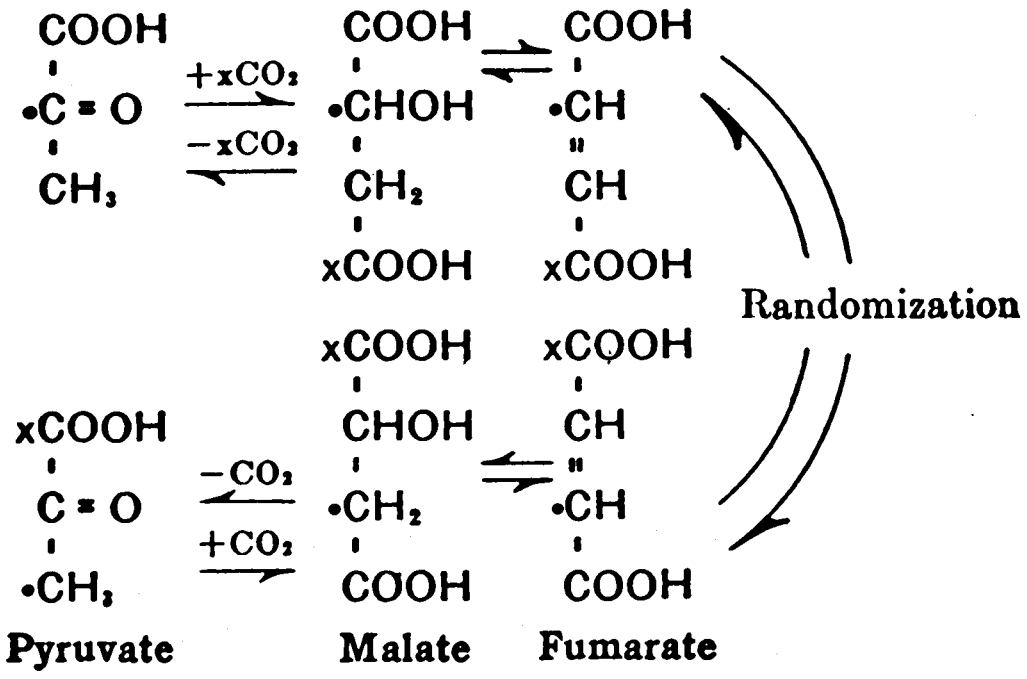

FIGURE 8.

DISPOSITION OF ISOTOPICALLY LABELED CARBON DIOXIDE ENTERING THE CITRIC ACID CYCLE.
White et al (226)

of this substrate by the kidneys obtained from parathyroid hormone-treated animals.

Table XXVI gives the comparison of the radioactive carbon dioxide evolved by the kidneys of control animals to that evolved by the kidneys of the PTH-treated animals for the various labeled substrates. The ratios of the total activities evolved are approximately 3 and 2 for acetate-1-C^{14} and citrate-1,5-C^{14} respectively and slightly greater than unity for glucose-1-C^{14} and succinate-2,3-C^{14}. The ratios of the specific activities of evolved $C^{14}O_2$ are again 3 and 2 for acetate-1-C^{14} and citrate-1,5-C^{14} and less than unity for glucose-1-C^{14} and succinate-2,3-C^{14}. The values for acetate and citrate indicate that the parathyroid hormone decreases the utilization of these substances by the kidneys. The values for glucose-1-C^{14} and succinate-2,3-C^{14} seem to indicate that the parathyroid hormone has little effect on the utilization of these substrates by the kidneys. It is tempting to speculate however, that the reason the ratios of specific activities of evolved $C^{14}O_2$ for glucose-1-C^{14} and succinate-2,3-C^{14} are less than one is perhaps a result of the parathyroid hormone shunting the metabolism of succinate and the methyl-carbon of acetate by the previously mentioned alternate routes.

The analysis of the incubation mixtures for the activity associated with the organic acids reveals some information which sheds some light on what may be occurring in the Krebs

cycle of the kidney as influenced by the parathyroid hormone.

The most striking features of these results, arising from incubating kidney tissue with acetate-1-C^{14} are: 1) the total acetate-1-C^{14} remaining is much less in the controls than in the experimentals and 2) the total activity in the individual organic acids is greater in the experimentals than in the controls. In spite of the decreased utilization of acetate-1-C^{14} by the experimentals, there is a general increased incorporation of activity into the organic acids. This finding supplements the data for the $C^{14}O_2$ evolution, that is, some area of the Krebs cycle is inhibited by the parathyroid hormone. The increased activity in the malic and isocitric acids in the experimentals may indicate that some common factor in the further metabolism of each is being affected by the parathyroid hormone. For example, the conversion of malate to pyruvate and of isocitrate to alpha-ketoglutarate are both triphosphopyridine nucleotide dependent reactions.

The organic acid profiles of the kidneys incubated with citrate-1,5-C^{14} demonstrate that citrate also is not as well utilized by the kidneys from PTH-treated animals. The amount of citrate-1,5-C^{14} remaining is much greater in the experimentals than in the controls and the organic acids in the experimentals contain more activity than the controls with large increases occurring in the fumaric and malic acids of the experimentals. Table XXVII shows the relationship between

acetate-1-C^{14} and citrate-1,5-C^{14} utilization to the incorporation of C^{14} from the various substrates into citrate. The control kidney homogenates oxidize more acetate-1-C^{14} and citrate-1,5-C^{14} than do the parathyroid hormone-treated kidneys. The incorporation of labeled acetate into citrate is either increased in the experimental kidneys (that is, the net production of citrate is increased) or once citrate is produced its further oxidation is inhibited. The data suggests that the latter is occurring.

TABLE XXVI

RATIOS OF $C^{14}O_2$ EVOLVED BY THE KIDNEYS OF CONTROL RATS TO THAT EVOLVED BY THE KIDNEYS OF PTH-TREATED RATS WHEN INCUBATED WITH C^{14}-LABELED SUBSTRATES

	Control/PTH-Treated	
Substrate	Total Activity Evolved	Specific Activity
Acetate-1-C^{14}	3.3	2.9
Citrate-1,5-C^{14}	2.3	2.3
Glucose-1-C^{14}	1.2	0.8
Succinate-2,3-C^{14}	1.1	0.9

TABLE XXVII

RELATIONSHIP BETWEEN C^{14}-LABELED SUBSTRATE UTILIZATION TO ACTIVITY INCORPORATED IN CITRATE

Substrate	Condition	% Substrate Remaining	Citrate (cpm)
Acetate-1-C^{14}	Control	3.5	14,000
Acetate-1-C^{14}	PTH-Treated	35	26,000
Citrate-1,5-C^{14}	Control	2.4	27,000
Citrate-1,5-C^{14}	PTH-Treated	24	280,000
Succinate-2,3-C^{14}	Control	2.9	31,000
Succinate-2,3-C^{14}	PTH-Treated	3.1	30,000
Glucose-1-C^{14}	Control	---	5,900
Glucose-1-C^{14}	PTH-Treated	---	6,000

Table XXVIII shows the ratios of radioactive carbon dioxide evolved to the radioactivity incorporated into citric, malic and fumaric acids using the various labeled substrates. These ratios are greater for the controls than for the experimentals when acetate-1-C^{14} or citrate-1,5-C^{14} are the substrates. The same results are obtained when succinate-2,3-C^{14} is the substrate although the difference between controls and experimentals are not nearly as pronounced. The ratios for acetate-1-C^{14} and for citrate-1,5-C^{14}, which are an indication of the metabolic turnover of the organic acids of the Krebs cycle, support the finding that the Krebs cycle in the kidney

is inhibited by the parathyroid hormone.

When acetate-1-C^{14} enters the Krebs cycle, the label appears in either the 1-or 5-positions of citrate (Figure 7). Even though no carbon atom of acetate is lost on one turn of the Krebs cycle, it is not coincidental that the effect of parathyroid hormone on the oxidation of acetate-1-C^{14} and citrate-1,5-C^{14} leads to similar results. The first carbon dioxide evolved in the cycle arises from the gamma-carboxyl of citric acid and occurs during the transformation of isocitric acid to alpha-ketoglutaric acid (104). The second carbon dioxide arises from the 1-or 5-positions of citric acid and occurs during the transformation of alpha-ketoglutaric acid to succinate acid (104. The observed differences in the $C^{14}O_2$ evolution in the oxidation of acetate-1-C^{14} and citrate-1,5-C^{14} may be a consequence of an effect of parathyroid hormone on one of these decarboxylation steps.

These observations could be related to a decreased isocitric dehydrogenase activity either as a result of a parathyroid hormone effect on the enzyme itself or on a cofactor of the enzyme system. A decreased isocitric dehydrogenase activity in bone after the injection of parathyroid hormone has been reported (87). It has been reported that parathyroid hormone also decreases the absorbancy of reduced triphosphopyridine nucleotide(TPNH) at 340 millimicrons (145). This author (32) in an <u>in vitro</u> study of the triphosphopyridine nucleotide-

TABLE XXVIII

RATIOS OF RADIOACTIVE CARBON DIOXIDE EVOLVED TO THE INCORPORATION OF ACTIVITY INTO CITRIC, FUMARIC AND MALIC ACIDS

Substrate	Condition	Carbon Dioxide/Acid (cpm/cpm) Citric	Fumaric	Malic
Acetate-1-C^{14}	Control	45	49	15
Acetate-1-C^{14}	PTH-Treated	7	13	4
Citrate-1,5-C^{14}	Control	--	79	15
Citrate-1,5-C^{14}	PTH-Treated	--	17	3
Succinate-2,3-C^{14}	Control	26	35	14
Succinate-2,3-C^{14}	PTH-Treated	24	25	12
Glucose-1-C^{14}	Control	5	5	2
Glucose-1-C^{14}	PTH-Treated	4	6	2

requiring enzyme systems of isocitric dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase found a slight inhibition by parathyroid hormone on all three enzyme systems. Subsequent studies indicated that the effect of the hormone was not due to any effect on the ability of TPNH to absorb light at 340 millimicrons.

Thus, it seems that the increased citrate in the kidney (103) following the administration of parathyroid hormone is a result of the inhibition of an active system for citrate

oxidation and not a result of an increased production of citrate. This inhibition of citrate oxidation would tend to maintain the elevated serum citrate which in turn could be a stimulus for bone resorption.

Sufficient data is not available to determine the exact area of the Krebs cycle in which the parathyroid hormone exerts its influence in the kidney. In order to accomplish this, the fate of the labels must be followed kinetically, more labeled substrates must be studied (for example, gamma-labeled citrate, glucose-2-C^{14}, acetate-2-C^{14} and succinate-1,4-C^{14}) and the exact location of the label in the organic acid products should be determined.

CHAPTER V

SUMMARY

The effect of parathyroid hormone on the utilization of acetate-1-C^{14}, glucose-1-C^{14}, succinate-2,3-C^{14} and citrate-1,5-C^{14} by kidney homogenates has been studied. The amount and specific activity of radioactive carbon dioxide evolved by the homogenates using these substrates was determined and the activity incorporated into the organic acids of the Krebs cycle was measured.

Homogenates of the kidneys obtained from parathyroid hormone-treated rats evolved less $C^{14}O_2$ than did those from control rats when incubated with either acetate-1-C^{14} or citrate-1,5-C^{14}. The specific activity of the evolved $C^{14}O_2$ was also proportionately less. Incubation with either glucose-1-C^{14} or succinate-2,3-C^{14} resulted in relatively slight differences in both the total and specific activities of evolved $C^{14}O_2$ between the kidneys from control and PTH-treated rats.

The C^{14}-activity incorporated into the organic acids of the kidney homogenates, after incubation with these substrates, revealed gross differences in the acid profiles of the homogenates when either acetate-1-C^{14} or citrate-1,5-C^{14} were the substrates. Kidneys obtained from parathyroid hormone-treated rats utilized significantly less acetate-1-C^{14} and cit-

rate-1,5-C^{14} than did the control kidneys. In addition, the amount of activity incorporated into the organic acids of the Krebs cycle was greater in the experimentals than in the controls in spite of the decreased utilization of these substrates.

The evidence presented supports the theory that the parathyroid hormone has a direct effect on the kidney. Furthermore, the evidence suggests that the direct effect of the parathyroid hormone on the kidney is a result of an inhibition of oxidation of substrates of the Krebs cycle in this tissue.

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APPENDIX

A. Glucose Determination

Glucose was determined by the method of Folin and Wu (54). 2.0 ml of a standard glucose solution was placed in a Folin-Wu sugar tube. 2.0 ml of alkaline copper reagent was added and the contents were mixed without allowing the solution to go above the constricted portion of the tube. The tube was heated in at least four inches of rapidly boiling water for exactly eight minutes. The tube was then removed from the boiling water, placed in cold water and after five minutes, 2.0 ml of phosphomolybdic acid color reagent was added. The tube was again heated for five minutes in rapidly boiling water. The contents were then diluted to 25.0 ml with distilled water and mixed by inversion. A portion of the solution was transferred to a colorimeter tube and the optical density was determined within 30 minutes with a Klett-Summerson photoelectric colorimeter fitted with filter #42. The data for the standard glucose curve are given in Table XXIX.

The protein-free filtrates of blood or homogenates were prepared as follows: 1.0 ml of sample was added to 7.0 ml of distilled water followed by 1.0 ml of 2/3N sulfuric acid. The solution was mixed and then 1.0 ml of 10% sodium tungstate was added drop by drop with constant stirring. After 1 hour, the precipitated protein was removed by centrifugation and 2.0 ml of the supernatant was used for determination of glucose.

TABLE XXIX

GLUCOSE DETERMINATION

Glucose (mgm)	Number of Determinations	Optical Density*
0.16	6	95 ± 3
0.20	6	120 ± 4
0.40	6	251 ± 7
0.80	4	507 ± 5

* The values for the optical density are given with the Standard Deviation.

B. Phosphorus Determination

Phosphorus was determined by the method of Fiske and Subbarow (53) as follows: 5.0 ml of a standard phosphate solution in 10% trichloroacetic acid was placed in a graduated colorimeter tube. 1.0 ml of acid molybdate reagent was added and the contents of the tube were mixed. 0.4 ml of 1-amino-2-naphthol-4-sulfonic acid reagent was added and after dilution to 10.0 ml with distilled water the contents of the tube were mixed by inversion. After twenty minutes, the optical density was determined in a Klett-Summerson photoelectric colorimeter fitted with filter #66. The data for the phosphorus standard curve are given in Table XXX. The phosphate content of homogenates was determined as follows: 10 ml of cold 20% trichloroacetic acid was added to 10.0 ml of homogenate. After fifteen

minutes, the precipitate was removed by centrifugation and 5.0 ml of the supernatant was used for the determination of inorganic phosphate.

TABLE XXX

PHOSPHORUS DETERMINATION

Phosphorus (ug)	Number of Determinations	Optical Density*
4.0	12	31 ± 1
10.0	12	76 ± 3
16.0	12	124 ± 4
20.0	12	152 ± 7
24.0	6	173 ± 0

*The values for the optical density are given with the Standard Deviation.

C. Deoxyribonucleic Acid Determination

Deoxyribonucleic acid was determined by the method of Schneider (183). A stock solution of deoxyribonucleic acid in 5% trichloroacetic acid was prepared. A series of working standards was prepared by dilution of the stock solution with 5% trichloroacetic acid. A 1.0 ml aliquot of the working standard solution was placed in a pyrex test tube (15 x 150mm) with a scratch mark at 10.0 ml. 0.5 ml of 30% hydrogen peroxide and 0.5 ml of 10N sulfuric acid were added to the sample

and the tube was heated in a sand bath for 2 hours at a low temperature in order to drive off the water. Heating was continued for another hour at a high temperature to complete the digestion. The tube was removed from the sand bath and when it was sufficiently cooled, 5 ml of distilled water and 1.0 ml of acid-free molybdate reagent were added. The contents were mixed by shaking and 0.4 ml of aminonaphtholsulfonic acid color reagent was added. The contents were diluted to the 10.0 ml mark, mixed by inversion and transferred to a colorimeter tube. After twenty minutes, the optical density was determined in the Klett-Summerson photoelectric colorimeter fitted with filter #66. The data for the phosphorus content of the DNA standard dilution appear in Table XXXI.

The determination of deoxyribonucleic acid, based upon the colorimetric reaction of diphenylamine with deoxyribose, was as follows: 1.0 ml of the working standard deoxyribonucleic acid solution was mixed with 2.0 ml of diphenylamine reagent and heated for ten minutes in boiling water. The tubes were cooled for five minutes in the refrigerator and the contents were transferred to micro colorimeter tubes. The optical density was determined in a Klett-Summerson photoelectric colorimeter fitted with filter #60. The data for the DNA-diphenylamine determination appear in Table XXXII.

A standard curve was prepared relating optical density to micrograms of DNA-phosphorus. The practice of using the

organic phosphorus content of DNA as the reference rather than DNA weight has the advantage that DNA samples of varying degree of purity will yield the same curve when diphenylamine color intensity is plotted against DNA-phosphorus.

TABLE XXXI

PHOSPHORUS CONTENT OF DNA

DNA (ug)	Number of Determinations	Optical Density*	Phosphorus (ug)
50	13	32 ± 1	4
100	10	64 ± 2	8
150	6	95 ± 1	12
200	13	125 ± 2	16
250	6	159 ± 3	20

* The values for the optical density are given with the Standard Deviation.

D. Citric Acid Determination

A slightly modified method of Beutler and Yeh (13) was used for the estimation of citric acid. 1.0 ml of a standard solution of citric acid in 1N sulfuric acid was pipetted into a conical, graduated centrifuge tube. 0.25 ml of 18N sulfuric acid was added and the solution was diluted to 2.5 ml with distilled water. 0.1 ml of 1M potassium bromide and 0.5 ml of saturated potassium permanganate solution were added, the mixture was shaken briefly and then allowed to stand at room

TABLE XXXII

DNA DETERMINATION WITH DIPHENYLAMINE

DNA (ug)	Number of Determinations	Optical Density*
50	6	40 ± 1
100	9	81 ± 1
150	6	121 ± 3
200	9	160 ± 3
250	6	198 ± 1

*The values for the optical density are given with the Standard Deviation.

temperature for ten minutes. The tube was chilled in an ice bath for fifteen minutes and 3% hydrogen peroxide was added dropwise until the permanganate was decolorized. Weak permanganate solution was added until a faint violet or yellow brown color persisted for more than a few seconds. The total volume was adjusted to 5 ml with distilled water and 5.0 ml of isooctane was added. The tube was tightly stoppered with a rubber stopper and shaken for ten minutes on a mechanical shaker. 3.0 ml of the isooctane layer was siphoned off and pipetted into a clean micro colorimeter tube and 3.0 ml of thiourea solution was added. The colorimeter tube was again tightly stoppered and shaken on the mechanical shaker for fifteen minutes. The tube was inserted directly into the colorimeter and the optical density of the

yellow colored thiourea extract was determined with a Klett-Summerson photoelectric colorimeter fitted with filter #42. A turbidity reading was taken with filter #66. The reading with filter #66 was substracted from that with filter #42. The data for the standard citric acid curve are given in Table XXXIII.

TABLE XXXIII

CITRIC ACID DETERMINATION

Citric Acid (umoles)	Number of Determinations	Optical Density*
0.04	13	19 ± 2
0.10	13	52 ± 1
0.16	13	86 ± 3
0.20	13	106 ± 7
0.30	13	168 ± 5
0.40	13	230 ± 5

*The values for the optical density are given with the Standard Deviation.

E. Calcium Determination by the Method of Roe and Kahn (177)

A 5.0 ml aliquot of the supernatant from a homogenate precipitated with an equal volume of cold 20% trichloroacetic acid was placed in a 15 ml graduated conical centrifuge tube. 1.0 ml of 25% sodium hydroxide solution was added, the solution was mixed by shaking and allowed to stand for five minutes. 1.0

ml of 5% trisodium phosphate solution was added, the solution was thoroughly mixed and the tube was set aside for one hour. At the end of this time the precipitated calcium phosphate was centrifuged for two minutes and the supernatant was carefully poured off. The centrifuge tube was inverted on a clean piece of filter paper and allowed to drain for two minutes. The mouth of the tube was wiped with a clean cloth and the precipitate was washed with 5 ml of alkaline alcohol wash reagent. The wash reagent was delivered from a pipet with a fine blowing tip in order to break up the precipitate and to rinse down the sides of the centrifuge tube. If necessary, a stirring rod was used to break up the precipitate. The precipitate was again centrifuged for two minutes, the supernatant fluid was poured off and the tube was allowed to drain as before. After draining, the mouth of the tube was wiped dry and 2.0 ml of acid molybdate reagent was added to dissolve the precipitate and to form phosphomolybdate from the phosphate present. After solution was complete, the contents were diluted to 10.0 ml with distilled water and mixed well. 5.0 ml was transferred to a colorimeter tube, 0.4 ml of aminonaphtholsulfonic acid reagent was added, the volume was adjusted to 10.0 ml with distilled water, the solution was mixed by inversion and after twenty minutes the optical density was determined in a Klett-Summerson photoelectric colorimeter fitted with filter #66.

The mean optical density for seventeen determinations

of 5.0 ml of a standard calcium solution containing 0.10 mg calcium was 201 ± 8.

The calcium content of 5.0 ml of an unknown solution carried through the same procedure as the standard was determined by the formula:

$$\text{Mg Calcium in Unknown} = \frac{\text{Optical Density of Unknown}}{201} \times 0.1$$

F. Calcium by the Method of Ashley and Roberts (8)

1.00 ml of ethylenediamine tetraacetate solution(EDTA) was accurately pipetted into a 10 ml beaker. 0.2 ml of a 10.0 mg% calcium standard solution or a 0.2 ml aliquot of serum was added, followed by 1 ml of calcein indicator solution. Three drops of 1N sodium hydroxide and three drops of 1% sodium cyanide were added. A small magnet sealed in capillary glass was placed in the beaker and the beaker was placed on a magnetic stirrer. The excess EDTA was titrated with a 10.0 mg% calcium standard solution. The titration was performed under ultraviolet light while the solution was vigorously stirred. The titration was continued until a definite green fluorescence appeared. Blank titrations contained 0.2 ml of double-distilled water in place of the serum or standard calcium solution. Under these conditions, 0.78 ± 0.01 of 10.0 mg% calcium titrant was required for the titration of the blanks and 0.59 ± 0.01 ml was required for the titration of a 10.0 mg% standard calcium solu-

tion. The calcium concentration of an unknown solution was determined by the formula:

$$\text{Mg\% Calcium of Unknown} = \left(\text{ml of Titrant for Blank} - \text{ml of Titrant for Unknown}\right) \times 50$$

G. Self-Absorption of Barium Carbonate.

Increasing volumes of a solution of radioactive carbon dioxide in 10% sodium hydroxide were mixed with 5.0 ml of 10% barium chloride in the filter tower of a precipitation apparatus (Tracerlab E-8B) containing weighed discs of filter paper. The water was removed by suction and the barium carbonate was washed twice with small amounts of water. The precipitate was then washed three times with a 50% ethanol in acetone mixture (v/v) and two times with acetone alone. The precipitate was dried briefly by suction and then for at least one-half hour in the air. Each disc containing the barium carbonate was weighed and then transferred to a stainless steel planchet. The radioactivity was determined in a Tracerlab windowless gas-flow counter assembly.

From these data a method of correction for self-absorption was derived. A plot was made relating the specific activity (cpm/mg $BaCO_3$) to sample thickness (mg $BaCO_3/cm^2$). The curve was extrapolated to zero thickness to obtain the maximum specific activity. A self-absorption correction curve was then plotted (Figure 6) whose ordinate is the count rate expressed as

per cent of the maximum specific activity and whose abscissa is the sample thickness. These data are given in Table XXXIV.

TABLE XXXIV

SELF-ABSORPTION OF BARIUM CARBONATE

Radioactive Carbonate Solution (ml)	$BaCO_3$ (mg)	cpm	Specific Activity (cpm per mg $BaCO_3$)	Sample Thickness (mg $BaCO_3$ per cm^2)	Count Rate (% Maximum Specific Activity)
0.05	1	216	166	0.5	90
0.10	2	370	157	0.8	85
0.20	5	600	134	1.6	73
0.50	11	1160	106	3.0	57
1.0	21	1500	70	7.5	38
1.5	31	1620	52	11.0	28
2.0	41	1730	42	14.4	23
2.5	48	1720	35	17.0	19
3.0	61	1780	29	21.6	16

The values are the averages of triplicate determinations.

H. **Reagents**.

Alkaline-Alcohol Wash.

10 ml of amyl alcohol was added to 58 ml of ethyl alcohol and diluted to 100 ml with distilled water. Two drops of 1% phenolphthalein solution was added

and then sufficient 5% sodium hydroxide solution was added dropwise until a distinct pink color was obtained.

Alkaline Copper Reagent.

40 grams of Na_2CO_3 was dissolved in 500 ml of distilled water. 7.5 grams of tartaric acid was added and when solution was complete, 4.5 grams of $CuSO_4 \cdot 5H_2O$ was added. After the cupric sulfate was dissolved, the solution was diluted to 1000 ml with distilled water.

Aminonaphtholsulfonic Acid Reagent.

0.5 grams of recrystallized 1-amino-2-naphthol-4-sulfonic acid was dissolved in 195 ml of 15% sodium bisulfite and 5 ml of 20% sodium sulfite. The solution was stored in a brown bottle and prepared fresh every two weeks.

Calcein Indicator.

A stock solution was prepared by dissolving 250 mg of powdered calcein indicator into 4.0 ml of 1N NaOH and then adding 30 ml of distilled water. A working calcein solution was prepared by diluting 1.0 ml of the stock solution to 100 ml with distilled water.

Diphenylamine Reagent.

1.0 gram of recrystallized diphenylamine was dissolved in 200 ml of glacial acetic acid and to this was added 5.5 ml of concentrated H_2SO_4.

Ethylenediamine Tetraacetate Solution (EDTA).

0.1875 grams of reagent disodium dihydrogen ethylenediamine tetraacetate was dissolved in 250 ml of distilled water.

Molybdate Reagent (acid-free).

25 grams of ammonium molybdate was dissolved in 1000 ml of distilled water.

Molybdate Reagent (acid).

25 grams of ammonium molybdate was dissolved in 200 ml of distilled water. The solution was poured into a 1 liter volumetric flask containing 300 ml of 10N H_2SO_4 and then diluted to 1000 ml with distilled water.

Phosphomolybdic Color Reagent.

To 35 grams of molybdic acid and 5 grams of sodium tungstate in a 1 liter beaker was added 200 ml of 10% NaOH and 200 ml of distilled water. The solution was boiled vigorously for 30 minutes, cooled and then 125 ml of 85% phosphoric acid was added. The solution was then diluted to 500 ml with distilled water.

Thiourea Solution.

Two grams of sodium borate was dissolved in 100 ml of 4% thiourea in water. If the solution was opalescent, it was filtered. The final pH was 9.2.

APPROVAL SHEET

The dissertation submitted by John C. Colla has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 24, 1962
Date

Maurice V. FitzHenry
Signature of Adviser