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In Vitro Studies of Parathyroid Hormone Effects on Enzyme Systems

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IN VITRO STUDIES OF PARATHYROID HORMONE EFFECTS ON ENZYME SYSTEMS

by

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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.

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He is co-author of the following publication: "The Use of Carbon-14-Labeled Material in Malting and Brewing Research", A.S.B.C. Proc. 26 (1957).

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CHAPTER I

INTRODUCTION

One of the striking effects of the injection of the parathyroid hormone is the elevation of the serum citrate (L'Heureux, 1953). The origin of this citrate is still open to question, but can arise from one of three possibilities. First, the oxidation of citrate by the organism is hindered or inhibited, secondly, the production of citrate is accelerated, and thirdly, storehouses of citrate, mainly bone, are being dissolved. All three possibilities have been either directly or indirectly investigated and to date none can be categorically excluded from thinking and research.

That the hormone acts by inhibiting the utilization of citrate by the organism was first given credence by Neuman and his co-workers (1956), who stated that the parathyroid hormone decreased the ability of reduced triphosphopyridine nucleotide to absorb light at 340 millimicrons *in vitro*. They postulated that this was perhaps what was also occurring *in vivo*. Since it is known that the pyridine nucleotides are intimately involved in the citric acid cycle and carbohydrate metabolism, it is readily seen that any interference with them could easily lead to an accumulation of citrate in the organism. That is, the parathyroid hormone, by blocking oxidation cycles, would promote acid accumulation from glycolysis. Unfortunately,

no experimental evidence was given for this hypothesis. Reichert (1950) extended these investigations and indeed was able to show an *in vitro* destruction of the ability of reduced triphosphopyridine nucleotide to absorb light at 340 mu. Over a 6 hour period the destruction was about 15 per cent. However, he demonstrated that this effect was not due to the calcium mobilizing activity of the hormone preparation because a pepsin digest of the hormone, physiologically inactive, increased the effect from 2 to 3 times.

Other evidence in support of this inhibition theory was obtained by Laskin and Engel (1956) who found that following the administration of hormone, respiration in bone slices was markedly reduced despite the addition of dextrose, pyruvate, and some of the di- and tricarboxylic acid intermediates of the citric acid cycle. They were able to show a depression of the succinic dehydrogenase system.

A somewhat similar study, this time with kidney mitochondria, was undertaken by DeLuca and Steenbock (1957). They, however, used vitamin D which is known to have some of the same metabolic manifestations as the parathyroid hormone. In their work they incubated the mitochondria with and without vitamin D and various Krebs cycle substrates and found that the vitamin had an inhibitory effect on citrate and isocitrate oxidation. They also demonstrated the same effect on glutamate oxidation, but none on the oxidation of alpha-ketoglutarate, succinate, beta-hydroxybutyrate, and pyruvate in the presence of oxalace-

tate. They noted that only the triphosphopyridine nucleotide systems were affected.

DeLuca et al (1959) next investigated the effect of vitamin D upon the enzyme systems of disrupted mitochondria. They found no effect on aconitase, TPN isocitric dehydrogenase, DPN isocitric dehydrogenase, TPNH cytochrome C reductase, DPNH cytochrome C reductase, and pyridine nucleotide transhydrogenase activities. Next they studied the effect of the vitamin on kidney mitochondria prepared in two different ways. One preparation was obtained from isotonic sucrose and the other from hypertonic sucrose. Mitochondria prepared by these methods differ from each other in both structure and permeability. They found that kidney mitochondria from hypertonic sucrose were more affected by vitamin D than kidney mitochondria prepared from isotonic sucrose. Both types of mitochondria resulted in a lower rate of oxidation of citrate in the presence of vitamin D. The conclusion was drawn that vitamin D influenced the kidney mitochondrial structure and permeability. Further evidence for this postulate was obtained by the use of mitochondrial swelling agents which reversed the vitamin effect.

Dixon and Perkins (1953) tested the levels of some of the enzymes of the citric acid cycle in bone to see if the rise in serum citrate did come from this storehouse of citrate. Large doses of parathyroid hormone were given to test animals and the levels of citrogenase, aconitase, and isocitric dehy-

drogenase in the bone of treated animals were compared to the levels of control animal bone. They found no difference in the enzyme levels of bone between the treated and untreated animals.

The third possibility that the production of citrate in the organism is accelerated would obtain if carbohydrate metabolism were shunted into the citric acid cycle pathway at a faster rate than in normal metabolism. Blanchet (1952) demonstrated the hypoglycemic effect of parathyroid extract. The percent decrease in blood glucose was directly related to the USP units of parathyroid hormone administered. Olmer and Paillas (1936) reported that high blood levels of glucose in diabetic patients were significantly lowered following injections of parathyroid hormone. Notario and Maduri (1956) showed that guinea pigs treated for twenty days with one, two, and four units of parathyroid hormone daily showed at the two lower doses, lower blood sugar and increased glycogen deposits, but at the highest dose a progressive decrease in the glycogen deposits and little change in the blood sugar. Barbieri and Martinotti (1955) demonstrated that short treatment of guinea pigs with parathyroid extract caused in the islets of Langerhans a slight increase in the number of alpha-cells; prolonged treatment caused a decrease in these cells and a marked increase in beta-cells. Thus, it would seem that the parathyroid hormone does indeed have an effect on carbohydrate metabolism.

Another piece of evidence which supports either the

theory of accelerated production or inhibition of citrate was presented by Lehman (1959). He incubated bone slices, obtained from normal animals and animals that had received doses of parathyroid hormone, in the presence of pyruvate-2-C^{14}. Analyses were then made of the labeled citrate after a 30 minute incubation. He found three times the amount of labeled citrate in the bone obtained from the animals which had received the hormone than in the bone from the control animals. Since the incubation time was relatively small, this must mean that the increase in citrate arose from either an acceleration of citrate production or an inhibition of the oxidation of the labeled citrate produced. The rise in bone citrate could not have been due to a dissolution of the bone mineral.

Statement of the Problem

The purpose of this thesis is to determine whether the parathyroid hormone has an *in vitro* effect on some of the enzyme systems which are known to be involved in the metabolic fate and production of citric acid.

Some individual enzymes, related to citric acid and carbohydrate metabolism, and which require TPN in their systems, will be studied and the parathyroid effect on them will be determined. The oxidation of substrates by kidney homogenates, and kidney mitochondria prepared and treated in various ways will also be studied and the effect of parathyroid hormone on them will be determined.

If an effect on an enzyme system could be demonstrated, this might well lead to a non-biological assay for the hormone. Another consequence of the demonstration of such an effect would perhaps be to elucidate the loci of the physiological action of the hormone in its role as a metabolic regulator.

CHAPTER II

EXPERIMENTAL

The reader is referred to the Appendix for the chemicals and preparation of reagents.

Since the experimental work is divided into two major phases, spectrophotometric and manometric, the specific methods and procedures will be given with each experimental section. It is hoped that this procedure will facilitate the reader in following the course of this research.

Spectrophotometric

In this phase of the thesis the enzymes which will be studied are enzymes related to glycolysis and citric acid oxidation and which require TPN in their systems. The enzymic activities will be followed in the Model DU Beckman spectrophotometer at room temperature by observing the change in light absorption at 340 millimicrons due to the formation of TPNH.

Isocitric Dehydrogenase

The first enzyme studied was isocitric dehydrogenase. This enzyme is intimately involved in the citric acid oxidation cycle in two places. It requires TPN for its first reaction and the two reactions it catalyses are:

a) Isocitrate + TPN $\longleftrightarrow$ Oxalsuccinate + TPNH

b) Oxalsuccinate $\xleftrightarrow{Mn^{++}}$ alpha-Ketoglutarate + CO_2

The enzyme was not commercially available and therefore was prepared by the method of Siebert et al (1957).

One-half gram of acetone powder of pig heart was homogenized with 12.5 ml of 0.01M phosphate buffer containing 0.001M Versene, pH 7.4. The homogenizer was a glass-made student preparation. Homogenization was continued for 10 minutes at 0-5°C. In all subsequent operations the temperature was never allowed to go above 5°. After homogenization the mixture was stirred for an additional 10 minutes. At the end of this time the homogenate was transferred to a plastic centrifuge tube and centrifuged in the International Refrigerated centrifuge for 15 minutes at a force of 24,000 x gravity. The sediment was discarded. The supernatant was clear and pale yellow. To it was added 1.3 grams of sodium chloride, slowly and with stirring. Then, again slowly and with stirring, 22 ml of saturated ammoniun sulfate solution containing 0.001 M Versene were added. The mixture was allowed to stand 30 minutes and then it was centrifuged for 20 minutes at 24,000 x gravity. The precipitate was discarded. 37 ml of saturated ammonium sulfate solution containing 0.001M Versene were added slowly and with stirring to the pale yellow supernatant. The mixture was allowed to stand for 15 minutes. At this point the

mixture was divided into three centrifuge tubes. The mixture was centrifuged for 45 minutes at 24,000 x gravity. The precipitate contained the isocitric dehydrogenase. Two tubes were drained of the supernatant and quickly stored in the freezer compartment of the refrigerator. The precipitate in the third tube was taken up with 1 ml of 0.01M phosphate buffer containing 0.001M Versene, pH 7.4. This enzyme solution was assayed immediately.

Assay of Isocitric Dehydrogenase

Experiment 1.

The assay system was that of Siebert et al (1957). Silica cells of 1 cm contained 1.00 ml of 0.1M Tris buffer containing 0.001M Versene, 0.2ml of 0.02M magnesium sulfate, 0.2 ml of 0.0015M TPN, 0.5 ml of 0.08M dl-isocitrate, 1.00 ml of 0.1 per cent gelatin containing 0.01M Tris and 0.001 M Versene, and 0.01 to 0.05 ml of enzyme solution. Water was added to make a final volume of 3.0 ml. The reaction was initiated by the addition of substrate. Controls contained an extra aliquot of water in place of substrate. After the addition of substrate, the contents of the cells were mixed once by inversion and readings were taken at 1/2 minute intervals.

The results are plotted on Figure I and the data appear in Table XXVIII in the Appendix. The activity of the

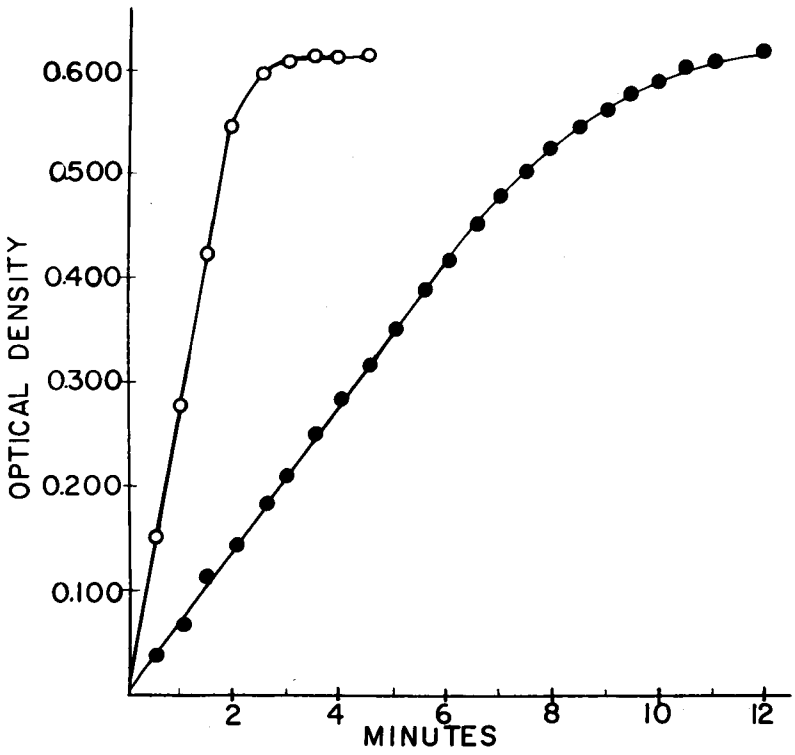

FIGURE I

ASSAY OF ISOCITRIC DEHYDROGENASE

—●—● 0.01 ml Enzyme, —○—○—
0.04 ml Enzyme.

enzyme is proportional to the concentration. This is apparent by a comparison of the time it takes to reach equilibrium and by the rate of the linear portions of the curves.

Experiment 2.

The enzyme preparation from experiment 1 was frozen, stored in the freezer compartment of the refrigerator, thawed two days later, and reassayed. The results of this experiment appear in Table XXIX in the Appendix. From Figure II it can be seen that the activity is still proportional to the enzyme concentration. Also, the enzyme does not appear to have lost any activity on freezing and thawing.

Effect of Parathyroid Extract on Isocitric Dehydrogenase

Experiment 3.

In this experiment the effect of 0.1 ml of Lilly's Parathyroid Extract (expiration date: August 1, 1958; Lot No. 5078-678089) was determined on 0.02 ml of enzyme solution. The enzyme preparation was the same one used in experiment 2. All the conditions were identical with those of experiment 2, with the exception of the blank which contained 0.1 ml of hormone. The data appear in Table XXX in the Appendix. The data for the 0.02 ml enzyme activity of experiment 2 and the 0.02 ml enzyme activity in the presence of 0.1 ml parathyroid extract of experiment 3 are plotted on Figure III.

There appears to be a slight inhibition of the isocitric

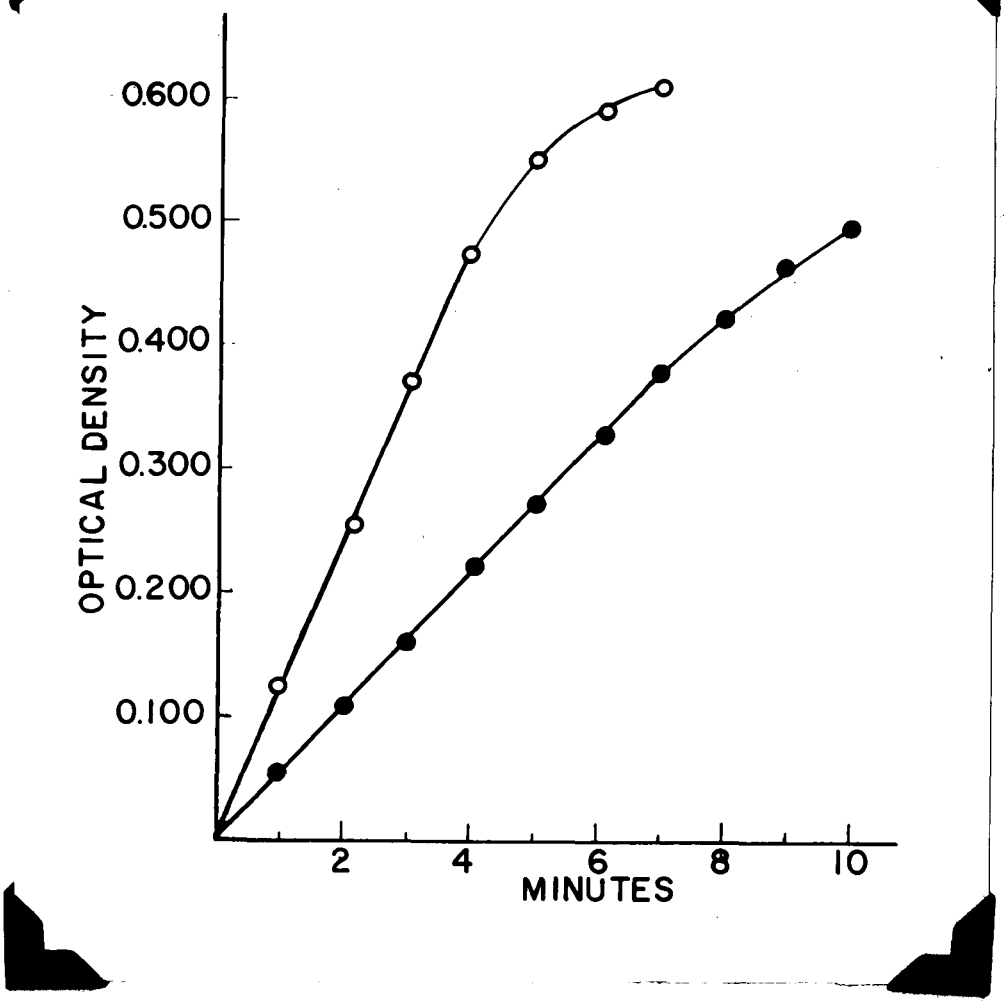

FIGURE II

ASSAY OF ISOCITRIC DEHYDROGENASE
AFTER FREEZING AND THAWING

—●—●— 0.01 ml Enzyme, —○—○—
0.02 ml Enzyme.

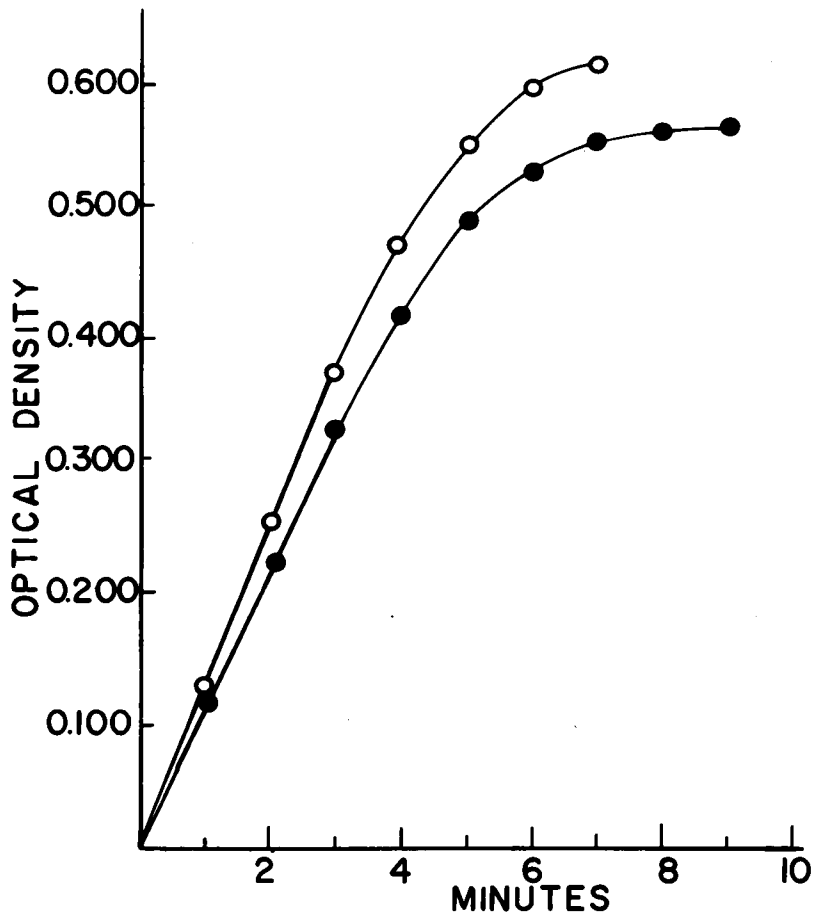

FIGURE III

EFFECT OF PARATHYROID EXTRACT ON ISOCITRIC DEHYDROGENASE

—o—o— 0.02 ml Enzyme, —●—● 0.02 ml Enzyme with 0.1 ml parathyroid extract.

dehydrogenase by the parathyroid extract. This is apparent not by the rates of the two separate reactions, because they seem to be almost identical, but in the final attainment of equilibrium.

Lyophilisation of Isocitric Dehydrogenase

The precipitates in all the tubes from the experiment in which isocitric dehydrogenase was prepared were combined with the aid of 3 ml of 0.01M phosphate buffer containing 0.001M Versene. This solution was quickly frozen and lyophilised. The total weight of the dry powder was 127 mgs. Protein was determined by the method of Warburg and Christian (1957). The enzyme preparation was found to contain a total of 2.84 mgs. protein.

Experiment 4.

An enzyme solution was prepared which contained 9.25 mgs. of lyophilised isocitric dehydrogenase preparation per ml of solution and this was assayed to see if the enzymic activity still remained. 0.1 ml and 0.2 ml of solution were assayed; otherwise all conditions were identical with the previous experiments. The results, given in Table XXXI in the Appendix, are plotted on Figure IV.

Buffered Hormone Effect on Isocitric Dehydrogenase

Difficulty was encountered with the solubility of the parathyroid extract at pH 7.4. Melius (1957) found that gradient dialysis of the parathyroid extract against a 0.1M

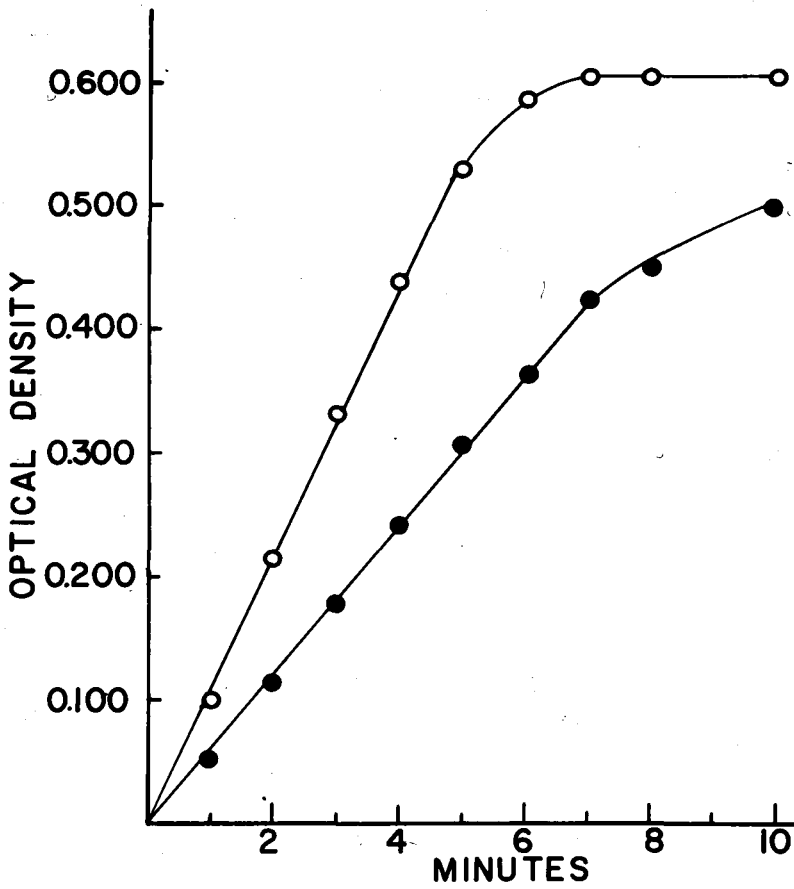

FIGURE IV

ASSAY OF LYOPHILIZED ISOCITRIC DEHYDROGENASE

—●—●— 0.1 ml Enzyme, —○—○— 0.2 ml Enzyme.

phosphate buffer led to a partition of activity between the soluble portion of the protein and the protein that precipitated during the dialysis. Thus, it was possible to dialyse the extract, have parathyroid activity remaining in solution, and at the same time eliminate the turbidity which formerly hindered the spectrophotometric studies. The dialysis procedure was as follows:

10 ml of Lilly's Parathyroid Extract (expiration date: August 1, 1958; Lot No. 5078-678089) was dialysed against one liter of 0.1M phosphate buffer, pH 7.4, for 3 hours in the deepfreeze. The solution outside the dialysis tube was continually mixed with a magnetic stirrer. The buffer was changed at the end of 3 hours and dialysis was continued overnight for an additional 18 hours. The precipitate which formed was removed by centrifugation in the International Refrigerated centrifuge at a force of 20,000 x gravity for 15 minutes and at 0°C. The supernatant was pale yellow and was at a pH of 7.4. In the subsequent experiments this hormone preparation will be referred to as Buffered Hormone.

Experiment 5.

Because the hormone was now in a phosphate buffer, it was convenient to change the assay system for isocitric dehydrogenase. The method now used was that of Colowick and Kaplan

(1957). Silica cells contained 2.6 ml of 0.1M phosphate buffer, pH 7.4, 0.1 ml of 0.0015M TPN, 0.1 ml of 0.1M magnesium chloride, 0.1 ml of 0.08M dl-isocitric acid, and 0.1 ml of enzyme solution. When buffered hormone was added to the cells an equivalent amount of phosphate buffer was omitted. The enzyme solution contained 13.7 mgs. of lyophilized isocitric dehydrogenase preparation per ml of 0.1M phosphate buffer, pH 7.4. Table I gives the experimental results of the effect of two concentrations of buffered hormone on 0.1 ml of enzyme solution.

There appears to be a slight increase in the rate of reaction of isocitric dehydrogenase by the 1.0 ml of buffered hormone. However, 2.5 ml of buffered hormone has no effect.

Experiment 6.

In this experiment the enzyme concentration was increased and the hormone concentration was decreased. The enzyme solution was the same as that in experiment 5. The data are given in Table XXXII in the Appendix and are plotted on Figure V. Here it appears that the hormone is inhibiting the enzymic reaction in the linear portion of the curve.

Experiment 7.

This experiment was performed to test a constant hormone concentration with different enzyme concentrations. A new enzyme solution was prepared which contained 11.1 mgs. of lyophilized isocitric dehydrogenase preparation per ml of 0.1M

TABLE I

BUFFERED HORMONE EFFECT ON
ISOCITRIC DEHYDROGENASE

Time (mins.)	Optical Density		
	0.1 ml Enzyme	With 1.0 ml buffered hormone	With 2.5 ml buffered hormone
1.0	0.043	0.047	0.057
2.0	0.084	0.094	0.097
3.0	0.126	0.140	0.135
4.0	0.172	0.191	0.182
5.0	0.214	0.235	0.229
6.0	0.254	0.279	0.276
7.0	0.29[illegible]	0.32[illegible]	0.311
8.0	0.330	0.361	0.351
9.0	0.372	0.396	0.390
10.0	------	0.427	0.420
11.0	0.431	0.450	0.430
12.0	0.448	0.462	0.452
13.0	0.463	0.474	0.472

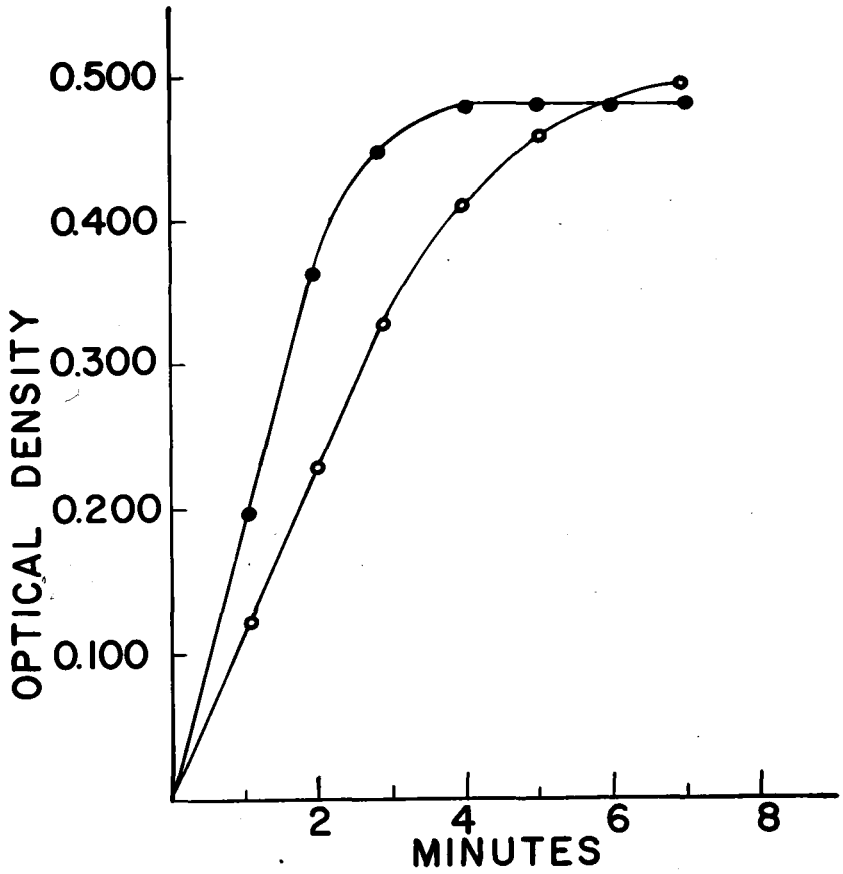

FIGURE V

BUFFERED HORMONE EFFECT ON ISOCITRIC DEHYDROGENASE

—●— 0.4 ml Enzyme, —○— 0.4 ml Enzyme with 0.5 ml buffered hormone.

phosphate buffer, pH 7.4. All other conditions were identical with those of experiment 6. The results are given in Table II.

It seems that the constant hormone concentration activates the enzymic reaction at the lower concentrations of enzyme. At the higher concentration of enzyme there is no appreciable effect of hormone.

Glucose-6-Phosphate Dehydrogenase

The next enzyme studied was glucose-6-phosphate dehydrogenase. It is a part of the pentose oxidation shunt. During the enzymic process, TPN is converted to TPNH. The reaction it catalyses is:

D-glucose-6-phosphate + TPN $\leftrightarrow$ 6-phophogluconolactone + TPNH

The enzyme used was: (Sigma) Zwischenferment, crude, 200 Kornberg units per gram, Lot No. 116-138. A solution of enzyme was prepared by dissolving 11 mgs. in 2 ml of 0.1 per cent sodium bicarbonate, pH 7.4.

Buffered Hormone and Glucose-6-Phosphate Dehydrogenase

Experiment 8.

The contents of the cells for the glucose-6-phosphate dehydrogenase assay are given in Table III. The data, Table XXXIII in the Appendix, plotted on Figure VI are corrected for protein and hormone concentration at zero time.

There seems to be a slight inhibition of the enzyme system by the buffered hormone.

TABLE II

EFFECT OF BUFFERED HORMONE
ON ISOCITRIC DEHYDROGENASE

Time (mins.)	Optical Density		
	0.1 ml Enzyme	0.2 ml Enzyme	0.3 ml Enzyme
1.0	0.038	0.070	0.100
2.0	0.076	0.146	0.196
3.0	0.114	0.221	0.293
4.0	0.162	0.298	0.378
5.0	0.202	0.366	0.447
6.0	0.238	0.418	0.487
7.0	0.278	0.455	0.498
8.0	0.315	0.472	0.502
9.0	0.351	0.479	0.505
10.0	0.384	0.478	0.506

TABLE II (Cont'd)

Time(mins.)	Optical Density		
	0.1 ml Enzyme with 0.5 ml buffered hormone	0.2 ml Enzyme with 0.5 ml buffered hormone	0.3 ml Enzyme with 0.5 ml buffered hormone
1.0	0.046	0.070	0.100
2.0	0.089	0.152	0.187
3.0	0.131	0.239	0.280
4.0	0.177	0.319	0.370
5.0	0.216	0.388	0.442
6.0	0.264	0.444	0.481
7.0	0.305	0.480	0.499
8.0	0.347	0.495	0.502
9.0	0.383	0.502	0.503
10.0	0.416	0.503	0.503

TABLE III

CONTENTS OF CELLS FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY

Materials	Cells					
	1.	2.	3.	4.	5.	6.
0.1 M phosphate buffer, pH 7.4	1.0ml	1.0ml	1.0ml	1.0ml	0.5ml	0.5ml
0.0015% TPN	0.2	0.2	0.2	0.2	0.2	0.2
Glucose-6-phosphate solution	---	0.1	---	0.1	---	0.1
Enzyme solution	0.1	0.1	0.2	0.2	0.1	0.1
Water	1.7	1.6	1.6	1.5	1.7	1.6
Buffered hormone	---	---	---	---	0.5	0.5

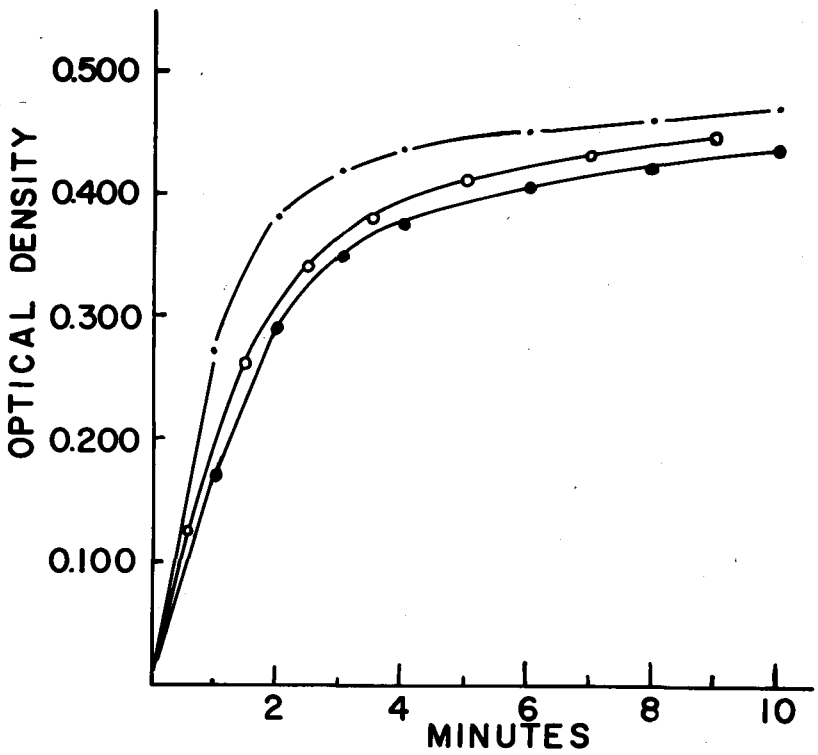

FIGURE VI

GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND BUFFERED HORMONE

—o—o— 0.1 ml Enzyme, —·— 0.2 ml Enzyme, —●—● 0.1 ml Enzyme with 0.5 ml buffered hormone.

Experiment 9.

The enzyme solution was too concentrated so it was diluted 1 to 5 with 0.1 per cent sodium bicarbonate buffer, pH 7.4. Three concentrations of enzyme were assayed along with the same three which contained 0.5 ml of buffered hormone. The data given in Table XXXIV in the Appendix and plotted on Figure VII were also corrected for protein and hormone concentration at zero time.

The hormone again appears to inhibit the enzyme activity. However, since the inhibition is of the same magnitude at all three enzyme concentrations, it would seem that this is not a true inhibition but a manifestation of some other unaccountable factor.

6-Phosphogluconic Dehydrogenase

The third TPN requiring enzyme studied was 6-phosphogluconic dehydrogenase. This enzyme is also part of the pentose oxidation shunt. The reaction it catalyzes is:

6-phosphogluconate + TPN $\longleftrightarrow$ D-ribulose-5-phosphate + TPNH

Any blocking of its mechanism could easily lead to an accumulation of citric acid from glycolysis.

The enzyme was obtained commercially from Sigma. It was very crude; Lot No. 47-673. An enzyme solution was prepared by dissolving 4 mgs. of the powdered preparation in 2 ml of 0.1 per cent sodium bicarbonate, pH 7.4. The enzyme solution was clear.

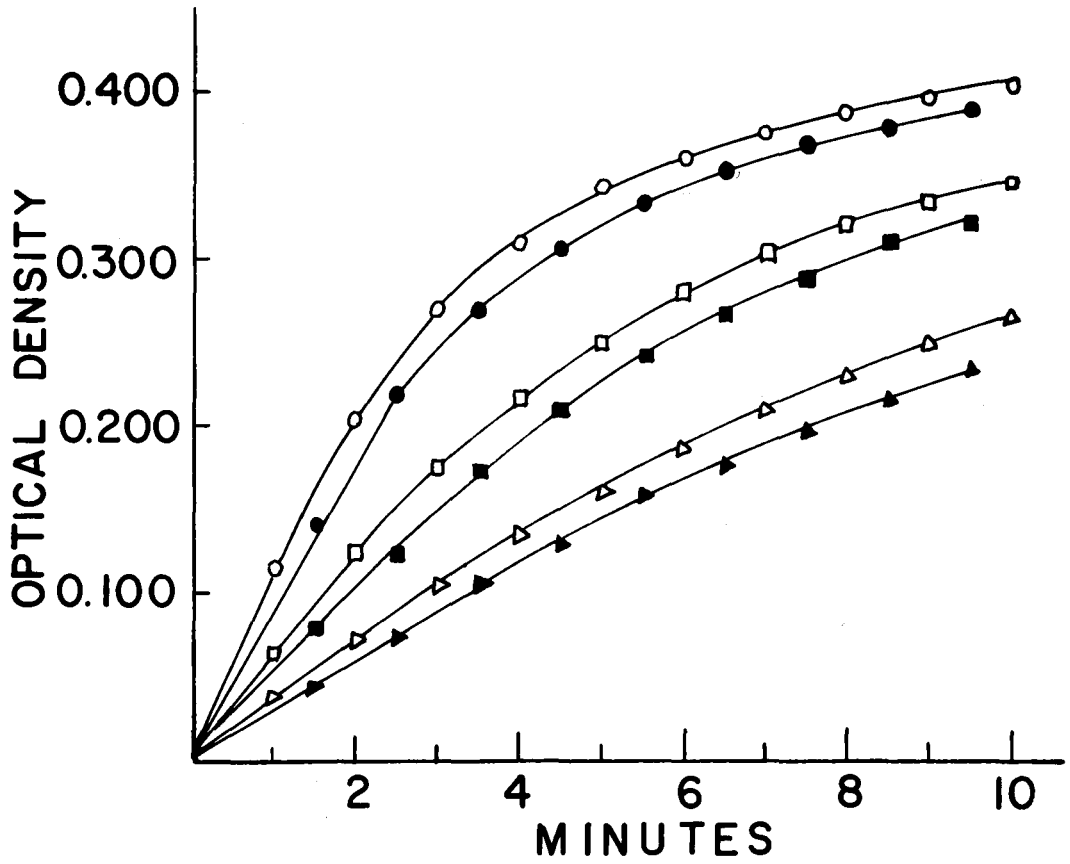

FIGURE VII

GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND BUFFERED HORMONE

—△—△— 0.1 ml Enzyme, —▲—▲— 0.1 ml Enzyme with 0.5 ml buffered hormone, —□—□— 0.2 ml Enzyme, —■—■— 0.2 ml Enzyme with 0.5 ml buffered hormone, —○—○— 0.3 ml Enzyme, —●—●— 0.3 ml Enzyme with 0.5 ml buffered hormone.

Buffered Hormone and 6-Phosphogluconic Dehydrogenase

Experiment 10.

The experimental procedure and reagents were the same as that for the glucose-6-phosphate dehydrogenase system except for enzyme and substrate. Two concentrations of enzyme were followed spectrophotometrically along with the same two concentrations which contained 0.4 ml of buffered hormone in the system. The results of this experiment, corrected for protein and hormone concentration at zero time, are given in Table IV.

It appears that buffered hormone is inhibiting the 6-phosphogluconic dehydrogenase system, but as with the glucose-6-phosphate dehydrogenase, the inhibition is of a constant nature for the two levels of enzyme concentration.

Manometric

It was decided that perhaps the systems studied spectrophotometrically were too restricted to show any gross effect of parathyroid hormone on them. Therefore, efforts were turned towards multi-enzyme systems. If the hormone acted on many individual enzyme systems only in a small way, there was the possibility that by studying the multi-enzyme systems a marked effect of parathyroid hormone would obtain.

The multi-enzyme systems studied were rat kidney homogenates and rat kidney mitochondria. Because of the turbidity of such preparations, they could not be studied spectro-

TABLE IV

BUFFERED HORMONE AND 6-PHOSPHOGLUCONIC DEHYDROGENASE

Time(min.)	Optical Density			
	0.1 ml Enzyme	0.1 ml Enzyme with 0.4 ml buffered hormone	0.2 ml Enzyme	0.2 ml Enzyme with 0.4 ml buffered hormone
0.5	0.045	0.042	0.071	0.070
1.0	0.081	0.079	0.126	0.122
1.5	0.115	0.107	0.173	0.166
2.0	0.144	0.134	0.212	0.201
2.5	0.171	0.156	0.240	0.229
3.0	0.192	0.176	0.269	0.252
3.5	0.212	0.192	0.288	0.271
4.0	0.230	0.208	0.307	0.288
4.5	0.246	0.223	0.323	0.304
5.0	0.261	0.237	0.338	0.319
6.0	0.287	0.259	0.363	0.344
7.0	0.310	0.278	0.382	0.362
8.0	0.325	0.296	0.397	0.378
9.0	0.341	0.312	0.409	0.390
10.0	0.354	0.327	0.418	0.401

photometrically. The course of the enzymic reactions was followed by determining the oxygen uptake in a Warburg apparatus.

Warburg flasks were calibrated with mercury by the method of Umbreit et al (1957). Manometers were calibrated with water. A micrometer type manometer calibrator was used. Brodie's solution with acid fuchsin as the dye was used for the manometer fluid. All buffers, substrates, and cofactors were pipetted into the main compartment of the Warburg vessels. All enzymes were pipetted into the sidearms. A 10 per cent alkali solution was put in the center cups of the vessels to absorb any carbon dioxide produced during respiration. A 2 cm square piece of filter paper was folded accordion fashion and it was inserted in the center well to aid in the absorption of carbon dioxide. Before the papers were inserted, however, the rims of the center cups were covered with a thin coat of lanolin to prevent the alkali from spilling over into the main compartment. Manometer to flask joints were also greased with lanolin to make air tight seals. The flasks were then connected to the manometers, inserted into the bath, and equilibrated for a few minutes with the stopcocks open. The stopcocks were closed and equilibration was continued for another few minutes. The contents of the sidearms were then tripped into the main compartments of the vessels at 15 second intervals. The flasks were equilibrated a short while and then readings were taken.

The various equilibration times will be given with each series of experiments.

Unless otherwise indicated, all values given in the tables will represent decreases in pressure, that is, oxygen uptake. Furthermore, all values will be corrected for flask and manometer constants, barometric pressure changes, and vapor pressure changes.

The temperature of the Warburg bath was maintained at 30°C throughout all determinations. Flasks were shaken at a rate of 100 oscillations per minute.

The procedures, data, and figures for nitrogen, phosphorus, and cytochrome C standardizations appear in the Appendix.

For the purpose of evaluation of the various effects of parathyroid hormone preparations on the enzyme systems, it was arbitrarily decided that a difference of more than 5 per cent in the oxygen uptake of the enzyme in the absence of hormone and enzyme in the presence of hormone would constitute an effect. The means of the determinations at the termination of each experiment were the values used. For example, if the oxygen uptake of a hypothetical system at the end of 30 minutes were 100 microliters and the oxygen uptake of the same system in the presence of hormone were 80 microliters at the end of 30 minutes, the percentage difference between them would be 20/100 x 100 = 20. Since this is over 5 per cent, the hormone would be said to inhibit this hypothetical enzyme system.

Effect of Hormone Extract on Kidney Homogenate Respiration

White albino rats of the Sprague-Dawley type were killed with ether. The kidneys were quickly removed, trimmed of fat and their protective membrane, weighed, minced with a sharp scalpel, and then homogenised with 9 volumes of 0.25M sucrose solution in a Porter-Elvehjem homogeniser for 3 minutes. The temperature was maintained at 0-5°C. A portion of the homogenate was heated for 15 minutes in a boiling water bath in order to inactivate all enzymes. The boiled homogenate was used as the control.

Experiment 11.

The method of Umbreit (1957) was used for the first experiment. When citrate was used as substrate there was no respiration by the kidney homogenate, and therefore, fumarate and pyruvate were used to initiate respiration. The hormone preparation was untreated Lilly's Parathyroid Extract (expiration date: August 1, 1959; Lot No. 1146-706177). The contents of the flasks are given in Table V.

Flasks were equilibrated 3 minute with the stopcocks open and 3 minutes with them closed. The homogenates were tripped into the main compartments at 15 second intervals and equilibration was continued for another 7 minutes. The first readings were taken as zero time. The values in Table VI represent the means of duplicate determinations.

It appears that the untreated hormone extract is

TABLE V

FLASK CONTENTS FOR EFFECT OF PARATHYROID EXTRACT ON KIDNEY HOMOGENATE RESPIRATION

Materials	Flasks		
	1.	2.	3.
0.1M phosphate buffer, pH 7.4	0.3 ml	0.3 ml	0.3 ml
0.1M Na fumarate	0.06	0.06	0.06
0.1M Na pyruvate	0.06	0.06	0.06
0.12M $MgCl_2$	0.1	0.1	0.1
0.01M ATP	0.3	0.3	0.3
Water	0.01	0.01	0.71
0.5% sucrose	0.97	0.97	0.97
Boiled kidney homogenate	0.4	---	---
Active kidney homogenate	---	0.4	0.4
Hormone extract	---	---	0.1
10 per cent KOH	0.2	0.2	0.2

TABLE VI

EFFECT OF PARATHYROID EXTRACT ON KIDNEY HOMOGENATE RESPIRATION

Time(mins.)	Oxygen Uptake(microliters)	
	Homogenate	With 0.1 ml hormone
0	0	0
2	5	5
4	10	11
6	12	13
8	14	17
10	17	21
13	23	27
16	25	31
18[a]	29 (29-27)	36 (36-37)
20	33 (32-33)	39 (38-39)
25	38 (37-39)	50 (49-50)

a. The values in parenthesis are the actual values of the duplicate determinations.

activating the respiration of kidney homogenate.

Experiment 12.

Experiment 11 was repeated and this time, for the purpose of an inactive hormone control, the effect of pepsin digested parathyroid hormone was also determined. The hormone was inactivated by pepsin as follows:

> Seven ml of Lilly's Parathyroid Extract (expiration date: August 1, 1959; Lot No. 1146-706177), pH 4.0, were adjusted to pH 2.0 with 2N hydrochloric acid. The adjusted extract was then incubated with 2 mgs. of crystalline pepsin for 17 hours at 37°C. At the end of incubation, the pH was adjusted to 8 with 2N sodium hydroxide to destroy any residual pepsin activity. Five minutes later the pH was readjusted to 4.0 with 2N hydrochloric acid. This material will be referred to as pepsin inactivated hormone. When not in use it was kept frozen in the freezer compartment of the refrigerator.

Another kidney homogenate was prepared with 0.25M sucrose solution as previously described. The conditions of the experiment were identical with those of experiment 11 except that equilibration times were different. These were 4 minutes with the stopcocks open and 4 minutes with the stopcocks closed. Zero readings were then taken 9 minutes after the homogenate was tripped into the main compartment. The respi-

ration data appear in Table VII. Again the values represent the mean of duplicate determinations.

Both the pepsin digested hormone and untreated hormone extract are inhibiting the respiration.

Experiment 13.

Experiment 12 was repeated with 0.2 ml of active and pepsin inactivated hormone. A new kidney homogenate was prepared in 0.25M sucrose solution. All conditions, including equilibration times, were identical with those in experiment 12. The data is given in Table XXXV in the Appendix and the values represent the mean of duplicate determinations. Figure VIII is a plot of the data.

The inhibitions of both hormone preparations at this concentration is most striking. It is interesting to note that pepsin inactivated hormone decreased respiration by approximately 80 per cent and active hormone extract decreased it by approximately 50 per cent.

Effect of Active and Pepsin-Inactivated Hormone on Kidney Mitochondria Prepared from Isotonic Sucrose

The next undertaking was to purify the test system to see if any effects of hormone could be obtained on the specialized enzyme system in the mitochondria. It was thought that the mitochondria would oxidise citrate if it were the only substrate available for oxidation.

TABLE VII

EFFECT OF PARATHYROID EXTRACT AND PEPSIN-DIGESTED HORMONES ON KIDNEY HOMOGENATE RESPIRATION

Time(mins.)	Oxygen Uptake(microliters)		
	Homogenate	With 0.1 ml pepsin-inactivated hormone	With 0.1 ml active extract
0	0	0	0
3	10	9	6
5	13	11	9
8	22	20	16
10	28	25	21
15	42	34	31
20	54	44	40
25	66	53	48
30	76	61	51
35	86	67	63
40[a]	95 (89-100)	73 (69-76)	70 (69-71)
50	111 (103-119)	82 (80-84)	77 (77-81)
60	121 (112-130)	88 (85-90)	84 (81-86)

a. The values in parenthesis are the actual values for the duplicate determinations.

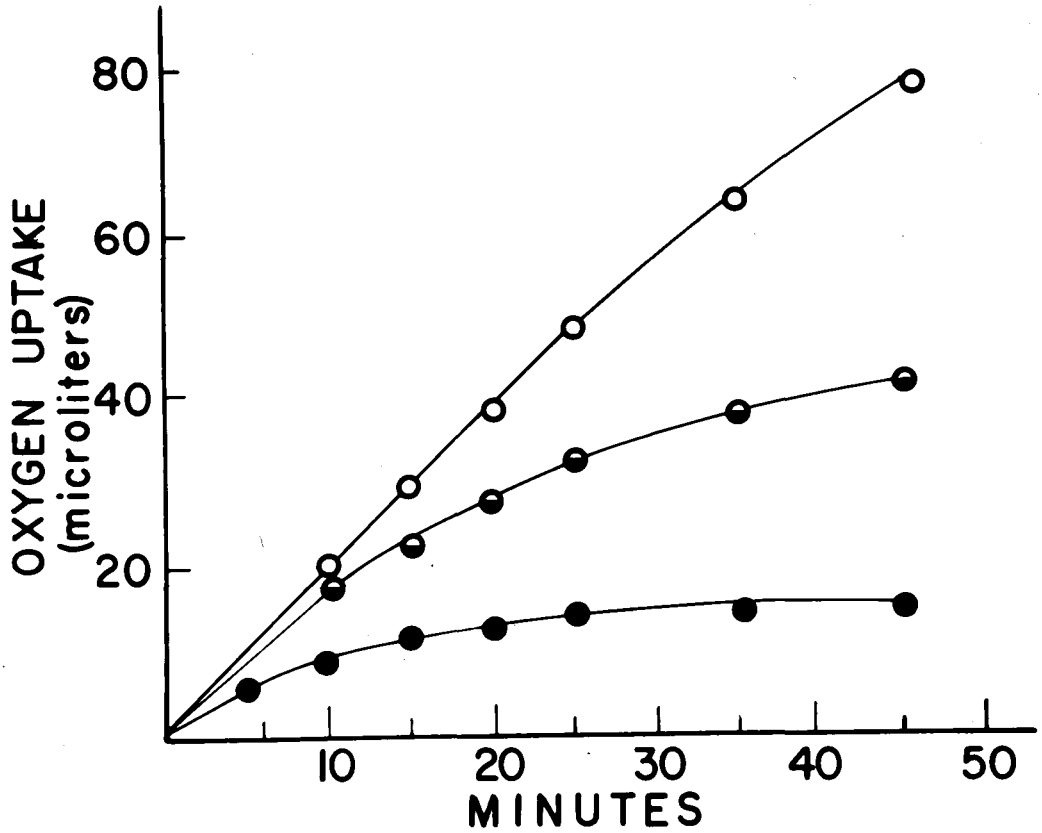

FIGURE VIII

EFFECT OF PARATHYROID EXTRACT AND PEPSIN-DIGESTED HORMONE ON KIDNEY HOMOGENATE RESPIRATION

—o—o— Homogenate, —o—o— Homogenate with 0.2 ml parathyroid extract, —●—● Homogenate with 0.2 ml pepsin-digested hormone.

Kidney mitochondria were prepared from 0.25M sucrose solution as described by Schneider (1948), and Schneider and Hogeboom (1950), as follows:

> The 0.25M sucrose kidney homogenate was centrifuged for 10 minutes at 2000 r.p.m. (head 840) in the International Refrigerated centrifuge set at 0°C. The supernatant was saved and stored in the refrigerator. The sediment was resuspended in 2.5 ml of 0.25M sucrose solution per original 10 ml of homogenate and it was rehomogenized for 2 minutes and centrifuged as before. The supernatant from this fraction was also saved. The sediment was resuspended a second time, homogenized, and centrifuged. The supernatant from this fraction and the two previous ones were combined and then centrifuged for 10 minutes at 9200 r.p.m. (head 296) and at 0°C. The pellet of mitochondria was washed with 2.5 ml of sucrose solution per original 10 ml of homogenate and the mitochondria were resedimented by centrifugation. The pellet was washed once more and the mitochondria were collected again by centrifugation at 9200 r.p.m. The final pellet was taken up with 2.5 ml of 0.25M sucrose solution per 10 ml of original homogenate. A portion was heated for 15 minutes in a boiling water bath to inactivate the enzymes.

Experiment 14.

The active and inactive mitochondria were then assayed using citrate as substrate. Cofactors, listed in Table VII, were added to the flasks. Equilibration was for 3 minutes with the stopcocks open and 3 minutes with them closed. The mitochondria and hexokinase in the sidearms were tripped at 15 second intervals into the main compartments and zero readings were taken after a final equilibration of 4 more minutes.

There was no respiration by the mitochondria when citrate was used as substrate.

Experiment 15.

Succinate was then tried as substrate for the mitochondria. All the conditions were the same as when citrate was used. The results of this experiment are given in Table IX. The values represent the means of duplicate determinations.

It seems that the active hormone does not affect the respiration of kidney mitochondria prepared from isotonic sucrose. The inhibition of respiration by pepsin inactivated hormone is still present.

Pepsin Inhibition Study

Experiment 16.

An attempt was made to determine the type of inhibition which was being exhibited by the pepsin inactivated hormone on succinate oxidation by kidney mitochondria. At the same

TABLE VIII

FLASK CONTENTS FOR EFFECT OF ACTIVE AND PEPSIN-INACTIVATED HORMONES ON KIDNEY MITOCHONDRIA RESPIRATION

Materials	Flasks			
	1.	2.	3.	4.
0.1M phosphate buffer, pH 7.4	0.4 ml	0.4 ml	0.4 ml	0.4 ml
0.2M $MgCl_2$	0.1	0.1	0.1	0.1
0.4M Na citrate	0.1	0.1	0.1	0.1
0.25M sucrose	1.0	1.0	1.0	1.0
0.1M glucose	0.4	0.4	0.4	0.4
0.01M ATP	0.3	0.3	0.3	0.3
1 x 10^{-4}M cytochrome c	0.2	0.2	0.2	0.2
Water	0.3	0.3	0.2	0.2
Hexokinase	0.1	0.1	0.1	0.1
Boiled Mitochondria	0.1	---	---	---
Active Mitochondria	---	0.1	0.1	0.1
Pepsin-inactivated hormone	---	---	0.1	---
Active hormone	---	---	---	0.1
10 per cent KOH	0.2	0.2	0.2	0.2

TABLE IX

EFFECT OF ACTIVE AND PEPSIN-DIGESTED HORMONES ON THE RESPIRATION OF KIDNEY MITOCHONDRIA PREPARED FROM ISOTONIC SUCROSE

Time(mins.)	Oxygen Uptake(microliters)		
	Mitochondria	With 0.1 ml pepsin-inactivated hormone	With 0.1 ml active hormone
0	0	0	0
3	11	6	8
8	32	23	32
13	53	40	54
18	72	54	75
23	86	69	92
28	105	82	106
38	139	110	139
48[a]	165 (163-166)	135 (134-136)	165 (159-171)
53	178 (177-178)	149 (148-150)	179 (173-185)
58	190 (188-191)	160 (159-160)	189 (182-195)

a. The values given in parenthesis represent the actual values of the duplicate determinations.

time it was decided to test the active hormone extract again.

A fresh preparation of kidney mitochondria was prepared from isotonic sucrose solution. The mitochondria were assayed at 3 concentrations of succinate (20, 40, and 80 micromoles). Two concentrations of pepsin inactivated hormone (0.1 ml and 0.2 ml) and two concentrations of active hormone (0.1 ml and 0.2 ml) were also assayed with the three concentrations of substrate. The mitochondrial concentration was the same in all flasks. All other conditions were identical with those of experiment 15. The excess substrate and hormones were added in place of water. The means of the duplicate determinations are given in Tables XXXVI to XL in the Appendix.

The values of the means at 5 minutes were plotted by the method of Lineweaver and Burk (1934). Figure IX shows that pepsin is inhibiting the ensyme system. It appears that the inhibition is of the uncompetitive type. In this type of inhibition, it is assumed that one still has

$$E + S \longleftrightarrow ES \longrightarrow P + E$$

but that

$$ES + I \longleftrightarrow ESI \quad \text{(inactive)}$$

The data for the active hormone is inconclusive. At the concentration of 0.1 ml hormone there is virtually no effect on respiration at all levels of substrate concentration. Except for the effect of the 0.2 ml hormone concentration on the

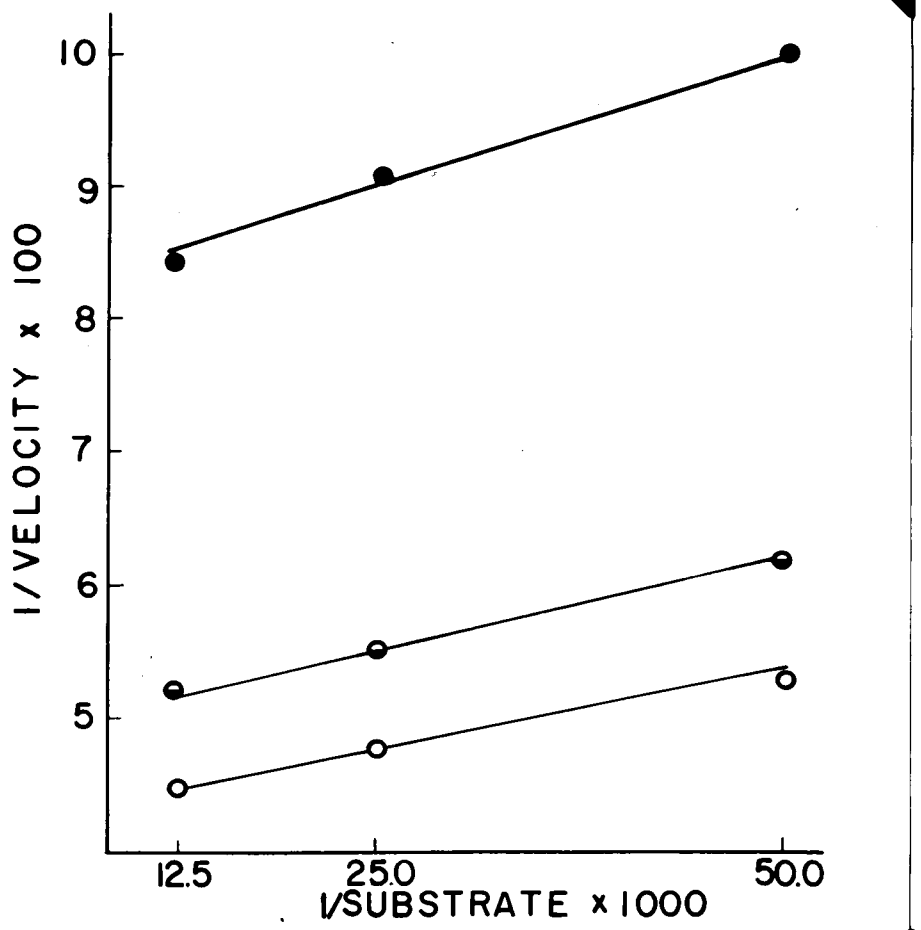

FIGURE IX

INHIBITION OF MITOCHONDRIA RESPIRATION BY PEPSIN-INACTIVATED HORMONE

—o—o— Mitochondria, —o—o— Mitochondria with 0.1 ml pepsin-inactivated hormone, —●—● Mitochondria with 0.2 ml pepsin-inactivated hormone.

highest concentration of succinate, the hormone does not seem to affect the respiration of kidney mitochondria prepared from isotonic sucrose.

Effect of Active and Inactive Hormones on Lyophilized Mitochondria Prepared from Hypertonic Sucrose

The next enzyme system tested was rat kidney mitochondria prepared from hypertonic sucrose. Ziegler and Linnae (1958) reported that mitochondria prepared from hypertonic sucrose were morphologically intact and that the oxidation of certain substrates of the citric acid cycle, mainly citrate, could not be studied by conventional manometric techniques, because they were inaccessible to their respective dehydrogenases in the intact mitochondria. However, when the mitochondria were sonically irradiated, all the substrates of the cycle were oxidized by the disrupted mitochondria. Since the necessary equipment for sonic irradiation was not available to the author, it was thought that disruption of the intact mitochondria could be obtained by lyophilization and then re-solution of the mitochondria in hypotonic media. The next series of experiments was undertaken with this goal in mind.

It was also believed that the pepsin inactivated hormone was not a valid control for an inactive hormone. It is true the pepsin digested hormone is physiologically inactive, but the final product of digestion is in reality not a modified hormone but a host of small peptides. Therefore, the hormone

was inactivated by two other means; both modified the hormone but the basic protein nucleus remained intact. The two methods of inactivation were oxidation and treatment with formaldehyde.

The method of Hogeboom et al (1948) was used to prepare mitochondria from 0.88M sucrose solution. It is as follows:

> A 10 per cent kidney homogenate was prepared in 0.88M sucrose solution with a Porter-Elvehjem homogenizer. Homogenization was continued for 3 minutes. The homogenate was centrifuged in the International Refrigerated centrifuge for 10 minutes at 600 x gravity and 0°C. The supernatant was saved. The sediment was resuspended in 2.5 ml of 0.88M sucrose solution per 10 ml of original homogenate and it was rehomogenised for 3 minutes. The second homogenate was centrifuged again for 10 minutes at 600 x gravity. The supernatants from both centrifugations were combined and centrifuged 3 times for 10 minutes at 600 x gravity. Each time the supernatant was saved. The mitochondria was collected from the supernatant by centrifugation at 23,000 x gravity for 20 minutes. The mitochondrial pellet was washed 3 times with 0.88M sucrose solution. After each washing the mitochondria were resedimented by centrifugation for 20 minutes at 23,000 x gravity. The final pellet was taken up with 3 ml of water and it was quickly frozen and lyophilised.

It was hoped that when the dry powder was taken up into hypotonic solution for assay that the mitochondria would disrupt and release their enzymes. This preperation will be referred to as lyophilized mitochondria prepared from hypertonic sucrose.

Oxidized parathyroid hormone was prepared by a modification of the method of Rasmussen (1958). He reported that oxidized parathyroid hormone was physiologically inactive and that the full activity could be restored by reduction with cysteine. Rasmussen (1958) used a highly purified and active parathyroid hormone in his studies. Reichert (1958) demonstrated that the Lilly's Parathyroid Extract was also physiologically inactivated when oxidized by the same procedure. The oxidation of the hormone is as follows:

0.06 ml of 30 per cent hydrogen peroxide was added to a 5 ml vial of Lilly's Parathyroid Extract (expiration date: May 1, 1960; Lot No. 2098-723416). The solution was allowed to stand for 30 minutes at room temperature. At the end of 30 minutes the solution was quickly frozen and lyophilized. The dry powder was then reconstituted to 5 ml with water. This preparation of hormone is referred to as lyophilized oxidized hormone in subsequent experiments.

In order to determine if oxidation changed any characteristics of the parathyroid hormone, the ultraviolet spectrum of oxidized hormone was compared with that of the active hormone. 0.2 ml of oxidized lyophilized and reconstituted hormone was diluted to 3 ml with 0.0001N hydrochloric acid. Similarily, an aliquot of lyophilized and reconstituted active hormone was diluted with the same acid solution. The spectrum of each was determined using 3 ml of the acid as blank. Then using the active hormone as blank, the spectrum of the oxidized hormone was determined. This second procedure was in reality measuring the differences in the spectrums of the two hormones. The spectrums of active and oxidized hormones are shown in Figure X and the plot of the differences in the spectrums is in Figure XI.

Figure X demonstrates that the oxidized hormone spectrum crosses that of the active in two places. This is evidence that some group, most probably tyrosine, has undergone change during oxidation. Figure XI demonstrates the appearance of a maximum at 240 millimicrons in the spectrum of the oxidized hormone almost quantitatively equal to the decrease at 270 millimicrons. This is further evidence that a group on the parathyroid hormone had undergone change during oxidation.

Formaldehyde inactivated parathyroid hormone was prepared by a modified procedure of the method of Shetlar et

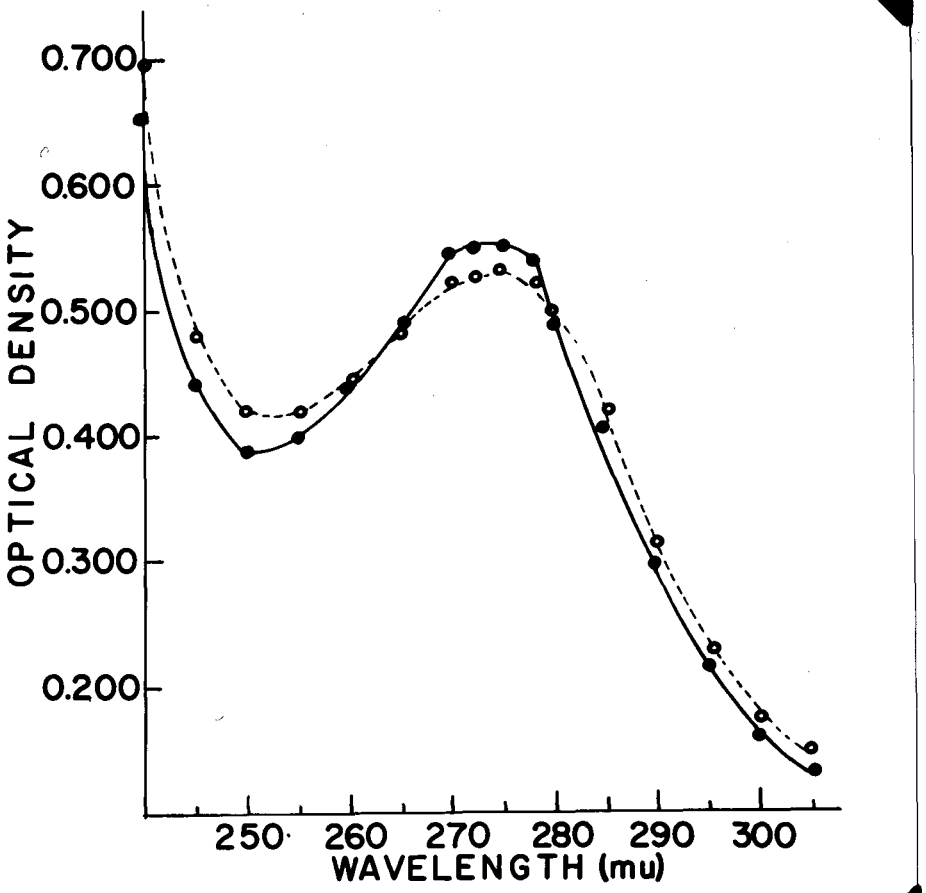

FIGURE X

ULTRA-VIOLET SPECTRA OF ACTIVE AND OXIDISED PARATHYROID HORMONES

—●—●— Active hormone, -·-o-·-o-·- Oxidised hormone.

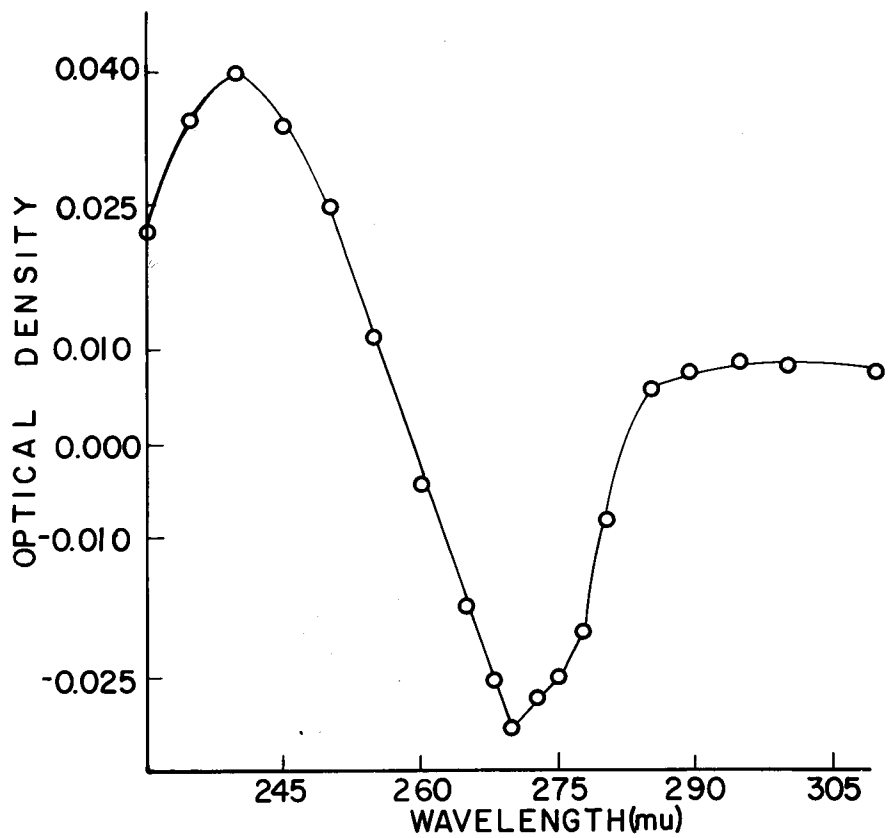

FIGURE XI

DIFFERENCE IN SPECTRA BETWEEN
ACTIVE AND OXIDIZED HOMOGENATES

al (1956) given below:

> 0.05 ml of 37 per cent formaldehyde was added to a 5 ml vial of Lilly's Parathyroid Extract (expiration date: May 1, 1960; Lot No. 2098-723416). The solution was allowed to stand at room temperature for 100 hours. At the end of this time the contents of the vial were quickly frozen and lyophilized. The dry powder was reconstituted to the original volume with 5 ml of water. The material did not dissolve even upon the addition of a small amount of 0.1N hydrochloric acid. A fine suspension was prepared by manual homogenization in a tapered glass homogenizer. This preparation is referred to as lyophilized formaldehyde hormone.

Since the formaldehyde treated hormone and oxidized hormone were lyophilized, a vial of Lilly's Parathyroid Extract (expiration date: May 1, 1960; Lot No. 2098-723416) was also lyophilized and reconstituted to the original volume with 5 ml of water. This step was taken to insure that the same volatile compounds were removed upon lyophilization.

Experiment 17.

An enzyme solution was prepared from the lyophilized 0.88M sucrose mitochondria. This solution contained 270 micrograms nitrogen per 0.2 ml. The first substrate tested was citrate; the contents of the manometric vessels are given in

Table X.

The equilibration times were 3 minutes with the stopcocks open, 3 minutes with the stococks closed, and 7 minutes after the enzyme solution and hexokinase were tripped into the main compartment.

Again citrate was unable to initiate respiration.

Experiment 18.

In this experiment succinate was tried as substrate for the enzyme solution. All conditions, otherwise, were identical with those of experiment 17. The results of this experiment are given in Table XI.

There appears to be an inhibition of respiration by lyophilized active, formaldehyde, and oxidized hormones. The inhibition by the lyophilized formaldehyde hormone is much more pronounced than that of the active or oxidized hormones.

Experiment 19.

Experiment 18 was repeated with the respiration being allowed to proceed for a longer time. The values given in Table XII represent the means of duplicate determinations.

Again there is an inhibition of respiration by all the preparations of lyophilized hormones. The formaldehyde hormone still inhibits more than the active or oxidized hormones.

Experiment 20.

It was thought that the addition of the small amount

TABLE X

CONTENTS OF FLASKS FOR RESPIRATION OF LYOPHILIZED MITOCHONDRIA PREPARED FROM HYPERTONIC SUCROSE

Materials	Flasks				
	1.	2.	3.	4.	5.
0.1M phosphate buffer, pH 7.4	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml
0.2M $MgCl_2$	0.1	0.1	0.1	0.1	0.1
0.1M glucose	0.4	0.4	0.4	0.4	0.4
1 x 10^{-4}M cytochrome c	0.4	0.4	0.4	0.4	0.4
0.01M ATP	0.3	0.3	0.3	0.3	0.3
0.4M Na citrate	0.1	0.1	0.1	0.1	0.1
Lyophilized active hormone	---	---	0.2	---	---
Lyophilized formaldehyde-treated hormone	---	---	---	0.2	---
Lyophilized oxidized hormone	---	---	---	---	0.2
Water	0.9	0.9	0.7	0.7	0.7
Hexokinase	0.1	0.1	0.1	0.1	0.1
Boiled enzyme	0.2	---	---	---	---
Active enzyme	---	0.2	0.2	0.2	0.2
10 per cent KOH	0.2	0.2	0.2	0.2	0.2

TABLE XI

EFFECT OF LYOPHILIZED ACTIVE, FORMALDEHYDE-TREATED, AND OXIDIZED HORMONES ON LYOPHILIZED MITOCHONDRIA PREPARED FROM HYPERTONIC SUCROSE

Time(mins.)	Oxygen Uptake(microliters)			
	Lyophilized Mitochondria	Lyophilized Mitochondria with Lyophilized Hormones		
		Active	Formaldehyde-treated	Oxidized
0	0	0	0	0
3	12	10	7	11
6	23	21	14	19
9	36	33	20	31
12	49	44	31	43
15	58	50	36	50
18	70	60	46	63
21[a]	78 (78-78)	71 (70-71)	50 (48-52)	69 (69-69)
24	86 (86-86)	79 (79-79)	56 (54-58)	78 (77-78)
27	97 (97-97)	87 (86-87)	61 (59-62)	84 (84-84)

a. The values in parenthesis are the actual values of the duplicate determinations.

TABLE XII

EFFECT OF LYOPHILIZED ACTIVE, FORMALDEHYDE-TREATED, AND OXIDIZED HORMONES ON LYOPHILLIZED MITOCHONDRIA PREPARED FROM HYPERTONIC SUCROSE

Time(mins.)	Oxygen Uptake(microliters)			
	Lyophilized Mitochondria	Lyophilized Mitochondria with Lyophilized Hormones		
		Active	Formaldehyde-treated	Oxidized
0	0	0	0	0
3	10	7	8	6
6	23	17	18	15
9	36	28	25	22
12	48	37	32	31
15	60	46	42	40
18	70	59	52	52
21	85	69	60	62
24	97	78	67	70
27[a]	110 (108-111)	90 (86-94)	76 (73-78)	83 (74-91)
33	129 (126-131)	109 (105-113)	90 (88-91)	102 (94-109)
39	147 (143-150)	125 (118-131)	97 (96-98)	115 (106-125)

a. The values in parenthesis are the actual values of the duplicate determinations.

of hydrochloric acid to the insoluble lyophilized formaldehyde treated hormone, in the attempt to solubilise it, perhaps was contributing to the marked inhibition of respiration by this preparation. The remainder of this preparation was treated with 11 volumes of absolute ethyl alcohol followed by 12 volumes of ether (Shetlar 1956). The precipitate was collected by centrifugation and then dried over concentrated sulfuric acid in a refrigerated desiccator for 4 days. Upon reconstitution with water to the volume before precipitation, the dry powder still did not dissolve. The slurry was homogenized manually to an even constituency in a tapered glass homogeniser.

Experiment 19 was repeated with the modified lyophilised formaldehyde treated hormone. In this experiment the effect of pepsin treated hormone was also studied. All conditions were identical with those of experiment 19, except that a new enzyme solution was prepared from the lyophilised 0.3M sucrose mitochondria. The new enzyme solution contained 230 micrograms nitrogen per 0.2 ml. The results are given in Table XIII. The values represent the means of duplicate determinations.

The effect of formaldehyde treated hormone now was radically changed. It did not affect the respiration. Lyophilised active and oxidised hormones still inhibited the respiration. Pepsin inactivated hormone, as it always had, also still inhibited respiration.

TABLE XIII

EFFECT OF LYOPHILIZED ACTIVE, MODIFIED FORMALDEHYDE-TREATED, AND OXIDIZED, AND PEPSIN-TREATED HORMONES ON LYOPHILIZED MITOCHONDRIA PREPARED FROM HYPERTONIC SUCROSE

Time(mins.)	Oxygen Uptake(microliters)				
	Lyophilized Mitochondria	Lyophilized Mitochondria with Hormones			
		Active	Modified Formaldehyde-treated	Oxidized	Pepsin-treated
0	0	0	0	0	0
3	6	5	5	4	4
6	12	10	11	10	8
9	18	17	18	16	13
12	25	23	25	23	15
15	30	28	32	26	20
18	37	34	39	31	25
21	42	38	43	37	28
24	48	45	52	41	33
27	56	50	58	46	37
30	61	56	65	52	41
33	67	62	70	57	47
39[a]	81 (81-82)	73 (70-76)	85 (81-89)	60 (64-70)	56 (54-57)
45	93 (90-96)	83 (79-86)	97 (93-100)	78 (73-82)	64 (63-64)
57	113 (110-115)	100 (98-102)	116 (112-120)	95 (91-99)	78 (76-80)

a. Values in parenthesis are the actual duplicate values.

Effect of Active and Inactive Hormones on Lyophilized Mitochondria Prepared from Isotonic Sucrose

Another enzyme preparation was turned to in an effort to oxidise citrate. Since mitochondria prepared from isotonic sucrose solution are already morphologically changed (Ziegler and Linnae 1958), it was thought that by lyophilising a preparation of mitochondria prepared from isotonic sucrose an even greater modification would be obtained when they were taken up into solution again.

Also, there were differences observed in the effects of the various parathyroid hormones which could not be readily explained. These variations could be due to some unaccountable factor in the medium in which they were carried. An effort then was made to remove the protein material from solution by precipitation and redissolution in water. Thus, the variables in the carrier media could be eliminated.

Rat kidney mitochondria were prepared from 0.25M sucrose solution as previously described. Instead of taking up the final mitochondrial pellet in 0.25M sucrose, the final pellet was taken up with water, the preparation was then quickly frozen and lyophilised. A solution of enzyme was prepared from a portion of the dry powder by dissolving in 0.1M Phosphate buffer, pH 7.4. It was hoped that this procedure would disrupt the mitochondria and release its enzymes into solution and that these enzymes would oxidise citrate.

Four precipitated hormones were prepared. They were as follows:

Precipitated active hormone.

The protein in one vial of Lilly's Parathyroid Extract (expiration date: February 1, 1961; Lot No. 9159-737444) was precipitated with ethyl alcohol and ether by the method of Shetlar et al (1956).

Precipitated oxidized hormone.

The hormone in one vial of Lilly's Parathyroid Extract (expiration date: February 1, 1961; Lot No. 9159-73744) was oxidized by the method of Rasmussen (1958). The excess hydrogen peroxide was removed with catalase. The protein was precipitated by the method of Shetlar et al (1956).

Precipitated formaldehyde treated hormone.

One vial of Lilly's Parathyroid Extract (expiration date: February 1, 1961; Lot No. 9159-737444) was treated with formaldehyde as previously described. The protein material was precipitated by the method of Shetlar et al (1956).

Precipitated pepsin digested hormone.

One vial of Lilly's Parathyroid Extract (expi-

ration date: February 1, 1962 Lot No. 9159-737444) was digested with pepsin as previously described. The protein was precipitated with ethanol and ether by the method of Shetlar et al (1956).

The precipitated proteins were collected by centrifugation in the International Clinical centrifuge set at half speed. They were washed once with 5 ml of acetone and recentrifuged. They were then dried over a beaker of concentrated sulfuric acid and a beaker of calcium chloride for 4 days in a refrigerated desiccator. All the dried precipitates were quantitatively collected in tared vials and weighed. The weights and descriptions of the precipitates are given in Table XIV.

TABLE XIV

PRECIPITATED HORMONES

	Weight (Mgs.)	
Precipitated active hormone	32	yellow-brown
Precipitated formaldehyde treated hormone	32	"
Precipitated oxidized hormone	36	"
Precipitated pepsin digested hormone	13	greyish-white

Solutions of hormone were prepared which had a concentration of 6 mgs. of precipitated material per ml of water. Only the precipitated active hormone did not go readily into solution.

Experiment 21.

Because the mitochondria would probably be disrupted, it was thought not necessary to include hexokinase, glucose, ATP, and sucrose in the assay system. An enzyme solution was prepared from the lyophilised mitochondria; it was found to contain 150 micrograms of nitrogen per 0.2 ml aliquot. The components of the Warburg flasks are given in Table XV.

The vessels were equilibrated 3 minutes with the stopcocks open and 3 minutes with the stopcocks closed. The enzyme solution was tripped into the main compartment and equilibration was continued for 4 minutes before zero readings were taken.

There was no respiration when citrate was the substrate.

Experiment 22.

In this experiment succinate was tried as substrate for the enzyme solution. All other conditions were identical with those of experiment 21. The results of duplicate determinations are given in Table XVI.

There appears to be only a slight effect of the precipitated hormones on the respiration of this enzyme system.

TABLE XV

FLASK COMPONENTS FOR EXPERIMENT 21

Materials	Flasks					
	1.	2.	3.	4.	5.	6.
0.1M phosphate buffer, pH 7.4	2.0ml	2.0ml	2.0ml	2.0ml	2.0ml	2.0ml
0.2M $MgCl_2$	0.1	0.1	0.1	0.1	0.1	0.1
0.4M Na citrate	0.1	0.1	0.1	0.1	0.1	0.1
1 x 10^{-4}M cytochrome c	0.2	0.2	0.2	0.2	0.2	0.2
Water	0.4	0.4	0.2	0.2	0.2	0.2
Precipitated active hormone	---	---	0.2	---	---	---
Precipitated oxidized hormone	---	---	---	0.2	---	---
Precipitated formaldehyde-treated hormone	---	---	---	---	0.2	---
Precipitated pepsin-digested hormone	---	---	---	---	---	0.2
Boiled mitochondria	0.2	---	---	---	---	---
Active mitochondria	---	0.2	0.2	0.2	0.2	0.2
10 per cent KOH	0.2	0.2	0.2	0.2	0.2	0.2

TABLE XVI

EFFECT OF PRECIPITATED HORMONES ON LYOPHILIZED MITOCHONDRIA PREPARED FROM ISOTONIC SUCROSE

Time(mins.)	Oxygen Uptake(Microliters)				
	Lyophilized Mitochondria	Lyophilized Mitochondria with Precipitated Hormones			
		Active	Oxidized	Formaldehyde-treated	Pepsin-treated
0	0	0	0	0	0
2	7	8	7	6	6
4	13	14	13	12	12
8	29	28	27	27	27
12	44	41	41	41	40
16	57	52	52	53	53
20[a]	72 (69-75)	64 (62-65)	65 (61-68)	67 (62-71)	65 (61-68)
24	85 (82-87)	75 (73-76)	76 (74-78)	80 (76-84)	77 (73-81)
28	95 (91-99)	83 (81-85)	86 (83-89)	89 (84-95)	88 (83-92)

a. The values in parenthesis are the actual values of the duplicate determinations.

It is interesting to note that the pronounced effect of pepsin digested hormone on respiration has now disappeared.

Experiment 23.

A new precipitated active hormone was prepared as previously described. The final dry precipitate weighed 32 mgs. A 6 mgs. precipitate per ml of water was prepared and experiment 22 was repeated one week later.

The values in Table XVII are corrected for the nitrogen content of the new enzyme solution so that a comparison can be made between the activities of the enzyme solutions of this experiment and experiment 22.

There is no effect now of the precipitated hormones on the oxygen uptake of the enzyme system. The enzyme has lost approximately 50 per cent of its activity in one week.

In Vivo and In Vitro Effect of Hormone on Kidney Homogenate Respiration

Experiment 24.

An opportunity was presented to obtain the kidneys of rats which had received 500 USP units of Lilly's Parathyroid Extract (expiration date: February 1, 1961; Lot No. 9159-737444) one hour previous to sacrifice. The kidneys from these rats were homogenated in 0.25M sucrose solution. A homogenate was also prepared from the kidneys of normal rats. A portion of each homogenate was heated in a boiling water bath for 15

TABLE XVII

EFFECT OF PRECIPITATED HORMONES ON LYOPHILISED MITOCHONDRIA PREPARED FROM ISOTONIC SUCROSE

Time(mins.)	Oxygen Uptake(microliters)				
	Lyophilised Mitochondria	Lyophilised Mitochondria with Precipitated Hormones			
		Active II	Oxidised	Formaldehyde-treated	Pepsin-treated
0	0	0	0	0	0
2	3	3	4	4	5
4	7	8	9	8	9
8	15	16	18	17	19
12	23	25	27	26	27
16	3-	32	36	32	32
20[a]	37 (34-39)	38 (36-40)	39 (37-41)	39 (38-39)	38 (36-40)
24	42 (40-44)	42 (41-42)	43 (41-45)	41 (40-41)	42 (40-44)
28	46 (44-48)	48 (46-50)	48 (47-49)	45 (44-45)	45 (45-49)

a. The values in parenthesis are the actual values of the duplicate determinations.

minutes in order to inactivate the enzymes. The components of the Warburg vessels are given in Table XVIII.

Equilibration times were 3 minutes with the stopcocks open, 3 minutes with them closed, and 4 minutes after the homogenate was tripped into the main compartment. The results in Table XIX are the means of 5 determinations. They are all expressed as oxygen uptake per mg of homogenate nitrogen.

Respiration seems to be greater by the treated rat kidney homogenate than by the normal rat kidney homogenate. This is not consistent with the finding of Laskin and Engel (1956) that injections of hormone into rats causes an inhibition of the succinic dehydrogenase of bone. However, in this experiment kidneys were used, and also, the time of sacrifice of the animals after they received the dose of hormone was much shorter than in Laskin and Engel's study.

There was no effect of 20 USP units of parathyroid hormone on the respiration of normal rat kidney homogenate.

Effect of Precipitated Hormones on Kidney Homogenate Respiration

Experiment 25.

The precipitated hormones up to this point had not been tested with kidney homogenates. Initial work with untreated active hormone and pepsin digested hormone on kidney homogenates had shown an inhibition of respiration (experiments 12 and 13). However, in that series of experiments, pyruvate

TABLE XVIII

FLASK COMPONENTS FOR IN VIVO-IN VITRO EFFECT OF PARATHYROID HORMONE ON KIDNEY HOMOGENATE RESPIRATION

Materials	Flasks			
	1.	2.	3.	4.
0.1M phosphate buffer, pH 7.4	2.0 ml	2.0 ml	2.0 ml	2.0 ml
0.4M Na succinate	0.1	0.1	0.1	0.1
Water	0.5	0.5	0.3	0.5
Active hormone[a]	---	---	0.2	---
Boiled homogenate	0.4	---	---	---
Normal rat, kidney homogenate	---	0.4	0.4	---
Treated rat, kidney homogenate	---	---	---	0.4
10 per cent KOH	0.2	0.2	0.2	0.2

a. The 0.2 ml of active extract is 20 USP units of activity and it corresponds to the amount of hormone in 0.4 ml of treated rat, kidney homogenate on the basis of 500 USP units injected per volume of kidney homogenate.

TABLE XIX

IN VIVO AND IN VITRO EFFECT OF PARATHYROID HORMONE ON KIDNEY HOMOGENATE RESPIRATION

Time(mins.)	Oxygen Uptake(u liters/mg N)		
	Normal rat, kidney homogenate	Active hormone with normal rat, kidney homogenate	Treated rat, kidney homogenate
0	0	0	0
4	27	27	27
8	48	49	50
12	65	67	71
16	78	80	87
20	91	93	102
24	102	103	115
28	112	113	127
36	134	132	151
44[a]	150 3	150 2	171 3

a. The means of 5 determinations at 44 minutes are given along with the standard error of the mean.

and fumarate had been used as substrates. It was thought advisable then to see if the precipitated hormones exhibited an effect on kidney homogenate respiration when succinate was the substrate.

A rat kidney homogenate was prepared in 0.25M sucrose solution. The nitrogen content was 920 micrograms per 0.4 ml of homogenate. All conditions were the same as in experiment 24. The values given in Table XX are the means of duplicate determinations.

There was no effect of the precipitated active, formaldehyde treated, and pepsin hormones on kidney homogenate respiration. The effect of oxidised hormone was inhibitory.

Effect of Lyophilised Active and Oxidised Hormones on Kidney Mitochondria Prepared from Isotonic Sucrose

Experiment 26.

Active hormone had shown a slight inhibitory effect on the respiration of kidney mitochondria prepared from isotonic sucrose (experiment 16). Precipitated oxidised hormone had also shown a slight inhibitory effect on kidney homogenate respiration (experiment 25) and on lyophilised mitochondria prepared from isotonic sucrose (experiment 22). Lyophilised oxidised hormone was also inhibitory on the respiration of lyophilised mitochondria prepared from hypertonic sucrose (experiments 18, 19 and 20). The next experiment was undertaken to test lyophilised active and oxidised hormones on kidney

TABLE XX

EFFECT OF PRECIPITATED HORMONES ON KIDNEY HOMOGENATE RESPIRATION

Time(mins.)	Oxygen Uptake(microliters)				
	Homogenate only	Homogenate with Precipitated Hormones			
		Active II	Oxidized	Formaldehyde-treated	Pepsin-treated
0	0	0	0	0	0
4	26	23	23	26	28
8	47	42	42	48	48
12	64	59	58	66	67
16	78	72	71	83	82
20	92	86	84	94	95
24	103	99	96	106	108
28	114 (107-120)	109 (105-112)	105 (103-106)	117 (116-117)	119 (113-124)
36	136 (128-143)	131 (128-133)	125 (123-126)	138 (137-139)	141 (136-145)
44	155 (150-160)	148 (147-149)	143 (139-146)	158 (155-160)	161 (157-164)

a. The values in parenthesis are the actual values of the duplicate determinations.

mitochondria prepared from isotonic sucrose.

Rat kidney mitochondria were prepared from 0.25M sucrose solution by the method of Schneider (1950). The nitrogen content of 0.2 ml of mitochondrial solution was 400 micrograms. The flask components and equilibration times were the same as those in experiment 24. The values given in Table XXI are the means of 4 determinations.

Lyophilised active hormone has no effect on the respiration of mitochondria and lyophilised oxidized hormone is inhibitory.

Effect of Active Hormone Extract on the Oxidation of Various Substrates by Kidney Mitochondria Prepared from Hypertonic Sucrose

Experiment 27.

This experiment was performed to investigate the effect of active hormone extract, treated in no way, on the oxidation of various substrates by morphologically intact mitochondria.

Rat kidney mitochondria were prepared from 0.88 M sucrose solution by the method of Hogeboom et al (1948). The final mitochondrial pellet was taken up in 0.88 M sucrose solution. The nitrogen content of 0.1 ml of mitochondria suspension was 130 micrograms. The flask components are given in Table XXII. The final sucrose concentration was 0.5M. Under these conditions the mitochondria are structurally stable during the

TABLE XXI

EFFECT OF LYOPHILIZED ACTIVE AND OXIDIZED HORMONES ON KIDNEY MITOCHONDRIA PREPARED FROM ISOTONIC SUCROSE

Time(mins.)	Oxygen Uptake(microliters)		
	Mitochondria only	With Lyophilised Active Hormone	With Lyophilised Oxidised Hormone
0	0	0	0
4	20	23	16
12	56	62	50
16	75	80	65
20	90	96	80
24	105	112	96
28	123	128	110
36	149	151	136
44[a]	176 2	176 3	159 4

a. The means of the 4 determinations at 44 minutes are given along with the standard error of the mean.

TABLE XXII

CONTENTS OF WARBURG FLASKS FOR EXPERIMENT 27

Materials	FLASKS		
	1.	2.	3.
0.1M phosphate buffer, pH 7.4	0.5 ml	0.5 ml	0.5 ml
0.2M $MgCl_2$	0.1	0.1	0.1
0.1M glucose	0.4	0.4	0.4
1 x 10^{-4}M cytochrome c	0.2	0.2	0.2
0.01M ATP	0.2	0.2	0.2
0.4M Na substrate	0.1	0.1	0.1
1.76M sucrose	0.8	0.8	0.8
Water	0.5	0.5	0.3
Active hormone extract	---	---	0.2
Hexokinase	0.1	0.1	0.1
Boiled mitochondria	0.1	---	---
Active mitochondria	---	0.1	0.1
10 per cent KOH	0.2	0.2	0.2

assay (Ziegler and Linnae 1958).

Equilibration times were 3 minutes with the stopcocks open, 3 minutes with them closed, and 4 minutes after the mitochondria and hexokinase were tripped into the main compartment. The oxygen uptake was determined in triplicate for all substrates in the presence of hormone and for every mitochondria determination. The values in Tables XXIII to XXIV are the means of the triplicate determinations.

The substrates malate, alpha-ketoglutarate, and glutamate failed to produce respiration. The active hormone extract had no effect on fumarate and citrate oxidation and it appeared to activate the oxidation of pyruvate and succinate.

TABLE XXIII

EFFECT OF ACTIVE HORMONE EXTRACT ON KIDNEY MITOCHONDRIA RESPIRATION WITH FUMARATE AS SUBSTRATE

Time(mins.)	Oxygen Uptake(microliters)	
	Mitochondria only	Mitochondria with Hormone
0	0	0
4	2	2
8	2	3
12	4	5
16	4	4
20	5	5
24	5	5
32	8	8
44[a]	9 (7-11)	8 (6-10)

a. The values in parentheses give the ranges of the triplicate determinations.

TABLE XXIV

EFFECT OF ACTIVE HORMONE EXTRACT ON KIDNEY MITOCHONDRIA RESPIRATION WITH CITRATE AS SUBSTRATE

Time(mins.)	Oxygen Uptake(microliters)	
	Mitochondria only	Mitochondria with Hormone
0	0	0
4	2	3
8	5	6
12	6	7
16	8	9
20	10	11
24	11	13
32[a]	14 (13-14)	15 (14-17)

a. The values in parentheses give the ranges of the triplicate determinations.

TABLE XXV

EFFECT OF ACTIVE HORMONE EXTRACT ON KIDNEY MITOCHONDRIA RESPIRATION WITH PYRUVATE AS SUBSTRATE

Time(mins.)	Oxygen Uptake(microliters)	
	Mitochondria only	Mitochondria with Hormone
0	0	0
4	2	3
8	4	5
12	2	5
16	4	6
20	3	6
24	3	8
32[a]	3 (0-7)	8 (0-13)

a. The values in parenthesis give the ranges of the triplicate determinations.

TABLE XXVI

EFFECT OF ACTIVE HORMONE EXTRACT ON KIDNEY MITOCHONDRIA RESPIRATION WITH SUCCINATE AS SUBSTRATE

Time(mins.)	Oxygen Uptake(microliters)	
	Mitochondria only	Mitochondria with Hormone
0	0	0
4	6	6
8	11	13
12	19	21
16	25	29
20	32	37
24	39	46
42	70	80
44[a]	74 (67-78)	84 (82-87)

a. The values in parenthesis give the ranges of the triplicate determinations.

CHAPTER III

DISCUSSION AND SUMMARY

From recent studies of parathyroid hormone effects on the whole organism, and of the effects of vitamin D on the whole organism and on the particulate matter of cells, there were bits of evidence which led one to believe that perhaps the hormone would show some effect on specific enzyme systems removed from the influence of the whole organism. Attempts were made to clarify this issue. The results of this particular research, however, are inconclusive and on that basis, this hypothesis still remains unproven.

Both individual enzyme systems and multi-enzyme systems were used. The multi-enzyme preparations included rat kidney homogenates, rat kidney mitochondria obtained from isotonic sucrose and hypertonic sucrose, and rat kidney mitochondria obtained from isotonic and hypertonic sucrose and then lyophilised.

The effect of active and inactive hormones was determined on the various enzyme systems. Active hormone preparations included untreated hormone extract, dialysed hormone extract, lyophilized hormone extract, and hormone precipitated from a hormone extract. Inactive hormone preparations included a pepsin-digested hormone extract, a pepsin-digested hormone extract precipitated with ethanol and ether, hormone extracts

oxidized or treated with formaldehyde and then lyophilized, and hormone extracts oxidized or treated with formaldehyde and then precipitated from solution with ethanol and ether.

It is difficult to assess the results of the effects of active hormone on isocitric dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic dehydrogenase, because all the effects observed were nearly of the same magnitude in all three enzyme systems and at the different concentrations of the same enzyme. A possible explanation for this is that the hormone may have been affecting some component common to all three enzyme systems. The most plausible component would either be TPN or TPNH. That this is not unlikely, was shown by Reichert (1958) who, in a purely in vitro system of hormone and TPNH, was able to demonstrate a small effect of parathyroid hormone on the absorbency of TPNH over a period of 6 hours.

The parathyroid extract, commercially obtained, showed a definite inhibition of the respiration of kidney homogenates the three times it was tested when fumarate and pyruvate were used as substrates. When pepsin-inactivated hormone was used as a control-inactive hormone, there was a more pronounced inhibition. The parathyroid extract had only slight variable effects on the respiration of homogenates and of kidney mitochondria when succinate was used as the substrate. Pepsin-inactivated hormone still inhibited markedly in both these instances.

Lyophilized active and oxidized hormone preparations also inhibited the respiration of lyophilized mitochondria prepared from hypertonic sucrose when succinate was the substrate. The lyophilized oxidized hormone always appeared to inhibit more, than the active hormone preparation. Lyophilized oxidized hormone also inhibited the respiration of mitochondria prepared from isotonic sucrose while lyophilized active hormone had no effect.

With the exception of oxidized hormone, which inhibits, the precipitated hormone preparations had no effect on the respiration of kidney homogenates and lyophilized mitochondria when succinate was the substrate. It then seems plausible that some component, originally in the hormone medium as a contaminant or introduced in the process of inactivation of the various hormones, was producing the inhibitory effects of the other hormone preparations.

For example, the marked inhibitory effects of pepsin-inactivated hormone may well have been due to the presence of excess chloride ion. During the digestion of the hormone, hydrochloric acid was used to adjust and readjust the pH of the solution. When pepsin-inactivated hormone was precipitated from the medium in which it was digested and then taken up into distilled water the inhibitory effects of this preparation disappeared. The evidence points in this direction, also, with the formaldehyde-inactivated hormone. Because it was not soluble after lyophilization and reconstitution with water, a small

amount of hydrochloric acid was added in an attempt to solubilize it. This preparation also markedly inhibited respiration. When the hormone was precipitated and removed from the medium and retested, the inhibition disappeared.

The inhibitory effects of the oxidized hormone preparations cannot be rationalized at this time. The reader must be cautioned, however, that an effect was arbitrarily ascertained if respiration in the presence of hormone differed by more than 5 per cent from that of the enzyme system in the absence of hormone. The effects, therefore, may have only been a matter of which value was chosen to evaluate the results.

All attempts to oxidize citrate proved fruitless except when mitochondria prepared from hypertonic sucrose were used. The respiration, though, was very small and the active parathyroid extract had no effect on it. This difficulty in the oxidation of citrate by homogenates and mitochondrial preparations is not native to this research. Ziegler and Linnae (1958) found that in the study of mitochondrial-bound pyridinoproteins, the activities of the enzymes were highly variable, and that they were a function of the damage sustained by the mitochondria during preparation. Specifically with the oxidation of citrate, they demonstrated that fresh mitochondria and mitochondria frozen and then thawed did not oxidize citrate. Only when the mitochondria were sonically disrupted did the oxidation of citrate proceed. Thus, in the present study, it

is most probable that a disruption of the mitochondria was never obtained.

Another problem which undoubtedly had some bearing on the study was the purity of the hormone extract. It was not convenient or practical to initiate a further purification of the hormone. That remains a problem in itself. The parathyroid extract, commercially available, contains approximately 6 milligrams of protein material and 100 USP units of parathyroid activity per milliliter. This is approximately 16 USP units per milligram of protein. Rasmussen (1957) purified a crude parathormone B preparation by means of zonal electrophoresis on polyvinyl chloride and obtained a highly active component of approximately 200 units of activity per milligram of protein. More recently, Munson (1959) purified the commercially available extract by a new method using cellulose columns and he also obtained a hormone preparation with about 200 units of activity per milligram of protein. Applying a new method of extraction from fresh glands and using the new purification procedure, the same worker obtained a product which had greater than 500 units of activity per milligram of protein.

It would seem then that further work on the problem as a whole must either await a readily available hormone preparation of high purity or more concrete evidence for the direct effect of parathyroid hormone on specific enzyme systems.

CHAPTER IV

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APPENDIX

Chemicals and Preparation of Reagents

Acetone (Mallinckrodt) Analytical Reagent, Control 0610.

Adenosine-5-phosphate (Sigma) Disodium salt·$3H_2O$, from heart muscle, assay 90-100 per cent corrected for moisture. Lot No. 46-93. A 0.01M solution was prepared by dissolving 62.3 mgs. in 10 ml water.

Alpha-ketoglutaric acid (California Foundation for Biochemical Research) CFF. Lot No. 171465. A 0.4M solution of the sodium salt was prepared by dissolving an appropriate amount of acid in 0.4M sodium hydroxide.

Aminonapthosulfonic acid (Matheson Coleman and Bell) Technical. The acid was recrystallized as follows: heat 100 ml water to 90°C and dissolve in it 150 grams of sodium bisulfite and 10 grams of sodium sulfite. To this solution add 15 grams of the crude sulfonic acid and stir until all but the amorphous impurity has dissolved. Filter the hot solution through a large paper, cool the filtrate under the tap, and add to it 10 ml of concentrated hydrochloric acid. Filter with the aid of suction, wash with about 300 ml of water, and finally with alcohol until the washings are colorless. Dry the precipitate in air with the least possible exposure to light, powder, and transfer to a brown bottle. The color reagent for phosphorus determinations is prepared by dissolving 0.5 grams of the

recrystallized material in 195 ml of 15 per cent sodium bisulfite and 5 ml of 20 per cent sodium sulfite. The solution is kept in a brown bottle and prepared fresh every two weeks.

Ammonium molybdate·$4H_2O$ (Merck) Reagent. Lot No. 42113. The ammonium molybdate color reagent for phosphorus determinations was prepared by diluting 25 grams to 200 ml with water, adding 300 ml of 10N sulfuric acid, and diluting to 1 liter with water.

Ammonium sulfate (Baker) Reagent. Lot No. 90765. A saturated solution of ammonium sulfate was prepared by adding excess salt to 1 liter of water and allowing the mixture to equilibrate to 2°C in the refrigerator.

Brodie's solution. Prepared by dissolving 23 grams of sodium chloride, 5 grams of sodium cholate, and 100 mgs. of acid fuchsin in 500 ml of water.

Catalase (Sigma) Lyophilized, from beef liver, 40-50 milliequivalents of sodium perborate. Control 5216.

Cytochrome c (Sigma) From beef heart, free of ammonium sulfate and sodium chloride, assay 100 per cent corrected for 2 per cent water and based on a molecular weight of 16,500. Prepared solutions were standardized.

Dextrose (Mallinckrodt) Analytical Reagent. Control 6XM.

Dialysis tubing (Visking Company) 18/32 inch diameter.

Digestion mixture for nitrogen determinations. Contains 25

mgs. mercuric oxide, 25 grams potassium sulfate, and 100 ml of concentrated sulfuric acid per liter of solution.

Ethyl alcohol, absolute (U.S. Industrial Chemical Company) U.S.P.

Ether (Mallinckrodt) Analytical Reagent. Control 6CE.

Formaldehyde, 37 per cent (Mallinckrodt) Analytical Reagent. Control ACD.

Fuchsin, acid (Hartman-Leddon) Total dye content 59 per cent. Control 139.

Fumaric acid (Mathoson) Practical. The acid was recrystallized twice from water. A 0.4M solution of the sodium salt was prepared by dissolving an appropriate amount of the recrystallized acid in 0.4M sodium hydroxide.

Gelatin (Nutritional Biochemical Company) Control 975.

Glucose-6-phosphate (Sigma) Barium salt, approximately $7H_2O$. Lot No. 16-26. The sodium salt was prepared by dissolving 130 mgs. of the barium salt in 2 ml of water and then adding 25 mgs. of sodium sulfate. The mixture was shaken for 10 minutes, allowed to stand for 10 minutes, and then it was centrifuged to remove the barium sulfate. The supernatant was siphoned off and used for glucose-6-phosphate dehydrogenase assays.

L-(-)-Glutamic acid (Eastman Kodak) Control 1466. The sodium salt was prepared by dissolving an appropriate amount of the acid in 0.4M sodium hydroxide.

Hexokinase (Sigma) Crude, type II, from yeast, 50,000 K.U. units per gram at 25°C. Lot No. 88-615. Five mgs. per ml solutions were always prepared.

Hydrochloric acid, 37 per cent (Mallinckrodt) Analytical Reagent. Control 6FY.

Hydrogen peroxide, 30 per cent (Bakers) Reagent. Lot No. 90111.

dl-Isocitric acid lactone (California Foundation for Biochemical Research) CFR. Lot No. 120753. The lactone was converted to the sodium salt of isocitric acid by heating in 0.1N sodium hydroxide in a boiling water bath for 10 minutes.

Lanolin, anhydrous (Mallinckrodt) U.S.P.

dl-Malic acid (Mathieson) Technical. The acid was recrystallized twice from water. The sodium salt was prepared by dissolving an appropriate amount of the recrystallized acid in 0.4M sodium hydroxide.

Magnesium chloride•$6H_2O$ (Mallinckrodt) Analytical Reagent. Control 6MY.

Mercuric oxide (Mallinckrodt) U.S.P. Control 3HG-1.

Manganese sulfate•$1H_2O$ (Mallinckrodt) Analytical Reagent Control 6192.

Nessler's Reagent. Contained 140 grams potassium iodide, 200 grams mercuric iodide, and 100 grams sodium hydroxide per liter of solution. After preparation, the solution was allowed to stand for two days and then it was filtered

thru glass filter paper.

Nitrogen standard. Contained 4.719 grams of ammonium sulfate (dried in a desiccator) and 0.5 ml of concentrated sulfuric acid (as a preservative) per liter of stock solution.

Parathyroid extract (Lilly's) 100 USP units per ml. Lot numbers and expiration dates are given with each preparation used.

Pepsin (Armour and Company) Crystalline, porcine origin. Lot No. 100-145.

6-Phosphogluconate (Sigma) Barium salt. Lot No. 17-610. The sodium salt was prepared by dissolving 95.7 mgs. of the barium salt in 2 ml of water and then adding 35 mgs. of sodium sulfate. The mixture was shaken for 10 minutes, allowed to stand 10 minutes more, and then it was centrifuged to remove the barium sulfate. The supernatant was siphoned off and used as the substrate for 6-phosphogluconic dehydrogenase.

Pig heart (Worthington Biochemical Corporation) Acetone powder. Lot No. PHP22.

Potassium ferricyanide (Merck) Reagent. Lot No. 42270.

Potassium iodide (Mallinckrodt) Analytical Reagent. Control 3BC.

Potassium phosphate, monobasic (Baker and Adamson) Reagent. Lot No. P060.

Potassium sulfate (Baker) Analytical Reagent. Lot No. 61225.

Sodium bicarbonate (Baker and Adamson) Reagent. Lot No. D217.

Sodium bisulfite (Mallinckrodt) Analytical Reagent. Control AMB.

Sodium chloride (Mallinckrodt) Analytical Reagent. Control SAS.

Sodium cholcate(Merck) Soluble extract of ox-gall. Lot No. 42299.

Sodium citrate·$2H_2O$ (Mallinckrodt) Analytical Reagent. Control SAP.

Sodium hydrosulfite (Mallinckrodt) Analytical Reagent. Control UMG.

Sodium hydroxide (Baker) Reagent. Lot No. 9375.

Sodium phosphate, dibasic (Mallinckrodt) Analytical Reagent Control BCL.

Sodium phosphate, monobasic (Mallinckrodt) Analytical Reagent Control BDK.

Sodium succinate·$6H_2O$ (Matheson Coleman and Bell) Practical

Sodium sulfite (Mallinckrodt) Analytical Reagent. Control EJD.

Standard phosphorus solution. Prepared by dissolving 0.439 grams of dry monobasic potassium phosphate in 1 liter of water. A small amount of chloroform was added as preservative. This solution contained 0.1 mgs. of phosphorus in 1.0 ml. A working standard was prepared by diluting 5 ml of the stock solution to 100 ml with 10 per cent trichloroacetic acid.

Sucrose (Mallinckrodt) Analytical Reagent. Control STS.

Sulfuric acid, concentrated (Baker and Adamson) Reagent. Lot No. 4JOP221.

Trichloroacetic acid (Mallinckrodt) Analytical Reagent. Control CHT.

TRIS (trihydroxymethyl aminomethane) (Sigma) Sigma 7-9 Biochemical Buffer and Primary Standard. Lot No. T07-01.

TPN (triphosphopyridine nucleotide) (Sigma) Monosodium salt, from yeast DPN, assay 95 per cent based on H_2O. Lot No. 127-80-4.

Versene (disodium ethylene diamine tetraacetate·$2H_2O$) (Fisher) Reagent. Lot No. 771313.

Water. Distilled water was redistilled from an all glass pyrex apparatus and stored in a pyrex bottle. This doubly distilled water was used in the preparation of all reagents.

Nitrogen Determinations

Nitrogen was determined by a modification of the method of Nelson (1957). The standard curve obtained by his method was not linear over the range tested. The Nessler's reagent was prepared by a different method and readings were taken with the Klett-Summerson colorimeter using the green filter instead of the blue. Unknowns were read directly from Figure XII. The values represent the means of triplicate determinations. The data for the standard nitrogen curve is given

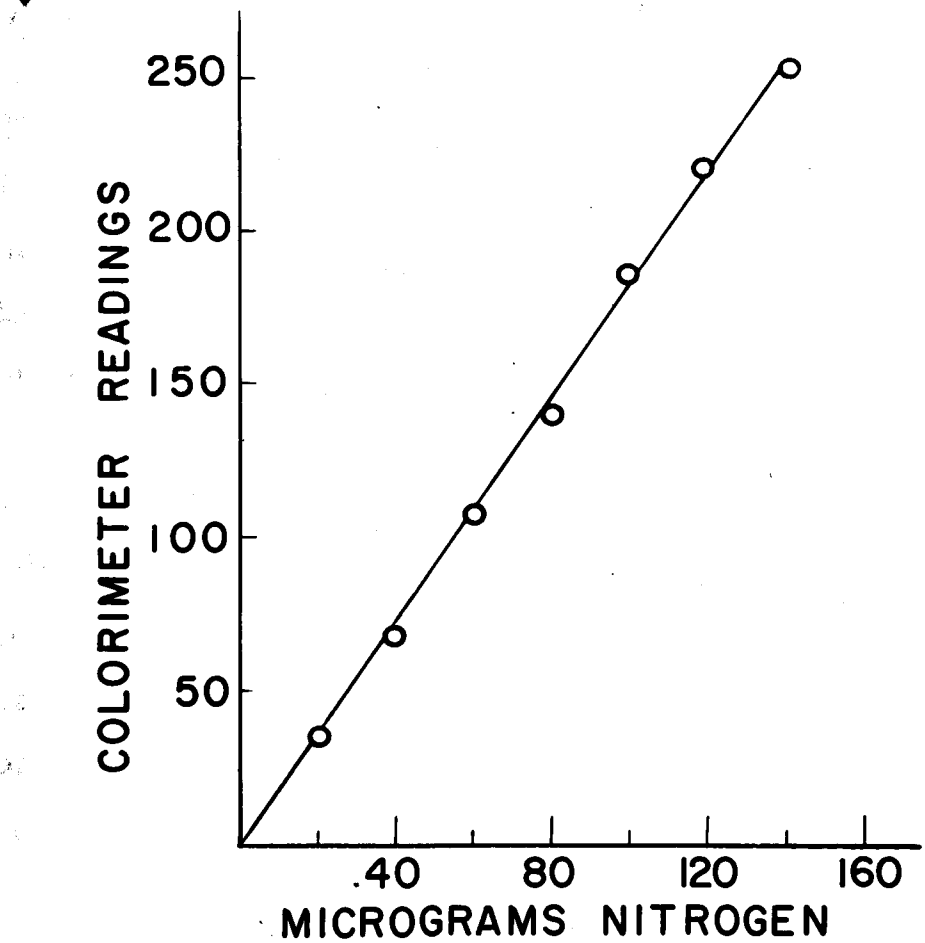

FIGURE XII

NITROGEN STANDARD CURVE

in Table XLIII.

Unknowns are diluted with digestion mixture and water so that 1 ml of diluted material contained 0.5 ml of digestion mixture and approximately one-tenth of the original unknown. For example, 0.4 ml of kidney homogenate was diluted with 5 ml of digestion mixture and 4.6 ml of water in a 10 ml volumetric flask. One ml of this material was taken for nitrogen determination. Duplicate unknowns were diluted and duplicate nitrogens were determined on each dilution.

Digestion was carried on in a sand bath on a hot plate for at least 3 hours. When black particles were present in the digestion tubes, 2 drops of 30 per cent hydrogen peroxide were added to all tubes and the digestion was continued for at least another hour. Digestion was performed in tubes calibrated with a 10 ml scratch mark so that the color development could be carried out directly in the digestion tubes. This eliminated any tranferring of samples.

Phosphorus Determinations

Inorganic phosphorus was determined by the method of Fiske and Subbarow (1925). Unknowns were read directly from the standard curve (Figure XIII). The data for triplicate determinations of phosphorus are given in Table XLIV.

Standardization of Cytochrome C.

Cytochrome c has a characteristic absorption spectrum

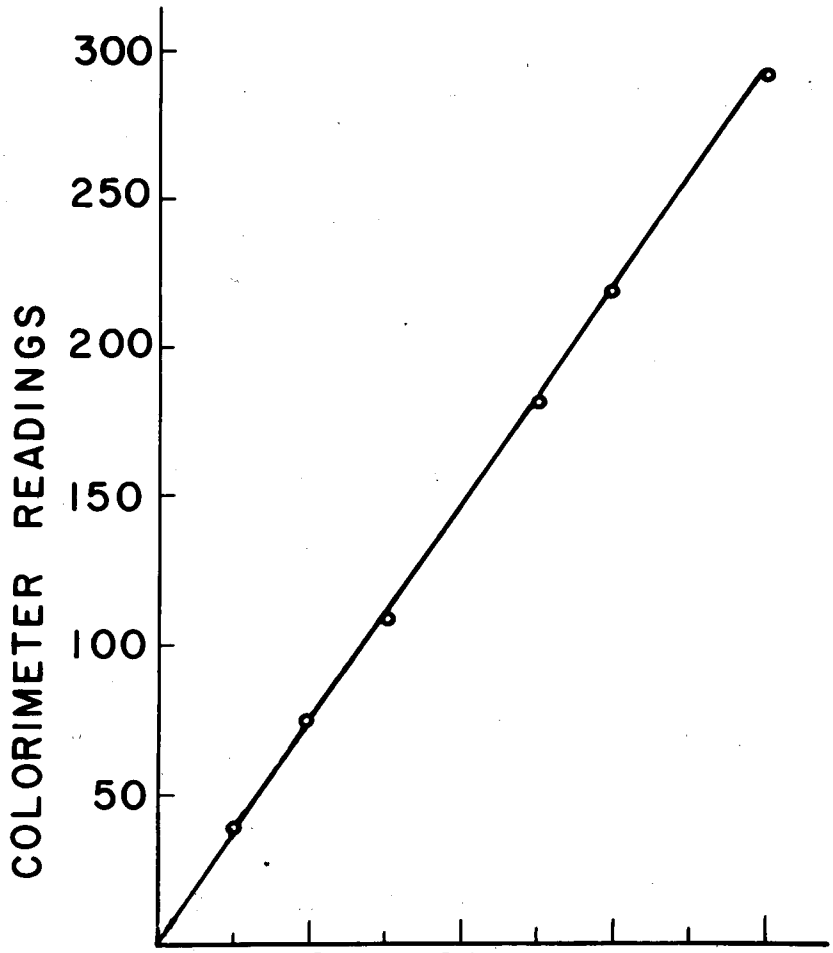

FIGURE XIII

PHOSPHORUS STANDARD CURVE

in the oxidized state and an equally definite absorption in the reduced state (Theorell, 1941, and Potter, 1941). The reduced form has a pronounced maximum at 550 millimicrons which is absent in the oxidized spectrum. The cytochrome can be oxidized with potassium ferricyanide and reduced with sodium hydrosulfite. Since the specific absorption coefficients are known for each form, one can measure the absorption at 550 millimicrons in both oxidized and reduced states, and the concentration can be calculated for each. Neither the ferricyanide nor the hydrosulfite absorb light at 550 millimicrons. The constants are as follows:

$a_R = 2.81$ (cm sq/mole) x 10^7 (Reduced cytochrome c at 550 mu)

$a_O = 0.90$ (cm sq/mole) x 10^7 (Oxidized cytochrome c at 550 mu)

An example of a cytochrome c standardization is given in Table XXVII.

An example calculation for oxidized cytochrome c is as follows:

The fundamental relation is $C = E/a$; the cells are 1 cm long.

$C = E/a = \frac{0.104}{0.90 \times 10^7} = 1.16 \times 10^{-8}$ moles per ml. There are 3.0 ml in the reaction mixture of which 0.4 ml were stock solution. Therefore, the stock solution contains :

$1.16 \times 10^{-8} \times 2.5 = 0.87 \times 10^{-7}$ moles/ml.

The calculations for the reduced cytochrome are the same except a_R is used.

TABLE XXVII

STANDARDIZATION OF CYTOCHROME C

Reaction Mixture		E (Log I_0/I)	Moles/ml (cell)	Moles/ml (stock solution)
Water	1.5ml			
0.1M phosphate, pH, 7.4	1.0ml			
Stock solution cytochrome	0.4ml			
1% $K_3Fe(CN)_6$	0.1ml	Oxidized state		
Final volume	3.0ml	0.104	0.116×10^{-7}	0.87×10^{-7}
Same solution plus 1.0 mgs. solid $Na_2S_2O_4$		Reduced state 0.321	0.114×10^{-7}	0.86×10^{-7}

Experiment 1

TABLE XXVIII

ASSAY OF ISOCITRIC DEHYDROGENASE

Time (mins.)	Optical Density	
	0.01 ml Enzyme	0.04 ml Enzyme
0.5	0.040	0.145
1.0	0.070	0.275
1.5	0.115	0.425
2.0	0.145	0.545
2.5	0.180	0.600
3.0	0.213	0.611
3.5	0.250	0.612
4.0	0.282	0.614
4.5	0.317	0.614
5.0	0.349	0.614
6.0	0.420	
7.0	0.478	
8.0	0.522	
9.0	0.562	
10.0	0.588	
11.0	0.610	
12.0	0.614	

xperiment 2

TABLE XXIX

ASSAY OF ISOCITRIC DEHYDROGENASE AFTER FREEZING AND THAWING

Time (mins.)	Optical Density	
	0.01 ml Enzyme	0.02 ml Enzyme
0.5	0.030	0.065
1.0	0.055	0.125
1.5	0.080	0.190
2.0	0.110	0.255
2.5	0.135	0.315
3.0	0.160	0.370
3.5	0.190	0.420
4.0	0.219	0.470
4.5	0.247	0.512
5.0	0.274	0.550
6.0	0.328	0.595
7.0	0.369	0.610
8.0	0.415	
9.0	0.455	
10.0	0.490	

Experiment 3

TABLE XXX

EFFECT OF PARATHYROID EXTRACT ON ISOCITRIC DEHYDROGENASE

Time (mins.)	Optical Density
	0.1 ml Active hormone with 0.02 ml Enzyme
0.5	0.065
1.0	0.115
1.5	0.170
2.0	0.225
2.5	0.275
3.0	0.325
3.5	0.370
4.0	0.415
4.5	0.450
5.0	0.490
5.5	-----
6.0	0.525
6.5	0.540
7.0	0.548
7.5	0.555
8.0	0.555
8*5	0.558
9.5	0.558

Experiment 4

TABLE XXXI

ASSAY OF LYOPHILIZED ISOCITRIC DEHYDROGENASE

Time (mins.)	Optical Density	
	0.1 ml Enzyme	0.2 ml Enzyme
1.0	0.052	0.097
2.0	0.115	0.212
3.0	0.179	0.333
4.0	0.243	0.442
5.0	0.307	0.530
6.0	0.365	0.587
7.0	0.422	0.602
8.0	0.450	0.605
9.0	0.497	0.607
10.0	0.605	-----

Experiment 6

TABLE XXXII

BUFFERED HORMONE EFFECT ON ISOCITRIC DEHYDROGENASE

Time (mins.)	Optical Density	
	0.4 ml Enzyme	0.4 ml Enzyme with 0.5 ml buffered hormone
0.5	0.101	0.071
1.0	0.201	0.124
1.5	0.289	0.177
2.0	0.365	0.231
2.5	0.422	0.282
3.0	0.456	0.331
3.5	0.472	0.373
4.0	0.477	0.410
4.5	0.480	0.437
5.0	0.480	0.461
5.5	0.480	------
6.0	0.478	0.477
6.5	0.478	0.485
7.0	0.478	0.486
7.5	0.478	0.487

Experiment 8

TABLE XXXIII

BUFFERED HORMONE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Time (mins.)	Optical Density		
	0.1 ml Enzyme	0.2 ml Enzyme	0.1 ml Enzyme with 0.5 ml buffered hormone
0.5	0.126	0.171	0.089
1.0	0.196	0.269	0.173
1.5	0.261	0.341	0.246
2.0	0.310	0.379	0.293
2.5	0.342	0.404	0.331
3.0	0.366	0.418	0.351
3.5	0.382	0.428	0.365
4.0	0.398	0.437	0.377
4.5	0.406	0.442	0.387
5.0	0.412	0.447	0.395
5.5	0.421	0.451	0.401
6.0	0.425	0.454	0.405
6.5	0.430	0.459	0.411
7.0	0.432	0.459	0.415
8.0	0.439	0.462	0.420
10.0	0.451	0.468	0.429

Experiment 9

TABLE XXXIV

BUFFERED HORMONE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Time (mins.)	Optical Density		
	0.1 ml Enzyme	0.2 ml Enzyme	0.3 ml Enzyme
0.5	0.025	0.037	0.060
1.0	0.041	0.063	0.116
1.5	0.057	0.097	0.165
2.0	0.075	0.125	0.206
2.5	0.091	0.150	0.242
3.0	0.107	0.174	0.271
3.5	0.121	0.196	0.296
4.0	0.135	0.215	0.310
4.5	0.149	0.233	0.330
5.0	0.162	0.251	0.344
5.5	0.174	0.265	0.353
6.0	0.187	0.278	0.362
6.5	0.198	0.291	0.371
7.0	0.209	0.302	0.378
7.5	0.219	0.312	0.385
8.0	0.230	0.321	0.390
8.5	0.239	0.328	0.395
9.0	0.248	0.335	0.399
9.5	0.256	0.341	0.402

TABLE XXXIV (Cont'd)

Time (mins.)	Optical Density		
	0.1 ml Enzyme with 0.5 ml buffered hormone	0.2 ml Enzyme with 0.5 ml buffered hormone	0.3 ml Enzyme with 0.5 ml buffered hormone
0.5	0.018	0.021	0.055
1.0	0.032	0.047	0.100
1.5	0.047	0.084	0.144
2.0	0.063	0.108	0.185
2.5	0.074	0.121	0.220
3.0	0.090	0.153	0.245
3.5	0.103	0.173	0.271
4.0	0.118	0.193	0.292
4.5	0.129	0.211	0.309
5.0	0.141	0.227	0.317
5.5	0.153	0.243	0.336
6.0	0.164	0.255	0.345
6.5	0.175	0.269	0.355
7.0	0.186	0.280	0.362
7.5	0.196	0.291	0.369
8.0	0.206	0.301	0.375
8.5	0.215	0.310	0.380
9.0	0.223	0.318	0.385
9.5	0.232	0.324	0.389

Experiment 13

TABLE XXXV

EFFECT OF ACTIVE AND PEPSIN-INACTIVATED HORMONES ON KIDNEY HOMOGENATE RESPIRATION

Time (mins.)	Oxygen Uptake (microliters)		
	Homogenate	Homogenate with 0.2 ml pepsin-inactivated hormone	Homogenate with 0.2 ml active hormone
0	0	0	0
5	11	6	11
10	20	9	18
15	31	12	23
20	39	13	28
25	49	17	33
35[a]	65 (63-67)	17 (17-18)	38 (32-45)
45	78 (75-81)	17 (17-18)	42 (34-49)
55	87 (84-92)	20 (20-20)	44 (37-52)

a. The values in parenthesis are the actual values of the duplicate determinations.

Experiment 16

TABLE XXXVI

INHIBITION STUDY.
MITOCHONDRIA RESPIRATION AT THREE CONCENTRATIONS OF SUCCINATE

Time (mins.)	Oxygen Uptake (microliters)		
	20 u moles	40 u moles	80 u moles
0	0	0	0
3	12	13	13
5	19	21	22
7	23	24	27
9	31	31	34
11	38	42	43
13	47	51	52
15[a]	50 (48-52)	53 (51-54)	56 (53-58)
20	66 (63-68)	71 (70-71)	76 (73-80)
25	71 (69-73)	86 (84-87)	95 (92-98)

a. The values in parenthesis are the actual values of the duplicate determinations.

Experiment 16

TABLE XXXVII

INHIBITION STUDY.
EFFECT OF 0.1 ML PEPSIN-INACTIVATED HORMONE ON KIDNEY MITOCHONDRIA RESPIRATION AT THREE CONCENTRATIONS OF SUCCINATE

Time (mins.)	Oxygen Uptake (microliters)		
	20 u moles	40 u moles	80 u moles
0	0	0	0
3	11	11	10
5	16	18	19
7	21	23	23
9	25	27	27
11	27	31	34
13	35	36	39
15[a]	39 (37-40)	41 (40-41)	45 (42-47)
20	51 (50-51)	56 (55-57)	58 (56-61)
25	60 (58-61)	66 (65-67)	67 (66-68)

a. The values in parenthesis are the actual values of the duplicate determinations.

Experiment 16

TABLE XXXVIII

INHIBITION STUDY.
EFFECT OF 0.2 ML PEPSIN-INACTIVATED HORMONE ON KIDNEY MITOCHONDRIA RESPIRATION AT THREE CONCENTRATIONS OF SUCCINATE

Time (mins.)	Oxygen Uptake (microliters)		
	20 μ moles	40 μ moles	80 μ moles
0	0	0	0
3	8	9	8
5	10	11	12
7	14	15	15
9	25	21	22
11	28	24	24
13	32	27	29
15[a]	39 (37-42)	31 (31-31)	34 (34-34)
20	48 (47-48)	43 (42-44)	43 (41-45)
25	59 (58-60)	50 (47-53)	54 (53-54)

a. The values in parentheses are the actual values of the duplicate determinations.

Experiment 16

TABLE XXXIX

INHIBITION STUDY,
EFFECT OF 0.1 ML ACTIVE HORMONE EXTRACT
ON KIDNEY MITOCHONDRIA RESPIRATION AT
THREE CONCENTRATIONS OF SUCCINATE

Time (mins.)	Oxygen Uptake (microliters)		
	20 u moles	40 u moles	80 u moles
0	0	0	0
3	11	10	8
5	20	19	19
7	27	25	25
9	31	30	30
11	43	44	44
13	49	51	52
15[a]	51 (48-54)	54 (51-57)	54 (53-54)
20	63 (60-65)	67 (64-70)	68 (67-69)
25	77 (77-77)	86 (82-90)	89 (88-89)

a. The values in parenthesis are the actual values of the duplicate determinations.

Experiment 16

TABLE XL

INHIBITION STUDY.
EFFECT OF 0.1 ML ACTIVE HORMONE EXTRACT ON KIDNEY MITOCHONDRIA RESPIRATION AT THREE CONCENTRATIONS OF SUCCINATE

Time (mins.)	Oxygen Uptake (microliters)		
	20 u moles	40 u moles	80 u moles
0	0	0	0
3	10	9	7
5	16	15	13
7	23	22	20
9	27	27	24
11	34	35	30
13	42	39	36
15[a]	47 (44-49)	50 (45-55)	44 (40-48)
20	58 (56-60)	65 (61-70)	57 (52-62)
25	71 (68-74)	80 (75-85)	69 (63-75)

a. The values in parenthesis are the actual values of the duplicate determinations.

TABLE XLI

ULTRA-VIOLET SPECTRA OF ACTIVE AND OXIDISED PARATHYROID HORMONES

Wavelength (mμ)	Optical Density	
	Active Hormone	Oxidised Hormone
240	0.658	0.700
245	0.446	0.482
250	0.390	0.422
255	0.398	0.418
260	0.438	0.443
265	0.493	0.482
270	0.550	0.525
272	0.550	0.528
275	0.555	0.535
278	0.539	0.525
280	0.500	0.490
285	0.410	0.419
290	0.305	0.317
295	0.217	0.230
300	0.164	0.178
305	0.137	0.150
310	0.122	0.135
320	0.107	0.119
340	0.081	0.089

TABLE XLII

DIFFERENCE IN SPECTRA BETWEEN OXIDIZED AND ACTIVE PARATHYROID HORMONES

Wavelength (mμ)	Optical Density
230	0.023
235	0.035
240	0.040
245	0.035
250	0.026
255	0.012
260	-0.004
265	-0.017
268	-0.025
270	-0.030
273	-0.027
275	-0.025
278	-0.020
280	-0.008
285	0.006
290	0.008
300	0.008
310	0.008
320	0.005
340	0.003

TABLE XLIII

DATA FOR STANDARD NITROGEN CURVE

Nitrogen (micrograms)	Colorimeter Readings	Mean	Corrected for blank
0	7		
0	6	7	0
0	8		
20	42		
20	43	43	36
20	43		
40	77		
40	76	76	69
40	76		
60	114		
60	113	114	107
60	114		
80	155		
80	153	152	145
80	148		
100	197		
100	189	192	185
100	191		
120	228		
120	232	228	221
120	224		
140	263		
140	257	260	253
140	259		

TABLE XLIV

DATA FOR STANDARD PHOSPHORUS CURVE

Phosphorus (micrograms)	Colorimeter Readings	Mean	Corrected for blank
0	6		
0	6	5	0
0	4		
5	44		
5	45	45	40
5	45		
10	81		
10	82	81	76
10	80		
15	117		
15	116	115	110
15	113		
25	187		
25	187	188	183
25	191		
30	222		
30	226	224	219
30	223		
40	299		
40	300	299	294
40	298		

APPROVAL SHEET

The thesis submitted by John C. Colla has been read and approved by three members of the Department of Biochemistry.

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

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 7, 1960
Date

Maurice V. St. Maurice
Signature of Adviser