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# In Vitro Effect of Parathyroid Gland Extract upon the Ultra-Violet Absorbtion of Reduced Coenzyme Ii

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# IN VITRO EFFECT OF PARATHYROID GLAND EXTRACT UPON THE ULTRA-VIOLET ABSORBTION OF REDUCED COENZYME II

by

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# LIFE

Leo Edmund Reichert, Jr., was born in New York City, New York, on January 9, 1932. He received his high school diploma from De La Salle Institute, New York City, New York, in June 1949.

From August 1950, to March 1952, he served on active duty with the United States Marine Corp. He graduated from Manhatten College, New York City, New York, in June 1955, with the degree of Bachelor of Science. During his senior year at Manhatten College, he received the Sinclair, Valentine and Company award in Chemistry.

In September, 1955, he began his graduate work in the Department of Biochemistry of Loyola University at the Stritch School of Medicine. He has held the position of Teaching Assistant in the Department of Biochemistry since his matriculation at the graduate school.

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# CHAPTER 1

## INTRODUCTION

The initial impetus for this work was a note published by Neuman and his associates (1956) in which it was stated that "Parathyroid extract was shown spectrophotometrically to destroy the chromophoric group (340 mu) of reduced coenzyme II in vitro rendering it practically non-absorbent." No experimental data or details as to the method employed in the work accompanied this categorical report. The authors felt that this observation could well indicate an in-vivo blocking of coenzyme II-linked reactions, with a resulting shunt of all glucose metabolism to citrate production. Such a situation could explain experimental findings that dramatic increases in citrate output from the femur of intact dogs occurred in the spongiosal blood almost immediately following injection of parathyroid extract. Neuman and his associates (1956) contended that this "blocking effect" provides and "intriguing" biochemical mechanism by which a parathyroid-controlled citrate gradient maintains a steady but supersaturated level of ionized calcium in the serum. This could also be involved in explaining previous findings of L'Heureux and Roth (1953) and others, that parenteral injection of parathyroid extract produces a rise in serum citrate levels.

No enzyme system has, to our knowledge, ever been shown to respond directly to parathyroid extract in vitro, and as far

as can be determined only one paper has been published on in-vivo effects of parathyroid extract upon enzyme systems.

Dixon and Perkins (1953) demonstrated the presence in bone of three enzymes of the Krebs Cycle which are involved in the formation and removal of citric acid, namely, citrogenase aconitase, and isocitric dehydrogenase. Citrogenase activity was found to be much higher than that of aconitase and isocitric dehydrogenase. Since citrogenase is the enzyme concerned in the formation of citric acid from acetyl coenzyme A, and isocitric dehydrogenase, the enzyme which may be considered as involved in the removal of citrate, it was suggested that a mechanism existed in bone which leads to a net production of citric acid. Perkins and Dixon (1953) then studied in-vivo effects of parathyroid extract upon citrogenase levels of bone. These workers found that massive doses of parathyroid extract injected into normal or parathyroidectomized animals did not produce any immediate rise in citrogenase activity. Further, the citrate content of the bones of both normal and parathyroidectomized rats did not differ. In evaluating this work, however, it should be pointed out that only a few animals were used for many of the measurements, and the parathyroid extract employed (Lilly) was dialyzed prior to injection. Work by Kenny (1957) has shown that dialysis markedly decreases the calcium mobilizing activity of his preparations, and work in

this thesis will show that prolonged dialysis of the Lilly preparation against distilled water completely removes the calcium mobilizing activity of the preparation.

Conclusive proof of an in vitro relationship between the calcium mobilizing principle of parathyroid gland extracts and the ultra-violet absorption of reduced coenzyme II as implied by Neuman and his associates (1956), would be of value not only in purification work on the parathyroid hormone, but also in attempts to better understand the mechanism of action of the hormone. These two areas of investigation are closely related in present day parathyroid hormone research.

A survey of the literature seems to indicate that researchers have obtained all the information now available by working with a heterogenous hormonal extract. Really significant advances seem to await the development of a homogeneous, pure parathyroid hormone preparation. Attempts to purify the hormone have been seriously hampered by the lack of a simple, rapid, and reliable means of assaying the biological potency of various parathyroid preparations, and this lack of a pure hormonal preparation has, in turn, seriously handicapped all attempts which have been made to understand the mechanism of operation of the hormone.

Were it possible to conclusively demonstrate that the calcium mobilizing principle of parathyroid gland extracts does

affect the absorption of reduced coenzyme II to a significant degree in vitro, the possibility of the development of a rapid, in vitro method for assaying the potency of parathyroid gland preparations arises, and assumes appreciable importance. The practical value of such a system and the impetus that it would give to all aspects of parathyroid hormone research can readily be appreciated by anyone familiar with this field. Not only would it be possible to do away with the tedious and time consuming animal assay procedures now in use, but the demonstration that a coenzyme and/or its associated enzyme systems respond directly to a hormone in vitro would cast additional light on the oft postulated mediation of enzyme systems via hormonal influences.

It appeared to us, therefore, to be of considerable importance to definitely evaluate this claim of purported hormonal action on reduced coenzyme II.

# CHAPTER II

## MATERIALS AND METHODS

In studying this purported hormonal effect on reduced coenzyme II, the following general approach was decided upon: (1) To determine whether or not the parathyroid preparation employed by Neuman and his associates (1956) actually does "destroy" the chromophoric group of reduced coenzyme II at 340 mu, and (2) to ascertain whether any effect observed is related to the calcium mobilizing activity of the extract employed.

Spectrophotometric Test System. It was first necessary to develop a test system whereby the reported hormonal effect could be studied. A search of the literature revealed that a somewhat analogous situation had been encountered by Conn et. al. (1952) in their studies on the influence of wheat germ oxidase upon the aerobic oxidation of reduced coenzyme II. The method employed by these workers was examined and modified to fit our experimental requirements.

Neuman and his associates (1956) utilized Eli Lilly and Company's commercial extract, "Solution Parathyroid Extract," as the hormonal preparation in their work. The commercial extract is now marketed as "injection Parathyroid,U. S. P.," and this was the hormonal extract employed in the experiments recorded here. Both preparations were assigned potencies of 100 U. S. P. units per milliliter of extract, and no indication

is given by the manufacturer or its regional representative, Mr. J. E. Brackett, that the method of preparation of the extract, or its properties, have been altered. The specific material used was Lot number 9058-674173, bearing an expiration date of October 1, 1957.

The reduced coenzyme II (TPNH) used in all experiments was triphosphopyridine nucleotide-sodium, reduced form, approximately 90% pure, Lot number 96-670, obtained from the Sigma Company, St. Louis, Mo.

The instrument used to measure the absorption of the reduced coenzyme at 340 mu was a Beckman model DU spectrophotometer, operating through a Sorenson Power Supply unit, and using a hydrogen lamp as the light source. Reactions were carried out in silica cells ( d 1.0 cm.) at room temperature, and in 0.1M potassium phosphate buffer, pH. 7.4. Absorbance measurements were recorded hourly, whenever possible, over a six hour test period.

The tube arrangement used in the spectrophotometric analysis is listed below.

| Control Tube A (Blank) | Control Tube B |
|---|---|
| 2.0 cc. Phosphate Buffer | 1.0 cc. Phosphate Buffer |
| 1.0 cc. Distilled Water | 1.0 cc. Distilled Water |
| | 1.0 cc. TPNH in Phosphate Buffer |

| Reaction Tube A (Blank) | Reaction Tube B |
|---|---|
| 2.0 cc. Phosphate Buffer | 1.0 cc. Phosphate Buffer |
| 0.5 cc. Distilled Water | 0.5 cc. Distilled Water |
| 0.5 cc. Extract | 0.5 cc. Extract |
| | 1.0 cc. TPNH in Phosphate Buffer |

The concentration of TPNH used was 0.2 mg. (0.270 micromole) per cc. of buffer. This concentration of TPNH was chosen because it was shown to produce an absorbance of slightly less than 0.400 optical density units on the spectrophotometer, and any decrease in absorbance could be followed through the most favorable range of the recording instrument. The selection of 0.5 cc. of parathyroid extract (50 U.S.P. units of activity) as a "test dose" was made since it was known to be of sufficient potency to produce a definite physiological response in rats - specifically, a rise in serum calcium - and because higher concentrations of parathyroid gland extract produced a turbidity in the silica cells. Sufficient potency to produce this physiological response was an important criterion, because it was immediately realized that an association of any possible spectrophotometric effect of the extract upon TPNH could not be properly evaluated with regard to the observations and hypothesis of Neuman and his associates (1956) unless this were so.

During the course of this work it became necessary to compare the spectrophotometric effect of various "treated"

preparations with that of the "untreated" commercial parathyroid extract. In these cases two additional reaction tubes were employed and prepared similarly to Reaction Tube A and B, with the exception that 0.5 cc. of the treated preparation was used instead of the untreated extract.

It was decided to express the effect of various parathyroid preparations upon the absorption of TPNH in terms of an arbitrarily designated "Activity Ratio." This "Activity Ratio" was defined as the total change in absorption of TPNH at 340 mu when it was present with the test material, divided by the total change in absorption of the coenzyme when it was not in contact with the test material.

Biological Assay Of Parathyroid Gland Extract. Administration of a potent parathyroid gland extract to experimental animals is known to produce a variety of responses, among them (a) an increase in serum calcium, (b) an increase in urinary calcium, (c) an increase in urinary phosphorus, (d) a decrease in serum phosphorus, (e) an elevation of plasma citrate, and, as indicated by more recent work, (f) elevation of serum glycoprotein and seromucoid levels (Shetlar et. al. 1956) and (g) an acceleration in development of musculas necrosis and mediocalcinosis (Lehr and Martin, 1956).

In this work we are singularly interested in correlating any observed spectrophotometric effect produced by the test

material on the absorption of TPNH at 340 mu, with the calcuim mobilizing potency of the material as manifested by its ability to increase the serum calcium levels in experimental animals. Only effect (a), therefore, was of immediate concern to us.

Reports from many workers have demonstrated the intact rats are quite refractory to administration of parathyroid gland extract, particularly with regard to the effect of the extract on serum calcium levels. For this reason, the accepted practice of utilizing parathyroidectomized rats for biological assay of extract potency was followed in this work.

In order to speed up the overall assay procedure, a departure was made from the assay methods previously employed in this laboratory. Parathyroidectomy was accomplished by cauterization rather than by surgical removal of the entire thyro-parathyroid apparatus, and serum samples for calcium analysis were obtained by cardiac puncture, instead of by tail bleeding. It is felt that concomitant use of these two techniques materially lessened the tediousness inherent in the biological assay of parathyroid gland extract potency.

Preparation And Care Of The Animals. As indicated, parathyroidectomy was accomplished by cauterization with a hot wire. Details of the procedure are given in the Appendix (page 58). Cauterization was found superior to the surgical removal of the thyro-parathyroid apparatus used by many workers

in that it (a) permitted destruction of the parathyroids with a minimum of damage to the thyroid gland, (b) practically eliminated bleeding as a source of trauma to the animal, and (c) in general, reduced the overall picture of shock to the animal necessarily associated with such an operation.

Following parathyroidectomy, the animals were allowed to recover for a period of at least 2 days before being prepared for assay. Throughout this work, white female rats of the Loyola colony were employed. Animals weighing 120-30 grams were chosen, and were maintained on a Purina Dog Chow diet, with tap water supplied ad libitum.

Preparation for assay consisted of fasting the animals for two nights and one day prior to the analysis of the serum. During the fasting period the animals were allowed tap water ad libitum.

Obtaining Serum Samples For Analysis. The actual assay procedure usually required one complete day. Blood was drawn in the morning, following the previously described fast period, by means of cardiac puncture. The details of this technique may be found in the Appendix (page 61). Immediately after the removal of the initial blood sample, 0.5 cc. of the material to be assayed was injected intraperitoneally into the animal. It was found that removal of 0.5 cc. of blood when using the cardiac puncture technique, provided ample quantities of serum

and did not adversely affect the animal.

Melius (1956) and others, have shown that rats respond maximally to injections of parathyroid preparations in six hours, and this amount of time was allowed to elapse before the final blood sample was taken, again by cardiac puncture.

Serum was obtained by centrifugation of the blood for five minutes in a clinical centrifuge. Serum samples taken prior to, and subsequent to, the six hour test period were simultaneously analyzed for calcium content.

Chemical Determination Of Calcium. Chemical analysis of the serum samples was performed according to the method of Natelson and Penniall (1955) with some modification. In this procedure, calcium in triethanolamine solution reacts with alizarin ( 1,2 -dihydroxyanthraquinone) in octanol solution, to give a purple-colored complex which is soluble in the octanol. After shaking to insure complete reaction, the mixture is centrifuged, the calcium alizarinate-octanol mixture (upper layer) is transferred to a colorimeter tube and the intensity of the color is measured in the Klett-Summerson photoelectric colorimeter using a number 56 filter (560 mu).

Magnesium has also been found to form a complex with alizarin, and this complex has 0.3 times the absorption of the calcium complex at 560 mu (Natelson and Penniall, 1955). Davies and Gordon (1954), however, found no change in serum magnesium

concentration after administration of 50 U.S.P. units of Lilly parathyroid gland extract to parathyroidectomized rats. In view of this fact, and since in this work we were not interested in absolute calcium levels, but only in the difference between initial and final serum calcium levels, it was felt that no correction need be made for the absorption of the magnesium alizarinate formed during this procedure.

The alizarin solution and the triethanolamine solution used were of the same concentration as in the original method. The modification consisted in using 6 mls. of the alizarin-octanol reagent instead of 3 mls., and in utilizing a 0.05 ml. serum sample for analysis instead of the 0.02 ml. serum sample prescribed by Natelson and Penniall (1955). For the extraction of the calcium alizarinate complex, it was found convenient to use 12 ml. glass stoppered centrifuge cones. Pipetting of serum samples was accomplished through use of 50 lamda micro-pipettes. Details of the calcium analysis are given in the Appendix (page 63).

# CHAPTER III

## EXPERIMENTAL RESULTS

### A. IN VITRO EFFECT OF PARATHYROID GLAND EXTRACT UPON THE ULTRA-VIOLET ABSORPTION OF REDUCED COENZYME II AT 340 mu.

Control Of Experimental Parameters. Before actual investigations of the effect of parathyroid gland extract upon the absorption of TPNH at 340 mu could be undertaken, a number of parameters were recognized as possible sources of error, and these were examined to ascertain the possible extent of their interference in our analysis of experimental results.

All silica cells used in this work were found not to absorb at 340 mu. Similarly, 0.1M potassium phosphate buffer solutions, when read against distilled water blanks, were found not to absorb at 340 mu. The buffer system utilized in the reaction tubes proved capable of maintaining a pH. 7.4 over a six hour period in the presence of 0.5 cc. of 0.01N HCl. Since no preparation would be utilized having a pH lower than 3, it was felt adequate buffering capacity was present in the reaction system.

Effect Of Parathyroid Gland Extract Upon The Absorbance Of TPNH at 340 mu. Table number 1 summarizes the results obtained for twenty five experiments on the effect of the parathyroid gland extract upon the absorbance of TPNH at 340 mu.

The data clearly indicates that the commercial extract utilized possesses the ability to catalyze the decrease in absorption of TPNH. This decrease is almost three times that observed in the TPNH control tubes. It is believed that this was the effect observed by Neuman and his associates (1956). However, in view of the previously mentioned heterogeneous nature of the extract employed, and considering the many types of physiological responses elicited by parathyroid gland extracts in experimental animals, an immediate association of this effect with the calcium mobilizing potency of the preparation is premature.

TABLE I

EFFECT OF PARATHYROID GLAND EXTRACT UPON THE ABSORBANCE OF TPNH AT 340 mμ.

| | TPNH Control Tube | | TPNH-Active Extract | | Activity Ratio |
|---|---|---|---|---|---|
| Expt. | Initial Absorbance | Decrease In Absorbance | Initial Absorbance | Decrease In Absorbance | |
| 1 | 0.355 | 0.037 | 0.353 | 0.088 | 2.5 |
| 2 | 0.338 | 0.033 | 0.354 | 0.068 | 2.1 |
| 3 | 0.319 | 0.036 | 0.310 | 0.104 | 2.8 |
| 4 | 0.373 | 0.048 | 0.345 | 0.105 | 2.2 |
| 5 | 0.378 | 0.031 | 0.378 | 0.091 | 2.9 |
| 6 | 0.433 | 0.035 | 0.433 | 0.109 | 3.1 |
| 7 | 0.394 | 0.044 | 0.382 | 0.127 | 2.9 |
| 8 | 0.351 | 0.031 | 0.348 | 0.082 | 2.6 |
| 9 | 0.312 | 0.029 | 0.303 | 0.074 | 2.6 |
| 10 | 0.357 | 0.031 | 0.360 | 0.084 | 2.7 |
| 11 | 0.347 | 0.034 | 0.357 | 0.096 | 2.8 |
| 12 | 0.355 | 0.037 | 0.353 | 0.088 | 2.4 |
| 13 | 0.361 | 0.039 | 0.327 | 0.101 | 2.6 |
| 14 | 0.354 | 0.034 | 0.358 | 0.073 | 2.1 |
| 15 | 0.323 | 0.037 | 0.311 | 0.104 | 2.8 |
| 16 | 0.319 | 0.036 | 0.310 | 0.104 | 2.9 |

TABLE I (con't.)

| Expt. | TPNH Control Tube: Initial Absorbance | TPNH Control Tube: Decrease In Absorbance | TPNH-Active Extract: Initial Absorbance | TPNH-Active Extract: Decrease In Absorbance | Activity Ratio |
|---|---|---|---|---|---|
| 17 | 0.338 | 0.033 | ----- | ----- | --- |
| 18 | 0.378 | 0.032 | 0.391 | 0.086 | 2.7 |
| 19 | 0.312 | 0.026 | 0.287 | 0.086 | 2.7 |
| 20 | 0.311 | 0.027 | 0.287 | 0.086 | 2.7 |
| 21 | 0.376 | 0.026 | 0.373 | 0.058 | 2.2 |
| 22 | 0.402 | 0.047 | 0.378 | 0.099 | 2.4 |
| 23 | 0.402 | 0.047 | 0.359 | 0.108 | 2.7 |
| 24 | 0.340 | 0.036 | 0.365 | 0.072 | 2.0 |
| 25 | 0.379 | 0.026 | ----- | ----- | --- |
| Mean (Std. dev.) | 0.354 (0.015) | 0.034 (0.005) | 0.351 (0.034) | 0.091 (0.016) | 2.7 (0.27) |

Effect Of A 0.2% Phenol Solution Upon The Ultra-Violet Absorption Of TPNH At 340 mu. The parathyroid gland extract utilized in this work was known to contain 0.2% phenol, added as a preservative. It was necessary to insure that this material did not affect the absorption of TPNH under our experimental conditions. The results obtained in Table 2 clearly show that the phenol present in the extract does not affect the absorption of TPNH at 340 mu.

TABLE 2

EFFECT OF A 0.2% PHENOL SOLUTION UPON THE ULTRA-VIOLET ABSORPTION OF TPNH AT 340 mu.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH-Control | 0.373 | 0.048 | --- |
| TPNH-Parathyroid Extract | 0.345 | 0.105 | 2.2 |
| TPNH-0.2% Phenol | 0.368 | 0.051 | 1.0 |

TABLE 2 (con't.)

| Expt. 2 | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH-Control | 0.325 | 0.031 | --- |
| TPNH-Parathyroid Extract | ----- | ----- | --- |
| TPNH-0.2% Phenol | 0.316 | 0.033 | 1.0 |
| | 0.312 | 0.031 | 1.0 |
| | 0.321 | 0.030 | 1.0 |
| | 0.316 | 0.030 | 1.0 |
| | 0.319 | 0.030 | 1.0 |

Effect Of Bovine Plasma Albumin On The Absorbance Of TPNH At 340 mu. The heterogeneity of the parathyroid gland extract employed, caused us to investigate the possibility that a non-specific protein-coenzyme interection could affect the absorbance of TPNH at 340 mu. A solution of Bovine Plasma Albumin (Armour, Lot number 66909) containing ten mgs. per cc., was prepared and utilized in the previously outlined test system. This concentration of protein represents a nitrogen level approximately 1.5 times that present in the corresponding volume of parathyroid extract. The results listed in Table 3 indicate that a non-specific protein material does not affect the absorbance of TPNH at 340 mu to a significant degree.

TABLE 3

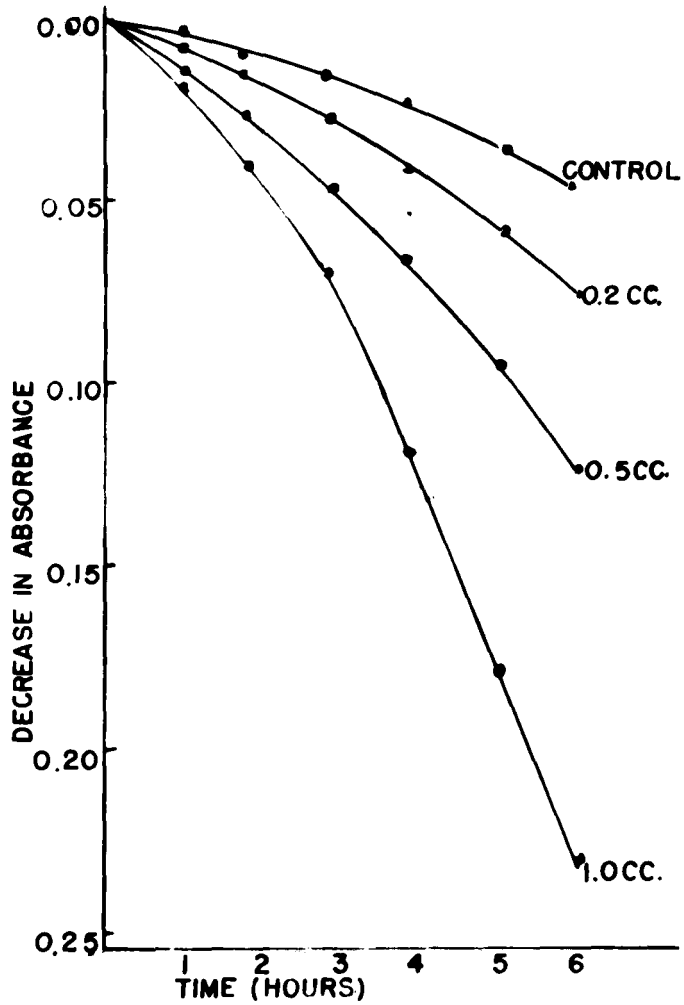
EFFECT OF BOVINE PLASMA ALBUMIN ON THE ABSORBANCE OF TPNH AT 340 mu.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH-Control | 0.386 | 0.033 | --- |
| TPNH-Bovine Plasma Albumin | 0.395 | 0.043 | 1.3 |
| | 0.388 | 0.041 | 1.2 |
| | 0.390 | 0.044 | 1.3 |

Effect Of Various Dose Levels Of Parathyroid Gland Extract Upon The Absorbance Of TPNH At 340 mu. The effect of graded amounts of parathyroid gland extract upon the ultra-violet absorption of reduced coenzyme II was recorded each hour over a six hour test period. Figure 1 shows the results obtained when 0.2, 0.5, and 1.0 cc. aliquots of the extract were allowed to react with TPNH under the usual experimental conditions. Each point represents the mean of three determinations.

It can be clearly seen that the rate of decrease in absorbance of TPNH at 340 mu over a six hour test period varies with the amount of extract employed in the reaction mixture.

Biological Assay Of Parathyroid Gland Extract. The parathyroid gland extract employed in the spectrophotometric work noted above was now assayed to establish its biological potency. Table 4 indicates the response of nine animals to 0,5 cc (50 U.S.P. units) of the extract. There can be little doubt that the material found to accelerate the decrease in absorbance of TPNH possesses calcium mobilizing potency.




EFFECT OF VARIOUS DOSE LEVELS OF
PARATHYROID GLAND EXTRACT UPON
THE ABSORBANCE OF TPNH AT 340 mu.

FIGURE 1

EFFECT OF VARIOUS DOSE LEVELS OF PARATHYROID GLAND EXTRACT UPON THE ABSORBANCE OF TPNH AT 340 mu.

TABLE 4

BIOLOGICAL ASSAY OF PARATHYROID GLAND EXTRACT.

| Animal | Initial Serum Calcium, mg % | Final Serum Calcium, mg % | Change In Serum Calcium, mg % |
|---|---|---|---|
| 1 | 12.8 | 15.3 | 2.5 |
| 2 | 12.0 | 13.9 | 1.9 |
| 3 | 13.4 | 15.0 | 1.6 |
| 4 | 7.4 | 12.6 | 5.2 |
| 5 | 12.1 | 15.0 | 2.9 |
| 6 | 12.0 | 14.4 | 2.4 |
| 7 | 10.5 | 11.2 | 0.7 |
| 8 | 5.8 | 8.1 | 2.3 |
| 9 | 10.1 | 13.5 | 3.4 |
| | | | 2.5 Mean |
| | | | (1.2) Std. Dev. |

Ultra-Violet Absorption Spectrum Of Parathyroid Gland Extract. The Ultra-Violet absorption spectrum of Lilly's Parathyroid gland extract was determined. The results are shown in Figure 2. The spectrum obtained closely resembles that recorded by Ross and Wood (1942) for their active parathyroid gland preparation. No variation could be found in the spectrum of the material after a six hour period.

The tube arrangement used in recording this spectrum is as follows:

Tube 1. (Blank)

2.0 cc. Potassium Phosphate Buffer, 0.1M, pH. 7.4
1.0 cc. Distilled Water

Tube 2.

2.0 cc. Potassium Phosphate Buffer, 0.1M, pH. 7.4.
0.8 cc. Distilled Water
0.2 cc. Parathyroid Gland Extract

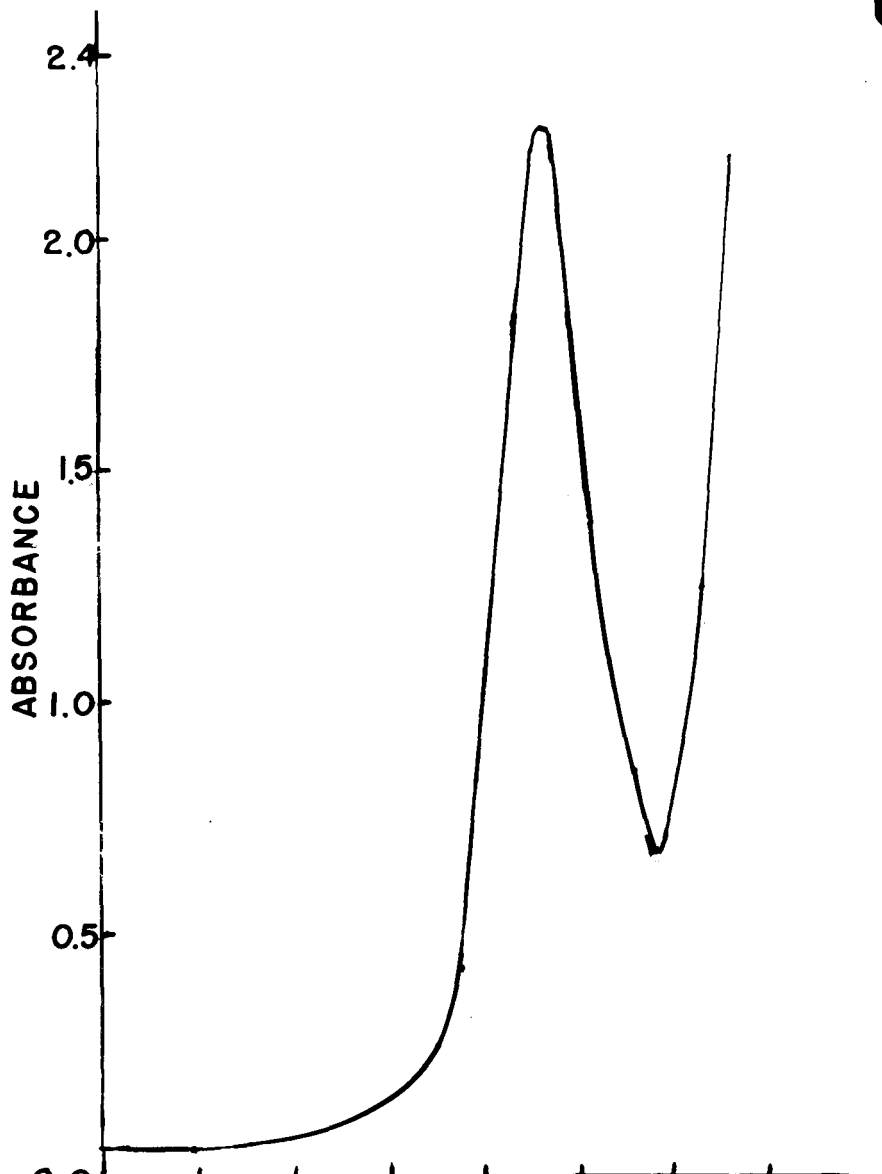



FIGURE 2

ULTRA-VIOLET ABSORPTION SPECTRUM OF PARATHYROID GLAND EXTRACT (Lilly)

Ultra-Violet Absorption Spectra Of Reduced Coenzyme II. The ultra-violet absorption spectrum of TPNH was recorded before and after a six hour interval. Figure 3 shows the spectra obtained. The charcteristic absorption peak at 340 mu indicates that the material used in this work is reduced coenzyme II. It will be noted that the decrease in absorption at 340 mu is accompanied by a corresponding increase in absorption at 260 mu, which is the absorption peak of oxidized coenzyme II. This would seem to indicate an aerobic oxidation of the TPNH initially in solution has taken place.

The tube arrangement employed in obtaining these spectra is as follows:

Tube 1. (Blank)

2.0 cc. Potassium Phosphate Buffer, 0.1M, pH. 7.4
1.0 cc. Distilled Water

Tube 2

1.0 cc. Potassium Phosphate Buffer, 0.1M, pH. 7.4
1.0 cc. Distilled Water
1.0 cc. TPNH in Potassium Phosphate Buffer, 0.1M, pH. 7.4

Ultra-Violet Absorption Spectra Of A Parathyroid Gland Extract-TPNH Reaction Mixture. It was felt that absorption spectrum of a parathyroid gland extract-TPNH reaction mixture recorded before and after a six hour interval might provide information as to the nature of the reaction taking place. The tube arrangement utilized in recording these spectra is as follows:

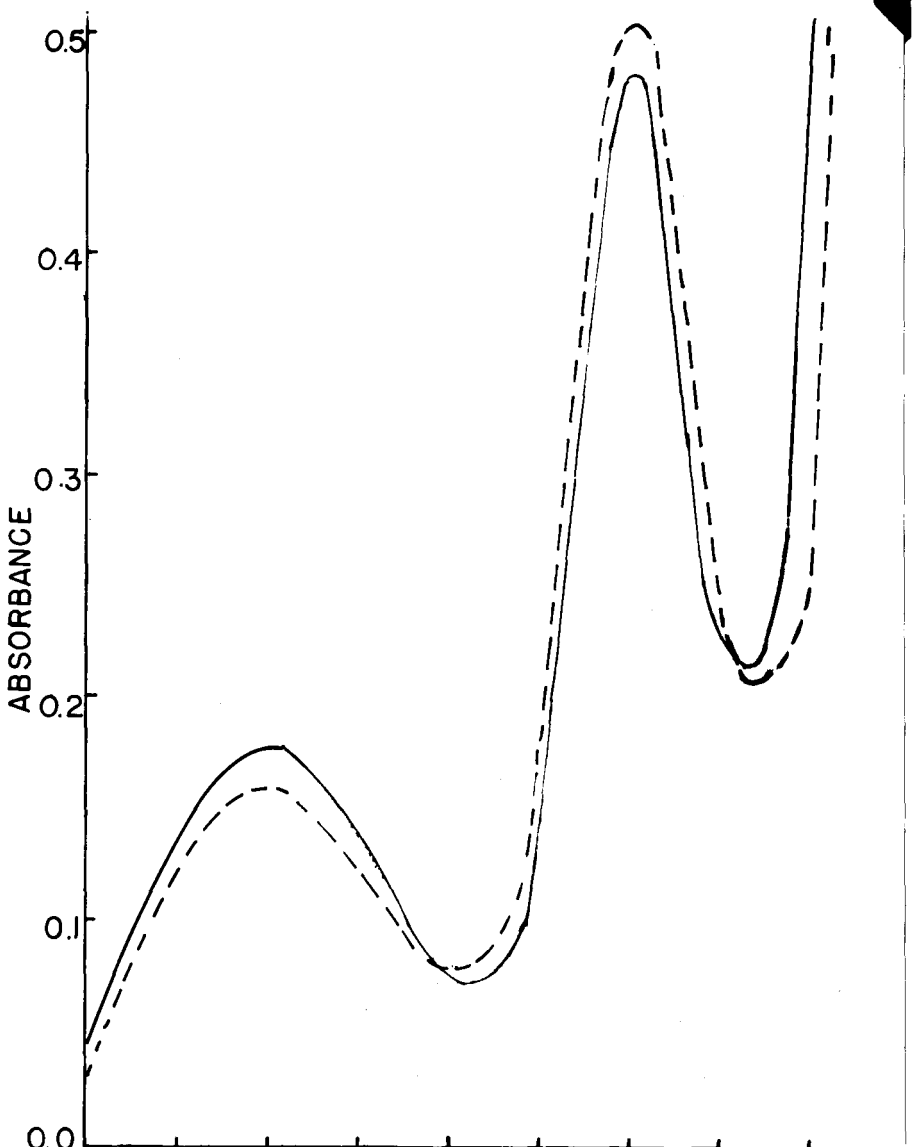



FIGURE 3

ULTRA-VIOLET ABSORPTION SPECTRA OF TPNH BEFORE AND AFTER A SIX HOUR TIME INTERVAL.

(Broken-line indicates spectrum recorded after the six hour time interval.)

Tube 1. (Blank)

2.0 cc. Potassium Phosphate Buffer, 0.1M, pH. 7.4
1.0 cc. Distilled Water

Tube 2.

1.0 cc. Potassium Phosphate Buffer, 0.1M, pH. 7.4
1.0 cc. TPNH in Potassium Phosphate Buffer, 0.1M, pH. 7.4
0.2 cc. Parathyroid Gland Extract
0.8 cc. Distilled Water

The spectra obtained are shown on Figure 4. It will be noted that no oxidized coenzyme II peak (260 mu) appears on the combined spectra, being overshadowed by the greater absorbance of the parathyroid extract at 275 mu. As expected, the drop in TPNH at 340 mu is still evident. The results give no suggestion of complex formation and/or disruption of the TPNH molecule occuring during the reaction period. The increase in absorbance at both 260 mu and 275 mu after six hours seems due to an extract catalized oxidation of TPNH which results in decrease in absorption at 340 mu and an increase in absorption at 260 mu as oxidized coenzyme is produced.

Definite association of the results obtained so far with the calcium mobilizing principle present in the parathyroid gland extract, however, has yet to be established.

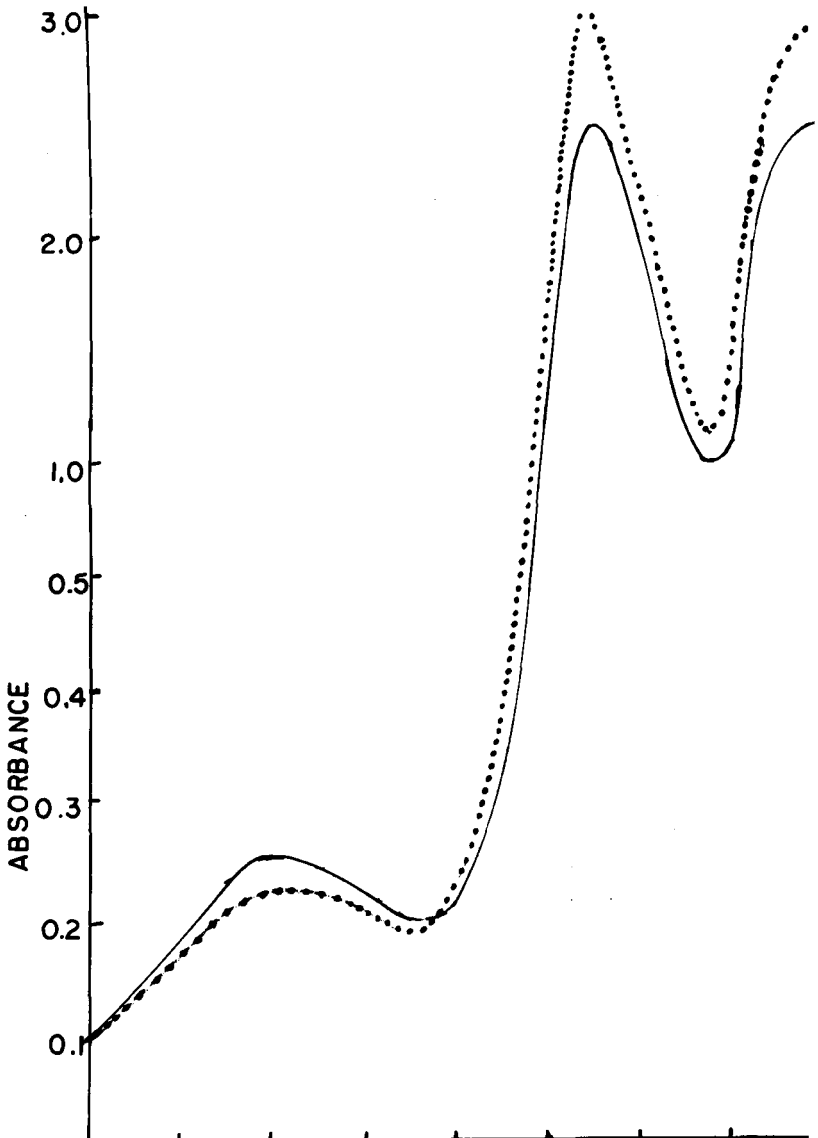



FIGURE 4

ULTRA-VIOLET ABSORPTION SPECTRA OF A PARATHYROID GLAND EXTRACT-TPNH REACTION MIXTURE BEFORE AND AFTER A SIX HOUR TIME INTERVAL

(Broken-line indicates spectrum recorded after the six hour time interval.)

## B. CORRELATION OF THE IN VITRO EFFECT OF PARATHYROID GLAND EXTRACT UPON THE ULTRA-VIOLET ABSORPTION OF TPNH WITH THE CALCIUM MOBILIZING POTENCY OF THE EXTRACT.

Experimental Approach To This Problem. As was stated previously, it was felt that the implication of Neuman and his associates (1956) that the calcium mobilizing principle shown to be present in the parathyroid gland extract was responsible for the accelerated decrease in absorbance of TPNH at 340 mu, required further experimental verification. The position that such a relationship existed would prove untenable if it could be shown (1) that biologically inactive parathyroid gland extract retained its ability to affect the absorbance of TPNH at 340 mu, or (2) if biologically active parathyroid gland extract could be shown not to affect the absorbance of TPNH under our experimental conditions. Experiments were designed to examine these possibilities.

In evaluating subsequent work, it should be remembered that we are singularly interested in determining whether such discrepancies between biological potency of the extract, and the spectrophotometric effect of the extract on TPNH could be observed. We were not primarily interested in obtaining information regarding the chemical nature of the parathyroid hormone, nor were we primarily interested in pursuing any sort of purification work with the extract.

Previous work has shown that 0.5 cc. of Lilly's parathyroid gland extract was capable of affecting the ultra-violet absorbance of TPNH at 340 mu to a significant degree, and was also capable of eliciting a significant calcium mobilizing response in our experimental animals. Our intention, then, was to treat this extract in various ways and observe the effect, if any, of this treatment on these two phenomena. Unless otherwise indicated, therefore, all spectrophotometric and biological assay work undertaken involved use of 0.5 cc. aliqouts of the treated extract preparation, and was performed under the previously defined experimental conditions.

Analysis Of "Aged" Parathyroid Gland Extract. A number of partially filled ampoules of Lilly's parathyroid gland extract bearing expiration dates no later than 1946, were pooled and the mixture analyzed for biological potency and spectrophotometric effect. Table 5 summarizes the data obtained. The results show that the biological and spectrophotometric responses elicited by this "aged" material are almost identical with those for preparations bearing an expiration date of October 1, 1957.

TABLE 5

BIOLOGICAL ASSAY AND SPECTROPHOTOMETRIC EFFECT OF "AGED" PARATHYROID GLAND EXTRACT.

a) Biological Assay

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% |
|---|---|---|---|
| 1 | 5.7 | 7.6 | 1.9 |
| 2 | 6.3 | 6.8 | 0.5 |
| 3 | 7.4 | 8.6 | 1.2 |
| 4 | 8.0 | 10.2 | 2.2 |
| 5 | 8.6 | 10.8 | 2.2 |
| 6 | 7.1 | 10.8 | 3.7 |
| 7 | 5.1 | 7.2 | 2.1 |
| 8 | 6.4 | 8.9 | 2.5 |
| 9 | 5.9 | 8.1 | 2.2 |
| | | | 2.1 Mean |
| | | | (0.8) Std. Dev. |

b) Spectrophotometric Effect.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.166 | 0.019 | --- |
| ** TPNH - Active Extract | 0.169 | 0.051 | 2.7 |
| TPNH - "Aged" Extract | 0.185 | 0.049 | 2.6 |
| | 0.177 | 0.044 | 2.3 |
| | 0.180 | 0.050 | 2.6 |

** In all subsequent work, "Active Extract" will refer to untreated Lilly "Injection Parathyroid, U.S.P.", expiration date October 1957.

Dialysis Of Parathyroid Gland Extract. It was decided to study the effect of dialysis upon the biological potency and spectrophotometric effect of parathyroid gland extract.

a) Dialysis Experiment 1. 10 mls. of active commercial extract were dialyzed against 200 mls. of distilled water for 25 hours at a temperature of 13-15°C. Changes of the water were made at 2, 4, 5½, 8½, 20½, and 23 hours. Dialysis was carried out over a magnetic stirrer to increase efficiency. Cellulose Dialyzing Tubing (A. H. Thomas, No. 4465-A2) was employed as the dialyzing membrane. The dialysand was lyophilized and reconstituted to original volume with distilled water. The final solution was adjusting to pH. 4.0 with 2N HCl. This is the pH of the active extract.

b) Dialysis Experiment 2. This experiment was carried out in the same manner as above, except that the material was dialyzed against 2 liters of distilled water with changes at 5½ and 23 hours, and the dialysand was reconstituted directly to original volume and pH without lyophilization.

The result of these two experiments are summarized in Table 6. It seems clear that dialysis of parathyroid gland extract against distilled water inactivated the biological potency and markedly reduced the ability of the extract to affect the absorbance of TPNH at 340 mu.

## TABLE 6

BIOLOGICAL ASSAY AND SPECTROPHOTOMETRIC EFFECT OF DIALYZED PARATHYROID GLAND EXTRACT.

a) Dialysis Experiment 1.

Biological Assay.

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% | |
|---|---|---|---|---|
| 1 | 8.2 | 8.4 | 0.2 | |
| 2 | 8.3 | 9.1 | 0.8 | |
| 3 | 9.4 | 9.3 | -0.1 | |
| 4 | 9.1 | 10.3 | 1.2 | |
| 5 | 8.6 | 8.3 | -0.3 | |
| 6 | 8.3 | 7.4 | -0.9 | |
| 7 | 9.6 | 8.7 | -0.9 | |
| 8 | 8.3 | 7.9 | -0.4 | |
| | | | 0.0 | Mean |
| | | | (0.7) | Std. Dev. |

Spectrophotometric Effect.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.323 | 0.037 | --- |
| TPNH - Active Extract | 0.311 | 0.104 | 3.0 |
| TPNH - Dialyzed Extract | 0.412 | 0.046 | 1.2 |
| | 0.440 | 0.043 | 1.1 |

TABLE 6 (con't.)

b) Dialysis Experiment 2.

Biological Assay.

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% |
|---|---|---|---|
| 1 | 5.4 | 7.0 | 1.6 |
| 2 | 6.7 | 6.3 | -0.4 |
| 3 | 5.3 | 5.5 | 0.2 |
| 4 | 6.6 | 6.9 | 0.3 |
| 5 | 6.8 | 6.7 | -0.1 |
| 6 | 5.7 | 6.9 | 1.2 |
| 7 | 5.8 | 6.8 | 1.0 |
| | | | 0.5 Mean |
| | | | (0.7) Std. Dev. |

Spectrophotometric Effect.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.378 | 0.032 | --- |
| TPNH - Active Extract | 0.391 | 0.086 | 2.7 |
| TPNH - Dialyzed Extract | 0.376 | 0.051 | 1.6 |
| | 0.374 | 0.057 | 1.8 |
| | 0.373 | 0.054 | 1.7 |

Formaldehyde Inactivation Of Parathyroid Gland Extract. The calcium mobilizing principle of Lilly's parathyroid gland extract was inactivated through use of formaldehyde by a method similar to the one employed by Shetlar et. al. (1956).

0.05 mls. of formalin were added to a 5 cc. ampoule of active extract and the mixture was incubated for 4 days at room temperature. After this time, 55 mls. of absolute ethanol and 60 mls. of diethyl ether were added, the resulting percipitate

being collected at the centrifuge. The material was dried under reduced pressure in a desiccator containing calcium chloride, concentrated sulfuric acid and paraffin, and then reconstituted to original volume with distilled water. The final solution was adjusted to pH. 4.0 with 2N HCl. Biological assay and spectrophotometric effect of the resulting material are shown on table 7.

The efficacy of the inactivation procedure is obvious from the biological assay data. Once again, the destruction of calcium mobilizing potency is accompanied by a decrease in the ability of the extract to accelerate a decrease in absorption of TPNH at 340 mu.

TABLE 7

BIOLOGICAL ASSAY AND SPECTROPHOTOMETRIC EFFECT OF FORMALDEHYDE INACTIVATED PARATHYROID GLAND EXTRACT.

a) Biological Assay.

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% |
|---|---|---|---|
| 1 | 7.8 | 8.9 | 1.1 |
| 2 | 7.8 | 8.9 | 1.1 |
| 3 | 11.6 | 11.5 | -0.1 |
| 4 | 11.1 | 12.2 | 1.1 |
| 5 | 12.0 | 12.1 | 0.1 |
| 6 | 11.1 | 10.9 | -0.2 |
| 7 | 7.1 | 6.7 | -0.4 |
| 8 | 6.3 | 6.6 | 0.3 |
| | | | 0.3 Mean |
| | | | (0.6) Std. Dev |

TABLE 7 (con't.)

b) Spectrophotometric Effect.

Expt. 1

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.351 | 0.031 | --- |
| TPNH - Active Extract | 0.348 | 0.082 | 2.6 |
| TPNH - Inactive Extract | 0.350 | 0.038 | 1.2 |

Expt. 2

| | | | |
|---|---|---|---|
| TPNH- Control | 0.312 | 0.029 | --- |
| TPNH - Active Extract | 0.303 | 0.074 | 2.6 |
| TPNH - Inactive Extract | 0.314 | 0.040 | 1.4 |

Expt. 3

| | | | |
|---|---|---|---|
| TPNH - Control | 0.357 | 0.031 | --- |
| TPNH - Active Extract | 0.360 | 0.084 | 2.7 |
| TPNH - Inactive Extract | 0.365 | 0.038 | 1.2 |

Expt. 4

| | | | |
|---|---|---|---|
| TPNH - Control | 0.355 | 0.037 | --- |
| TPNH - Active Extract | 0.353 | 0.088 | 2.4 |
| TPNH - Inactive Extract | 0.357 | 0.045 | 1.2 |

Effect Of Formaldehyde Upon The Ultra-Violet Absorption Of TPNH At 340 mu. Although formaldehyde inactivated parathyroid gland extract was shown not to affect the ultra-violet absorption of TPNH at 340 mu, a possible inhibitory action of the formaldehyde itself upon such an affect had to be considered.

A 0.01% formalin solution was prepared and adjusted to pH. 4.0 with 2N HCl. A 0.5 cc. aliquot of this preparation was allowed to react with TPNH under the usual experimental conditions.

The results obtained are listed in Table 8. No effect of formaldehyde, at the concentration used in the formaldehyde inactivation procedure, upon the absorbance of TPNH at 340 mu is indicated.

TABLE 8

EFFECT OF FORMALIN UPON THE ULTRA-VIOLET ABSORPTION OF TPNH AT 340 mu.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.356 | 0.025 | --- |
| TPNH - Formalin | 0.273 | 0.031 | 1.2 |
| | 0.274 | 0.033 | 1.3 |
| | 0.271 | 0.031 | 1.2 |
| | 0.277 | 0.032 | 1.2 |

Effect Of Ethanol-Diethyl Ether Upon Parathyroid Gland Extract. The effect of the ethanol-diethyl ether phase of the formaldehyde inactivation procedure upon the biological activity and spectrophotometric effect of parathyroid gland extract was now investigated.

An aliquot of active extract was treated with ethanol-ether in exactly the same manner as indicated in the formaldehyde inactivation procedure except that the material was not pre-treated with formalin.

The results obtained are summarized in Table 9. It can be seen that the biological potency of the extract has been markedly reduced, as has been the spectrophotometric effect of the

preparation. Neither decrease, however, is as pronounced as that observed following formaldehyde inactivation. The results seem to indicate that the component, (or components), of the parathyroid gland extract responsible for the ability of the preparation to mobilize serum calcium and affect the absorbance of TPNH at 340 mu is at least partially extractable with ethanol-diethyl ether.

TABLE 9

BIOLOGICAL ASSAY AND SPECTROPHOTOMETRIC EFFECT OF ETHANOL-DIETHYL ETHER TREATED PARATHYROID EXTRACT.

Biological Assay.

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% |
|---|---|---|---|
| 1 | 11.3 | 14.0 | 2.7 |
| 2 | 10.3 | 12.8 | 2.5 |
| 3 | 6.1 | 9.8 | 3.7 |
| 4 | 7.9 | 8.7 | 0.8 |
| 5 | 11.3 | 10.9 | -0.6 |
| 6 | 7.9 | 8.1 | 0.2 |
| 7 | 8.3 | 8.8 | 0.6 |
| 8 | 7.8 | 9.9 | 2.1 |
| 9 | 6.6 | 7.7 | 1.1 |
| 10 | 8.5 | 8.9 | 0.4 |
| 11 | 6.7 | 8.0 | 1.3 |
| 12 | 9.2 | 8.7 | -0.5 |
| 13 | 6.0 | 6.4 | 0.4 |
| 14 | 6.5 | 7.9 | 1.5 |
| 15 | 8.4 | 9.3 | 0.9 |
| 16 | 10.6 | 11.1 | 0.5 |
| 17 | 8.7 | 9.8 | 1.2 |
| 18 | 7.2 | 9.3 | 2.1 |
| 19 | 9.0 | 9.3 | 0.3 |
| | | | 1.1 Mean |
| | | | (1.1) Std. Dev. |

TABLE 9 (con't.)

Spectrophotometric Effect.

Expt. 1

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.355 | 0.037 | --- |
| TPNH - Active Extract | 0.353 | 0.088 | 2.5 |
| TPNH - Treated Extract | 0.355 | 0.063 | 1.7 |
| **Expt. 2** | | | |
| TPNH - Control | 0.338 | 0.033 | --- |
| TPNH - Active Extract | 0.354 | 0.068 | 2.1 |
| TPNH - Treated Extract | 0.351 | 0.049 | 1.5 |
| **Expt. 3** | | | |
| TPNH - Control | 0.319 | 0.036 | --- |
| TPNH - Active Extract | 0.310 | 0.104 | 2.8 |
| TPNH - Treated Extract | 0.310 | 0.062 | 1.7 |
| | 0.304 | 0.059 | 1.6 |
| | 0.313 | 0.064 | 1.8 |

Pepsin Inactivation Of Parathyroid Gland Extract. It was now decided to inactivate the calcium mobilizing principle of the parathyroid gland extract by pepsin digestion, and study the ability of the treated extract to affect the ultra-violet absorption of TPNH at 340 mu.

Parathyroid gland extract was adjusted to pH. 2.0 with 2N HCl. 0.1 mg crystalline pepsin, (Armour, Porcine Origin, Lot no. 108-145) was added per ml. of extract, and the mixture was incubated for 13 hours at 37°C. After incubation, the mixture was readjusted to pH. 4.0 with 2N NaOH and place under refrigeration until used.

Table 10 lists the results obtained from pepsin inactivation of two separate aliquots of active parathyroid extract. There can be little doubt of the efficacy of the pepsin digestion in both experiments. The important point, however, is the pronounced effect of this biologically inactive material upon the ultra-violet absorption of TPNH at 340 mu. It is difficult to reconcile any proposed association of the calcium mobilizing principle of the extract with the effect of the extract upon the ultra-violet absorption of reduced coenzyme II in light of these experimental data.

TABLE 10

BIOLOGICAL ASSAY AND SPECTROPHOTOMETRIC EFFECT OF PEPSIN-TREATED PARATHYROID GLAND EXTRACT.

a) Experiment 1

Biological Assay

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% |
|---|---|---|---|
| 1 | 9.8 | 9.8 | 0.0 |
| 2 | 7.7 | 7.1 | -0.6 |
| 3 | 6.7 | 6.0 | -0.7 |
| 4 | 6.5 | 5.6 | -0.9 |
| 5 | 8.0 | 6.9 | -1.1 |
| 6 | 7.6 | 6.2 | -1.4 |
| 7 | 6.0 | 5.3 | -0.7 |
| 8 | 8.1 | 7.3 | -0.8 |
| 9 | 7.9 | 6.6 | -1.3 |
| | | | -0.8 Mean |
| | | | ( 0.8) Std. Dev. |

TABLE 10 (Con't.)

a) Experiment 1 (con't.)

Spectrophotometric Effect

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.361 | 0.039 | --- |
| TPNH - Active Extract | 0.327 | 0.101 | 2.6 |
| TPNH - Pepsin Treated Extract | 0.354 | 0.137 | 3.5 |
| | 0.353 | 0.134 | 3.4 |
| | 0.361 | 0.135 | 3.6 |

b) Experiment 2

Biological Assay

| Animal | Initial Serum Calcium, mg% | Final Serum Calcium, mg% | Change In Serum Calcium, mg% | |
|---|---|---|---|---|
| 1 | 6.0 | 6.1 | 0.1 | |
| 2 | 7.3 | 7.5 | 0.2 | |
| 3 | 6.3 | 6.8 | 0.5 | |
| 4 | 10.0 | 9.9 | -0.1 | |
| 5 | 7.6 | 7.3 | -0.3 | |
| 6 | 7.7 | 8.0 | 0.3 | |
| 7 | 4.7 | 5.4 | 0.7 | |
| 8 | 5.8 | 4.5 | -0.7 | |
| 9 | 7.9 | 7.3 | -0.6 | |
| | | | 0.1 | Mean |
| | | | (0.1) | Std. Dev. |

Spectrophotometric Effect

Experiment 1

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.402 | 0.041 | --- |
| TPNH - Active Extract | 0.378 | 0.099 | 2.4 |
| TPNH - Pepsin Treated Extract | 0.376 | 0.132 | 3.2 |
| | 0.370 | 0.137 | 3.3 |
| | 0.373 | 0.132 | 3.2 |

TABLE 10 (Con't.)

Experiment 2

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.340 | 0.036 | --- |
| TPNH - Active Extract | 0.365 | 0.072 | 2.0 |
| TPNH - Pepsin Treated Extract | 0.388 | 0.115 | 3.2 |
| | 0.386 | 0.120 | 3.3 |
| | 0.400 | 0.119 | 3.3 |

Effect Of Pepsin Upon The Ultra-Violet Absorption Of TPNH At 340 mu. The possibility that the pepsin introduced in the pepsin inactivation procedure was responsible for the accelerated decrease in absorbance of TPNH at 340 mu, although extremely unlikely under our experimental conditions, still had to be considered.

1.0 mg. of crystalline pepsin (Armour, Porcine Origin, Lot no. 108-145) was added to 10 mls. of distilled water which had previously been adjusted to pH. 4.0 with 2N HCl. A 0.5 ml. aliquot of this solution was allowed to react with TPNH in the usual manner.

The results, outlined in Table 11, indicate quite clearly that the enzyme pepsin does not affect the absorption of TPNH at 340 mu under our experimental conditions.

TABLE 11

EFFECT OF PEPSIN UPON THE ULTRA-VIOLET ABSORPTION OF TPNH AT 340 mu.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.342 | 0.036 | --- |
| TPNH - Pepsin | 0.341 | 0.034 | 0.9 |
| | 0.344 | 0.036 | 1.0 |
| | 0.342 | 0,035 | 0.9 |

Absolute Ethanol-Diethyl Ether Treatment Of Pepsin-Inactivated Parathyroid Gland Extract. It will be recalled that work with untreated parathyroid gland extract indicated that the material in this preparation affecting the ultra-violet absorption of TPNH was at least partially extractable through treatment with ethanol-diethyl ether. An experiment was performed to determine whether such a situation also obtained with the pepsin-inactivated parathyroid gland extract just studied.

The results, summarized in Table 12, show an extremely pronounced decrease in spectrophotometric effect following ethanol-ether treatment of the biologically inactive preparation which had previously been found to effect the absorption of TPNH quite markedly.

TABLE 12

EFFECT OF ETHANOL-DIETHYL ETHER TREATMENT UPON THE SPECTROPHOTOMETRIC EFFECT OF PEPSIN-INACTIVATED PARATHYROID GLAND EXTRACT.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.354 | 0.034 | --- |
| TPNH - Active Extract | 0.358 | 0.078 | 2.3 |
| TPNH - Treated Pepsin | 0.336 | 0.040 | 1.2 |
| Inactivated Extract | 0.343 | 0.042 | 1.2 |
| | 0.343 | 0.043 | 1.3 |

Dialysis Of Pepsin-Inactivated Parathyroid Gland Extract.

An experiment was performed to determine whether the "component" of the pepsin-inactivated parathyroid gland extract responsible for affecting the ultra-violet absorption of TPNH at 340 mu was dialyzable against distilled water. It will be recalled that previous work indicated that dialysis of the active extract against distilled water resulted in a marked decrease in spectrophotometric effect.

10 mls. of pepsin-inactivated parathyroid gland extract were dialyzed against one liter of distilled water for 26 ½ hours at a temperature of 13-15°C. The water was changed at 4, 6, and 24 hours. Cellulose Dialyzing Tubing (A. H. Thomas, No. 4465-A2) was again employed as the dialyzing membrane. Dialysis was carried out over a magnetic stirrer to increase efficiency. The dialysand was reconstituted to original volume, and adjusted to pH. 4.0 with 2N HCl.

The spectrophotometric effect of this material is shown in Table 13. As expected, dialysis of the pepsin-inactivated preparation significantly reduces the ability of the material to effect the ultra-violet absorption of TPNH at 340 mu.

TABLE 13

SPECTROPHOTOMETRIC EFFECT OF DIALYZED PEPSIN-INACTIVATED PARATHYROID GLAND EXTRACT.

| Tube | Initial Absorbance | Decrease In Absorbance | Activity Ratio |
|---|---|---|---|
| TPNH - Control | 0.362 | 0.026 | --- |
| TPNH - Active Extract | 0.391 | 0.087 | 3.3 |
| TPNH - Dialyzed Pepsin-inactivated Extract | 0.366 | 0.056 | 2.2 |
| | 0.364 | 0.058 | 2.3 |
| | 0.366 | 0.056 | 2.2 |
| | 0.352 | 0.054 | 2.1 |

As an aid in evaluating this work, a summary of the data presented in this chapter will be found in Table 14, page 42.

## TABLE 14

### SUMMARY OF EXPERIMENTAL DATA

| Material | Mean Activity Ratio | Mean Change In Serum Ca., mg% |
|---|---|---|
| 1. 0.2% Phenol Solution | 1.0 | --- |
| 2. Bovine Plasma Albumin | 1.3 | --- |
| 3. Lilly's Injection Parathyroid, U.S.P. | 2.7 | 2.5 |
| 4. Dialyzed Active Extract, Expt. 1 | 1.2 | 0.0 |
| 5. Dialyzed Active Extract, Expt. 2 | 1.7 | 0.5 |
| 6. Formaldehyde-Inactivated Extract | 1.2 | 0.3 |
| 7. Ethanol-Diethyl Ether Treated Extract | 1.7 | 1.1 |
| 8. Pepsin-Inactivated Extract, Expt. 1 | 3.4 | -0.8 |
| 9. Pepsin-Inactivated Extract, Expt. 2 | 3.3 | 0.1 |
| 10. Pepsin | 1.0 | --- |
| 11. Ethanol-Diethyl Ether Treated, Pepsin-Inactivated Extract | 1.2 | --- |
| 12. Dialyzed, Pepsin-Inactivated Extract | 2.2 | --- |

# CHAPTER IV

## DISCUSSION AND CONCLUSIONS

Our experimental data indicates quite conclusively that a parathyroid gland extract - specifically, "Injection Parathyroid, U.S.P." (Lilly) - markedly affects the ultra-violet absorption of TPNH at 340 mu. TPNH in contact with this preparation for six hours at room temperature exhibits a reduction in absorbance 2.7 times that noted when the extract is omitted. It is felt that Neuman and his associates (1956) observed effects similar to those produced in our experiments, although their statement of a "destruction" of the chromophoric group indicates a more extensive phenomenon. However, on the basis of our experiments, their implication of a direct relationship between the effect of the parathyroid extract on the absorption of TPNH at 340 mu and on the serum calcium of laboratory animals appears premature.

A necessary corrolary to their position would be that any alteration of the calcium mobilizing potency of the active extract would always be accompanied by a corresponding and proportional variation in the effect of the material upon the ultra-violet absorption of TPNH. It was with this in mind, that this problem was undertaken. A variety of experiments were designed to establish the validity of this implied correlation between the calcium mobilizing potency of the parathyroid gland extract, and the spectrophotometric effect of the preparation on the absorption

of reduced coenzyme II at 340 mu.

The absorption peak at 340 mu observed in Figure 3 is due to the presence of reduced nicotinamide in the TPNH molecule. The TPNH itself is observed to undergo a small but significant decrease in absorption at 340 mu over the six hour reaction period. It is felt that this decrease in absorbance represents a simple aerobic oxidation of the reduced coenzyme. The rate of this oxidation is, of course, much too slow to be of physiological significance. The fact that it can occur, however, has, to our knowledge, never been questioned. Figure 3 shows that as the absorbance of TPNH at 340 mu decreases, there is a corresponding increase in absorbance at 260 mu, which is the absorbance peak for oxidized coenzyme II. This tends to support the contention that an oxidation reaction is occuring.

The ultra-violet absorption spectrum of the parathyroid gland extract utilized in this work, shown in Figure 2, closely resembles that recorded by Ross and Wood (1942) for their active parathyroid gland preparation. The absorption peak at 275 mu is due to the presence of the aromatic amino acids tyrosine, tryptophane, and phenylalanine in the extract. It should be pointed out that the 0.2% phenol added to the Lilly preparation as a preservative also absorbs at this wavelength. The presence of this preservative was a serious handicap in all attempts to record the spectra of treated preparations, since there was no way of knowing whether

the absorption peaks recorded were due to amino acids or simply to incomplete removal of the phenol. In general, the spectrum recorded for this extract is similar to those recorded for a number of simple proteins.

Figure 4 shows the spectra recorded of a parathyroid extract-reduced coenzyme II reaction mixture before and after the six hour test period. The peak at 340 mu is due to TPNH, that at 274 mu is due to the presence of the parathyroid gland extract. Disappearance of the oxidized coenzyme II peak at 260 mu is attributed to its being overshadowed by the absorption of the extract at 275 mu. It will be noted, however, that there is an increase in absorption after six hours at both 260 mu and 275 mu. In view of the fact that the spectrum of parathyroid gland extract alone was found not to change over the six hour reaction period, and since the decrease in absorption of TPNH at 340 mu was observed to produce an increase in absorption at 260 mu, it would seem that the general "upswing" noted in the spectrum of the mixture after the six hour reaction period is due primarily to oxidation of TPNH with the resultant production of oxidized coenzyme II. No complex formation or marked disruption of the TPNH molecule is indicated by these spectra. Rather, the evidence points to an oxidation of TPNH "catalyzed" by the parathyroid gland extract.

Dialysis of parathyroid gland extract against distilled

water for about 24 hours showed the component of the extract affecting the ultra-violet absorption of TPNH at 340 mu to be capable of passing through a cellophane dialyzing membrane. Compared to the original extract, the dialysand exhibited a marked decrease in spectrophotometric effect and was biologically inactive. The suggestion of an association between these two phenomena is strengthened by these data. A number of workers have noted the effect of dialysis upon the biological properties of parathyroid gland extracts. Kenny (1956) reports that he found both the calcium mobilizing and the phosphaturic "activities" to be "dialyzable to a considerable extent", but gives no further details. Melius (1957) utilized dialysis of parathyroid gland extract preparations against various buffers as a means of purification and reported no appreciable passage of the calcium mobilizing principle through cellophane dialyzing tubing. Davies and Gordon (1953) found the phosphaturic, but not the calcium mobilizing principle of his parathyroid extract to be dialyzable against dilute acetic acid. Our work resulted in almost a complete loss of the calcium mobilizing activity of the extract after dialysis against distilled water. We did not, however, assay the dialysate for possible calcium mobilizing activity.

As expected, the formaldehyde inactivation procedure followed proved effective in destroying the calcium mobilizing principle of the active extract. Again, this decreased biological activity was

accompanied by a decreased effect of the extract upon the ultraviolet absorption of TPNH at 340 mu.

Treatment of parathyroid gland extract with absolute ethanol-diethyl ether indicated that the factor affecting the absorbance of TPNH was soluble to a significant extent in these fat solvents. Loss of spectrophotometric effect was accompanied by a decrease in the calcium mobilizing activity of the treated extract. It will be noted, however, that the activity ratio of 1.7 obtained following treatment of the extract in this manner was the same as that obtained in Dialysis Experiment 2, yet the biological activity of the ethanol-ether treated extract was almost twice that of the dialyzed preparation. Here we see a discrepancy between calcium mobilizing ability, and spectrophotometric effect. The results of these two experiments are not, of themselves, considered as a sufficient basis for refuting the previously outlined contentions of Neuman and his associates (1956). They will, however, be referred to once again, in the light of other experimental data.

The decrease of biological activity of the extract following treatment with absolute ethanol-diethyl ether came as a surprise in view of the traditional concepts of workers in the field that the hormonal principle elicited by the parathyroid gland is protein in nature (Ross and Wood, 1942). Although some proteins are known to be soluble in such solvents, their number are few.

Our results do tend, however, to substantiate a rather startling report by Raoul et. al. (1955) who found in their work with parathyroid gland preparations, that all of the calcium mobilizing activity was extractable with either boiling absolute ethyl alcohol, or by a mixture of ethyl alcohol and diethyl ether at -40°C. No more direct substantiation of their work has, to our knowledge, been reported. It is to be emphasized that since in our work we did not assay the alcohol ether supernate for biological activity, we do not claim to have extracted the calcium mobilizing principle from the extract, but merely report a decrease of this activity in the treated preparation.

In evaluating the pepsin-inactivation experiments, we feel that the data prove conclusively the efficacy of this procedure in destroying the calcium mobilizing principle in the extract. Similarly, the ability of this treated preparation to catalyze a reduction in absorption of TPNH at 340 mu cannot be denied. The critical nature of these results is evident. We have taken a biologically active parathyroid gland preparation which has shown to unquestionably possess the ability to affect the ultraviolet absorption of reduced coenzyme II at 340 mu under our experimental conditions, and have destroyed its calcium mobilizing ability. If, as indicated by Neuman and his associates (1956), a correlation actually existed between the calcium mobilizing ability of the active preparation and its effect on

the ultra-violet absorption of TPNH at 340 mu, then we would expect the destruction of biological activity upon pepsin digestion to be accompanied by a reduction in the ability of the preparation to affect the ultra-violet absorption of the reduced coenzyme II. Not only did such a situation not obtain, but there was observed an actual increase in the ability of the extract to affect the absorption of TPNH. Furthermore, the pepsin itself was shown not to affect the ultra-violet absorption of the reduced coenzyme II. The inactivation procedure did not involve any manipulation of the extract which could likely cause a loss of any component of the extract, as, for example, dialysis or extraction. The conclusion seems inescapable that some biologically inactive degradation product of the pepsin digestion is affecting the absorbance of TPNH. Dialysis of the pepsin inactivated extract against distilled water markedly reduced the preparation's ability to affect the absorption of the coenzyme, while treatment with ethanol-ether virtually eliminated it. These results are similar to those observed when active extract was subjected to dialysis and extraction. It would appear that the moeity involved is similar in both the active and the pepsin inactivated extracts, being produced in the inactivated preparation as a result of the pepsin digestion. The fact that the moeity involved is a degradation product of pepsin digestion, and also its ability to pass through a cellophane dialyzing membrane, would

tend to preclude the material from being a protein or an extremely large polypeptide. The molecule involved also must possess some fat soluble grouping as indicated by its solubility in the alcohol-ether.

These experimental data would seem to render untenable the position that a direct association exists between the capacity of Lilly's parathyroid extract - "Injection Parathyroid, U.S.P." - to mobilize serum calcium, and catalyze a decrease in absorption of TPNH at 340 mu. These findings assume significance in the light of current thought on the mechanism of action of parathyroid hormone.

As stated previously, Neuman et. al. (1956) had found that a "dramatic increase" in citrate output from the spongiosal circulation of the femur of intact dogs occured following parenteral administration of parathyroid gland extract (Lilly). This was accompanied, of course, by a rise in serum calcium in the animal. These workers then observed that the parathyroid extract "destroyed the chromophoric group" of TPNH in vitro at 340 mu, and rendered it "practically non-absorbent". This was interpreted as giving evidence of a parathyroid controlled "blocking" of coenzyme II-linked reactions in vivo. Isocitric dehydrogenase is a coenzyme II-dependent enzyme, but exhibits very low activity in mature bone (Dixon and Perkins, 1952, and Neuman et. al., 1956). The metabolism of glucose-6-phosphate via the "Hexose Monophosphate Shunt" is also dependent upon a coenzyme II-linked enzyme, namely,

glucose-6-phosphate dehydrogenase. A "blocking" of the hexose monophosphate shunt reactions could channel glucose metabolism into the Krebs cycle, and since in bone the enzyme of the Krebs cycle concerned with the further metabolism of citrate, isocitric dehydrogenase, is "lacking" (Neuman, 1956) a pileup of citrate could result. This proposed blocking of coenzyme II linked reactions by hormonal action is the "intriguing biochemical mechanism" referred to by Neuman and his associates (1956) by which a "parathyroid controlled citrate gradient maintains a steady but supersaturated level of ionized calcium in the serum." Indeed, Strates, Neuman and Levinskas (1957) consider this to be an "established corollary" to their concept of bone mineralization. All of this, however, is apparently based on the initial assumption that the calcium mobilizing principle of Lilly's commercial parathyroid gland extract affects the absorbance of TPNH at 340 mu. Our experiments, again, would seem to render such a position untenable.

In conclusion, then, although a parathyroid controlled cellular mechanism may very well exist for maintaining a calcium ion gradient between blood and bone, and although this mediation may well involve participation of citric acid in some manner, the postulation of such a theory on the basis of the ability of the calcium mobilizing principle of Lilly's commercial parathyroid gland extract to destroy the chromophoric group of TPNH in vitro,

seems extremely premature and not in agreement with our experimental results.

# CHAPTER V

## SUMMARY

1. "Injection Parathyroid, U.S.P.", an active parathyroid gland extract commercially available from the Eli Lilly Company, was found to definitely affect the ultra-violet absorption of reduced coenzyme II at 340 mu.

2. Dialysis of this active commercial extract against distilled water resulted in a virtual elimination of the calcium mobilizing ability of the preparation and in a marked decrease in the ability of the preparation to affect the absorbance of TPNH at 340 mu. (Spectrophotometric Effect)

3. Treatment of the active extract with ethanol-diethyl ether resulted in a marked reduction of biological activity and a similar marked reduction in spectrophotometric effect.

4. Inactivation of the calcium mobilizing principle of the active extract through treatment with formaldehyde resulted in a virtual elimination of the spectrophotometric effect of this preparation.

5. Pepsin inactivation of the calcium mobilizing principle of the commercial extract resulted in an increased ability of the preparation to affect the absorption of TPNH at 340 mu. This is not to be expected if a correlation existed between the calcium mobilizing potency of the extract and its

spectrophotometric effect.

6. Both dialysis of the pepsin inactivated extract against distilled water and its extraction with ethanol-ether resulted in a decreased spectrophotometric effect.

7. Under our experimental conditions, no correlation was observed to exist between the calcium mobilizing principle of the commercial extract and the ability of the extract to decrease the absorption of TPNH at 340 mu.

8. Analysis of our experimental results seem to indicate that the material responsible for affecting the ultra-violet absorption of reduced coenzyme II at 340 mu is a dialyzable, biologically inactive moiety, which possesses some degree of solubility in typical fat solvents.

9. Any postulate regarding the mechanism of action of the parathyroid hormone based on the ability of the calcium mobilizing principle of Lilly's commercial parathyroid gland extract to decrease the absorption of TPNH at 340 mu would appear to be untenable in view of our experimental findings.

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# APPENDIX

## TECHNIQUE OF PARATHYROIDECTOMY BY CAUTERIZATION

The following technique was used routinely to parathyroidectomize female albino rats weighing 120-30 grams.

Ether was used as the anesthetic during the operation, which was carried out in the hood to facilitate removal of ether vapors. The animal was anesthetized in an ether jar and removed when, after rolling the animal over on its back, it ceased struggling to right itself. After removal from the jar the animal was placed on its back on a wooden board approximately 5 inches by ten inches, and secured by placing wide adhesive tape over each limb. A rubber band was placed about the incisor tooth in such a manner as to make the neck of the animal slightly taut, the incisor becoming the apex of a triangle formed by it and two nails imbedded in the board about two inches above the head of the animal. A small piece of cotton was made damp to the touch with ether and placed close to, but not in contact with the nose of the animal. Ether was kept at hand and periodically during the operation was added to the cotton, no more than five drops at a time. If, during the course of the operation, the animal developed pronounced ataxic type breathing, the cotton plug was removed from the nose area until breathing returned to normal. If, however, respiratory difficulties characterized by a wheezing or gasping sound developed, and did not cease after a

minute or two, the cotton plug was replaced and the operation completed as rapidly as possible. After the animal had been secured on the operating board, an incision was made along the midline of the neck from the cephalic boarder of the sternum to the mandible. Similar incisions are made through the various subcutaneous layers of connective tissue, hemostats were used as needed to hold the cut tissue to one side. Care was taken to avoid damage to the submaxillary salivary glands situated bilaterally to this midline incision. Any damage to these glands resulted in profuse bleeding. It was found convenient to feel for the trachea with a finger before making the incision, and then cutting along what was felt to be the midline of the trachea. In this way the initial incision sometimes separated the glands present right away. Once the muscle layer covering the trachea had been exposed, it was gently separated at the midline through use of a sharp probe or scalpel. The muscle flaps were retained by hemostats, and the exposed trachea cautiously freed of fascia. A muscle was noted lying adjacent to each lobe of the thyro-parathyroid apparatus. This was separated by blunt disection. Once this muscle had been retracted, it was usually possible to observe the parathyroid gland as a noticeable lighter-colored, bulbular projection located at the upper lateral edge of the thyroid. Identification was greatly facilitated through use of a 2.75X binocular loupe (Magni-Focuser). The parathyroid was

then cauterized with the eye tip of a cautery pencil. To insure complete removal, it was found best to cauterize the general area where the parathyroids were located. If the parathyroid glands could not be located, cauterization in the usual area generally proved effective. Care had to be taken not to use too hot an eye tip, since this would burn into the trachea, or destroy some other vital structure. Insertion of a blunt probe under the trachea, which was used to gently lift the thyro-parathyroid apparatus, proved helpful in allowing the operator to better distinguish and cauterize the parathyroid gland mass. Immediately after cauterization, all limb restraints were removed, and the wound was closed with from two to three wound clips. The animal revived in a minute or two following a return to its cage. If the animal developed a pronounced gasping or wheezing which did not subside within 15 minutes, it was destroyed.

## CARDIAC PUNCTURE TECHNIQUE

The following method was used routinely in obtaining blood for serum calcium analysis.

The animal is anesthetized with ether and placed on the operating board in the same manner as described above. The puncture is made utilizing a one cc. Luer type syringe, with a 25 or 26 guage needle. A larger needle is not advisable as it may seriously damage the heart. The needle is inserted at a point slightly lateral to the point of maximum palpitation as determined by touch. If the animal is prepared in the same manner for each cardiac puncture, it is often possible to "hit" the heart even though some difficulty is encountered in feeling the point of maximum palpitation. If the heart is missed in the initial puncture attempt, one should not probe around with the needle, but apply gentle lateral pressure to each side to see if the impulse of the heart can be felt on the needle. This will aid in judging the site of the next attempt. It is felt that for a beginner, it would be of great benefit to insert the needle into the animal, then destroy the animal with ether and observe the <u>in situ</u> location of the heart in relation to the position of the needle. This serves as an invaluable orientation. A number of rats withstood six successful heart punctures with no noticeable ill effects, and, in general, this method permitted rapid removal of 0.5 cc. of blood from the rat with very few

fatalities.

CALCIUM ANALYSIS OF SERUM-METHOD OF NATELSON AND PENNIALL (1955)

The reagents and materials used were prepared exactly as described by Natelson and Penniall (1955). The following is a detailed account of the technique shown to give reproducible results when using this method for serum calcium analysis.

One ml. of water is placed in a ground glass stoppered 12 ml. capacity, centrifuge cone. To this is added 50 lambda of serum, using a 50 lambda micropipette. The micropipetting seemed to be the source of most of the initial trouble encountered in obtaining reproducible results. The following procedure was found to be acceptable. The micropipette is attached to a tuberculin syringe by means of some rigid attachment, such as a plastic nozzle or a thick walled pressure tubing, about 3/4 inch in length. It should be possible to control the pipette using the syringe and only one hand. The micropipetting is inserted into the sample, and the sample is drawn up to point a bit above the 50 lambda mark. This sample is then discarded. The size of the sample will almost always be large enough to permit this with a 50 lambda (or less) pipette. The pipette is reinserted into the sample and again sample is drawn up to a point slightly above the mark. The outside nozzle of the micropipette is wiped of with Kleenex, and in this way the sample is usually drawn down to the mark by capillary action.

The sample is then ejected into the water in the centrifuge cone. The mixture in the centrifuge cone is used to rinse the micropipette twice. The micropipette is finally rinsed twice with distilled water, the rinse being ejected into the centrifuge cone. This procedure assures removal of all the serum from the walls of the micropipette. Pipetting of a second serum sample is accomplished in the same manner as just described, beginning with the rinse of the micropipette with the serum sample. After use, the micropipette is cleaned with detergent, distilled water, concentrated nitric acid, and again with distilled water. It is not necessary to dry the pipette if the above procedure is used. To the cones containing the serum and water, 2 mls. of Triethanol amine, and then 6 mls. of Alizarine-Octanol reagent are added. It was found convenient to add these reagents by burette for speed and accuracy. The tightly stoppered centrifuge cones are place on the shaking machine and shaken for 20 minutes. Following this, the cones are centrifuged for five minutes at 2000 rpm. at the clinical centrifuge. If cloudy supernates are obtained, the cones are re-centrifuged for another five minutes. It is important, however, that all the cones in a run are centrifuged for the same period of time. The supernate is drawn off using a 5 ml. graduated pipette controlled by mouth suction. About 6 mls. can usually be drawn off, but a minimum of 4 mls. are required for the colorimeter tubes. The

color developed in the supernates is measured at the Klett-Summerson colorimeter, using a number 56 filter (560 mu). This method involves a modification of the original Natelson and Penniall technique in that 50 lambda of sample are utilized, instead of 20 as suggested by these workers, and 6 mls. of the alizarin-octanol reagent are used instead of the original 3 mls. After use, the cones are cleaned with detergent, distilled water, acetone, dilute nitric acid, and distilled water. The colorimeter tubes are cleaned with detergent, distilled water, dilute nitric acid, and again with distilled water.

Figure 5 indicates the linear relationship between the concentration of serum calcium and the corresponding colorimeter reading.

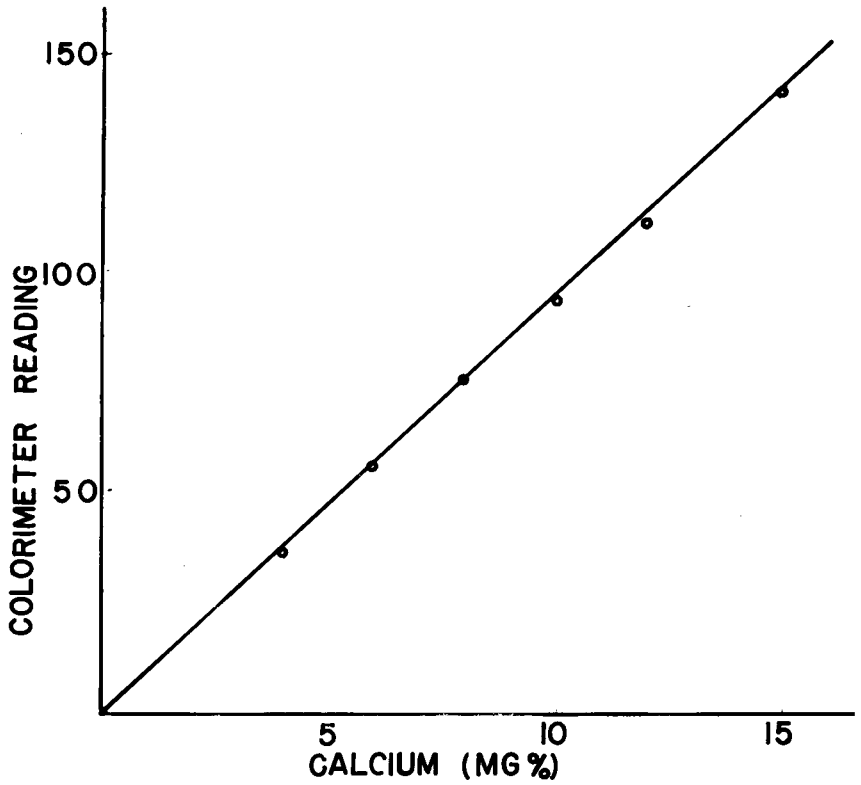


FIGURE 5

STANDARD CURVE FOR CALCIUM ANALYSIS

All Points Are The Mean Of At Least Four Determinations.

TABLE 15

ULTRA-VIOLET ABSORPTION OF PARATHYROID GLAND EXTRACT.

| Wavelength (mu) | Absorbance (slit 0.1 mm.) |
|---|---|
| 380 | 0.049 |
| 375 | 0.052 |
| 370 | 0.054 |
| 365 | 0.056 |
| 360 | 0.059 |
| 355 | 0.062 |
| 350 | 0.064 |
| 345 | 0.067 |
| 340 | 0.071 |
| 335 | 0.075 |
| 330 | 0.081 |
| 325 | 0.085 |
| 320 | 0.092 |
| 315 | 0.098 |
| 310 | 0.106 |
| 305 | 0.123 |
| 300 | 0.149 |
| 295 | 0.195 |
| 290 | 0.279 |
| 285 | 0.442 |
| 280 | 1.170 |
| 275 | 1.850 |
| 270 | 2.250 |
| 265 | 1.850 |
| 260 | 1.400 |
| 255 | 1.030 |
| 250 | 0.775 |
| 245 | 0.682 |
| 240 | 0.792 |
| 235 | 1.270 |
| 230 | 2.200 |
| 225 | 2.460 |

No change was observed in the spectrum after a 6 hour time interval. The data recorded were obtained using the tube arrangement listed on page 19, and are plotted in Figure 2, page 20.

TABLE 16

ULTRA-VIOLET ABSORPTION OF TPNH BEFORE AND AFTER A SIX HOUR TIME INTERVAL.

| Wavelength (mu) | Initial Absorbance (Slit 0.1 mm.) | Final Absorbance (Slit 0.1 mm.) |
|---|---|---|
| 380 | 0.042 | 0.036 |
| 375 | 0.061 | 0.052 |
| 370 | 0.082 | 0.072 |
| 365 | 0.104 | 0.094 |
| 360 | 0.126 | 0.114 |
| 355 | 0.150 | 0.133 |
| 350 | 0.165 | 0.148 |
| 345 | 0.176 | 0.158 |
| 340 | 0.180 | 0.162 |
| 335 | 0.176 | 0.159 |
| 330 | 0.167 | 0.150 |
| 325 | 0.153 | 0.137 |
| 320 | 0.136 | 0.122 |
| 315 | 0.117 | 0.106 |
| 310 | 0.101 | 0.092 |
| 305 | 0.086 | 0.083 |
| 300 | 0.077 | 0.080 |
| 295 | 0.071 | 0.082 |
| 290 | 0.073 | 0.092 |
| 285 | 0.088 | 0.116 |
| 280 | 0.143 | 0.172 |
| 275 | 0.233 | 0.265 |
| 270 | 0.355 | 0.387 |
| 265 | 0.452 | 0.480 |
| 260 | 0.482 | 0.502 |
| 255 | 0.450 | 0.457 |
| 250 | 0.380 | 0.392 |
| 245 | 0.300 | 0.301 |
| 240 | 0.239 | 0.239 |
| 235 | 0.213 | 0.209 |
| 230 | 0.223 | 0.286 |
| 225 | 0.269 | 0.515 |

The data recorded were obtained using the tube arrangement outline of page 21, and are plotted in Figure 3, page 22.

## TABLE 17

ULTRA-VIOLET ABSORPTION OF A PARATHYROID EXTRACT-TPNH REACTION MIXTURE BEFORE AND AFTER A SIX HOUR TIME PERIOD.

| Wavelength (mu) | Initial Absorbance (Slit 0.1 mm.) | Final Absorbance (Slit 0.1 mm.) |
|---|---|---|
| 380 | 0.088 | 0.090 |
| 375 | 0.110 | 0.106 |
| 370 | 0.132 | 0.126 |
| 365 | 0.157 | 0.147 |
| 360 | 0.182 | 0.170 |
| 355 | 0.206 | 0.190 |
| 350 | 0.225 | 0.206 |
| 345 | 0.240 | 0.218 |
| 340 | 0.250 | 0.225 |
| 335 | 0.240 | 0.225 |
| 330 | 0.247 | 0.221 |
| 325 | 0.236 | 0.211 |
| 320 | 0.225 | 0.209 |
| 315 | 0.213 | 0.197 |
| 310 | 0.206 | 0.195 |
| 305 | 0.206 | 0.204 |
| 300 | 0.224 | 0.230 |
| 295 | 0.264 | 0.282 |
| 290 | 0.350 | 0.380 |
| 285 | 0.532 | 0.585 |
| 280 | 1.260 | 1.320 |
| 275 | 2.170 | 2.240 |
| 270 | 2.500 | ----- |
| 265 | 2.250 | 2.270 |
| 260 | 1.800 | 1.980 |
| 255 | 1.440 | 1.510 |
| 250 | 1.150 | 1.220 |
| 245 | 0.982 | 1.040 |
| 240 | 1.030 | 1.100 |
| 235 | 1.470 | 1.540 |
| 230 | 2.320 | 2.700 |
| 225 | 2.560 | 2.950 |

The data recorded were obtained using the tube arrangement outlined on page 21, and are plotted in Figure 4, page 24.

TABLE 18

EFFECT OF VARIOUS DOSE LEVELS OF PARATHYROID EXTRACT UPON THE ULTRA-VIOLET ABSORBANCE OF TPNH AT 340 mμ.

| Material | Initial Absorb. | Decrease In Absorbance With Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| Expt. 1 | | 1 | 2 | 3 | 4 | 5 | 6 |
| TPNH-Control | 0.433 | 0.002 | 0.008 | 0.013 | 0.022 | 0.030 | 0.035 |
| TPNH-0.2 cc. Extract | 0.435 | 0.010 | 0.017 | 0.025 | 0.038 | 0.047 | 0.063 |
| TPNH-0.5 cc. Extract | 0.433 | 0.013 | 0.028 | 0.044 | 0.063 | 0.085 | 0.109 |
| TPNH-1.0 cc. Extract | 0.430 | 0.025 | 0.048 | 0.080 | 0.124 | 0.172 | 0.219 |
| Expt. 2 | | | | | | | |
| TPNH-Control | 0.394 | 0.007 | ----- | 0.019 | 0.030 | 0.038 | 0.044 |
| TPNH-0.2 cc. Extract | 0.396 | 0.011 | ----- | 0.038 | 0.050 | 0.065 | 0.079 |
| TPNH-0.5 cc. Extract | 0.382 | 0.016 | ----- | 0.056 | 0.077 | 0.102 | 0.127 |
| TPNH-1.0 cc. Extract | 0.402 | 0.020 | ----- | 0.083 | 0.140 | 0.191 | 0.230 |
| Expt. 3 | | | | | | | |
| TPNH-Control | 0.384 | 0.005 | 0.008 | 0.016 | 0.026 | 0.034 | 0.045 |
| TPNH-0.2 cc. Extract | 0.383 | 0.010 | 0.017 | 0.031 | 0.045 | 0.058 | 0.071 |
| TPNH-0.5 cc. Extract | 0.380 | 0.015 | 0.028 | 0.051 | 0.070 | 0.095 | 0.118 |
| TPNH-1.0 cc. Extract | 0.385 | 0.022 | 0.048 | 0.081 | 0.130 | 0.181 | 0.225 |

APPROVAL SHEET

The thesis submitted by Leo Edmund Reichert, Jr. 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.

November 14, 1957
Date

Maurice B. Visscher
Signature of Adviser