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Incorporation of Amino Acids Into Parathyroid Tissue Protein

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INCORPORATION OF AMINO ACIDS INTO PARATHYROID TISSUE PROTEIN

by

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LIFE

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In February 1963, he entered graduate work at New Mexico State Highlands University, and received a Master of Science degree in August 1964.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

"Endocrine glands and their hormones are a reminder that life is a highly improbable state of matter, dependent for its maintenance upon the success with which highly complex and sensitive chemical systems can continue their activities. This success can only be achieved if the constituent parts of these systems are continuously regulated in response to changing conditions within themselves and in the external environment. The progress of organic evolution has therefore depended upon the establishment of coordinating mechanisms. And the importance of the chemical agents known as hormones lies in the contributions that they have made to this fundamental requirement of life." (6)

The parathyroid glands were discovered in 1850, and were described as small compact yellow glandular bodies attached to the thyroid glands. Early in this century, MacCallum, Voegtlin, and Greenwald established the fundamental functions of this gland in calcium and phosphate metabolism, and Collip prepared the first biologically active and stable of the glands, and developed the first suitable assay method for the hormone which presented the final proof of the endocrine nature of this organ (43).

Anatomically, the parathyroid glands are developed from the entoderm of the branchial clefts, and are found as small

paired bodies in the region of the thyroid glands. The numbers and the position of these glands varies widely even between individuals of the same species. The number of the glands varies from one to four pairs; most commonly two pairs are seen. One pair, the internal parathyroids, may be imbedded entirely in the thyroids; the other pair, the external parathyroids, are usually found near the dorsolateral surface of the thyroids. However, accessory tissue may develop in widely scattered positions in the neck and upper thoracic region. Each gland is surrounded by a connective tissue capsule, and septa divide the gland into lobules. Microscopically, the parathyroid tissue resembles hyperplastic thyroid tissue. Although the parathyroid glands are the smallest of the endocrine organs, their total weight in an adult man is approximately 100 mg. They play a vital role in calcium homeostasis in higher vertebrates, and their removal, particularly in young animals, frequently leads to hypocalcemic tetany and death.

Removal of all the parathyroid tissue is normally followed by a fall in plasma calcium and a rise in plasma phosphate. Administration of parathyroid extract to humans with hypoparathyroidism caused a prompt increase in urinary phosphate and a fall in serum phosphate accompanied by slow rise in serum calcium. Early postulates (14) suggested that the primary action of the hormone was to promote phosphate execretion by the kidney, and that the rise in serum calcium was secondary to the fall in

serum phosphate. At the present time, it is recognized that the hormone acts on both kidney and bone through different mechanisms.

The primary function of the parathyroid glands is the precise regulation of the level of calcium ion in body fluids. Although gain and loss of calcium depends upon the balance between absorption from the intestinal tract and loss by excretion, the acute regulation of the plasma calcium level depends primarily on the vast reservoir of calcium present in the skeleton and the action of parathyroid glands. From the labile bone calcium pool, calcium may be withdrawn or added without the mediation of the parathyroid glands; however, precise homeostatic control requires the presence of functioning parathyroid tissue.

Gaillard (18) observed that when parathyroid extract or purified parathyroid hormone was added to the medium of tissue culture of bone, there was a rise in calcium and citrate in the medium, and an increase in the number and activity of osteoclasts. Stimulation of endogenous parathyroid hormone production by hypocalcemia or the administeration of parathyroid extracts to rats, dogs, and chicks caused a loss of bone mineral around large osteocytes buried in trabecular and cortical bones. This was associated with metachromatic staining changes, suggesting alteration in the properties of the bone matrix locally. Increase of hydroxyproline levels in plasma and urine following administration of parathyroid extracts has been demonstrated (37). Presumably this acid was released by breakdown of bone collagen and ground

substance. In the process of osteolysis, both inorganic and organic components of bone appeared to be released at the same time.

Parathyroid hormone increases the output of citrate by bone. This led to the formulation of a postulate that attributed calcium mobilization from bone to the solubilizing effect of chelation of calcium with citrate. However, citrate output is insufficient to account for all the calcium mobilized. A current theory is that parathyroid hormone increased production of both lactate and citrate by bone cells and the resulting fall in local pH is responsible for the solubilizing of the bone mineral (29). Studies with calcium-45 (10) show that the calcium mobilized by parathyroid hormone appeares to come from deep bone stores rather than from recently deposited calcium; instead of acting superficially, the hormone promotes resorption of the stable bone. This is somewhat similar to the action of vitamin D, and the two appear to act synergistically.

Parathyroid extract administration to humans leads to an abrupt and marked increase in the rate of phosphate excretion by the kidney and a progressive fall of serum phosphate. Many investigators believe that the effect of the hormone on urinary phosphate excretion takes place at the proximal renal tubule, by causing a decrease in the reabsorption of phosphate. In contrast, the hormone seems to promote calcium retention by increase in kidney reabsorption and intestinal uptake of calcium (37).

The parathyroids are also involved in magnesium metabolism.

Experimental magnesium deficiency in rats produces hypercalcemia, hypophosphatemia and increases the phosphate clearance by kidney. Parathyroidectomy results in a fall of plasma magnesium. Contimuous intravenous infusion of purified parathyroid hormone into rats causes a profound fall of urinary calcium and magnesium (23,24).

It has been observed by many workers that hyperplasia of the parathyroid glands occurs in conditions which tends to lower the plasma calcium level, such as, low calcium diet, vitamin D deficiency, a pregnancy and lactation. It seems logical that the level of calcium in blood would affect the function of the parathyroids. Serum obtained from the perfusate of low calcium blood through isolated thyroid-parathyroid gland apparatus from a dog when injected into normal or parathyroidectomized dogs caused a rise in serum calcium similar to that was obtained with injections of parathyroid extract (17). Copp and Davidson performed similar experiments in dogs (13) by perfusing the glands with high or low calcium blood in situ and simultaneously denervating the glands. The low calcium blood perfusate stimulated a remarkable rate of release or synthesis of the hormone, which in turn increased the blood calcium of the recipient dog to a level approaching that obtained in the same animal by continuous intravenous infusion of 20 U.S.P. units of parathyroid hormone per kilogram body weight per hour. In these experiments, glands were actually removed from the animal although the circulation

was still maintained. These experiments indicated a direct hormonal control of the parathyroid function by the level of calcium in the blood flowing through it. These direct and indirect indications appeared to provide an efficient feedback regulatory mechanism which controls the parathyroid hormone synthesis and secretion.

Roth and Raisz (44) reported that when parathyroid glands were cultured for 48 hours in a medium containing radioactive amino acids, a small amount of radioactivity was precipitable with trichloroacetic acid. The concentration of this labeled protein was inversely proportional to the calcium concentration in the medium. Morphological studies with the aid of electron-microscopy, showed that both protein and nucleic acid synthesis could be affected by the calcium concentration in the medium.

In addition inhibitors of protein synthesis such as, puromycin and actinomycin showed a decrease in incorporation of labeled amino acids into parathyroid tissue protein both in low-calcium and in high-calcium incubation media. However, a persistent difference of radioactivity released into the medium remained between the incubations in high- and low-calcium media. This difference showed a similar proportionality compared to the incubations which were carried out without the presence of inhibitors. These facts may indicate that calcium concentration in the medium directly affects the hormone secretory mechanism in parathyroid gland cells. And this does not require the production

of any new kind of RNA to provide transport or any kind of carrier proteins.

A most effective extraction method of the hormone from parathyroid glands was developed in 1959 by Aurbach (3) using concentrated phenol solution; the first pure preparations of the hormone were obtained with the additional aid of countercurrent distribution (4,40). This method gave a hormone with approximate potency of 200 U.S.P. units/mg. Further purification by gel filtration on Sephadex columns (5,33,41,42,43) improved the activity of the product to 2500 to 3000 U.S.P. units/mg. Carboxymethylcellulose chromatography further purified hormone materials were resolved into two components by electrophoresis in polyacrylamide gels at pH 2.8; both were biologically active and immunologically similar (34). They differed only slightly in their covalent structures. Heterogeneity in hormonal structures could be due to these two forms of parathyroid hormone being derived from the glands of different individuals, but also possible that both peptides could be present in the same animal.

The hormone is a polypeptide having a molecular weight of aproximately 9000. It contains one tyrosine, one tryptophane, two methionine, no cysteine and no carbohydrate. It is composed of 84 amino acids. An empirical structure of its amino acid composition is as follows:

Lys_{11}, His_4, Arg_5, Asp_7, Ser_7, Glu_{11}, Pro_3, Gly_5, Ala_6, Val_7, Met_2, $Ileu_3$, Leu_8, Tyr_1, Try_1, Phe_2, Glu_1.

The hormone is a single chain polypeptide with neither disulfide nor other intrachain bonds; however, optical rotatory dispersion studies showed that it possesses some areas of ordered three-dimensional structure. This has been attributed to noncovalent interactions (34). Since a large percentage of the functional groups of the protein molecule were shown readily accessible to solvent, this showed that large areas of the molecule do not have very much of a three-dimensional ordered structure. This contradictory result to the optical rotatory dispersion studies was explained as due to the effect of denaturation of the native hormone induced during the solvation of the molecule into concentrated urea (34).

Chemical and enzymatic degradation of the molecule and the amino acid analysis of the degraded products have given more information about the amino acid sequence within the chain. Repeated incubations of the polypeptide with carboxypeptidase and leucine aminopeptidase released amino acids from each terminal of the chain: 10 to 15 amino acids at the ends of the molecule. Alanine appeared to be the N-terminal and glutamine proved to be the N-carboxy terminal amino acid (31,34).

Serial analysis (34) showed the two peripheral portions of the molecule presumably have the following amino acid arrangement:

(1) NH_2 terminus:

H-Ala-Ser (Glu,Gly,Leu,Lys,His,Ile,Met,Phe)

(2) COOH-terminus:

(tyr,Gly,Arg) Try-Lys-His-Leu-Met-Glu-Ser-Phe-Ala-Val-Leu-Gln-COOH

Absolute sequences of the first twelve amino acids at the carboxyl-terminus and the first three at the amino terminus have been determined.

Use of tryptic digestion, cyanogen bromide reaction with methionine, peptic and chymotryptic cleavage as well as dilute hydrochloric acid hydrolysis broke down the polypeptide into various fragments at various positions and enabled Aurbach (34) to propose a working model structure. It is shown on figure-1.

Biological activity appeared associated with that portion of the native polypeptide which contained the fragment of the last portion of the molecule toward the COOH-terminus, with a composition of 20 amino acids. In this portion of the molecule are found amino acid residues essential to the biological activity of the hormone, namely, one of the methionine residues, the tyrosine residue and tryptophane. The last four amino acids can be removed without too much loss of its activity (34).

(tyr,lys) (Glu,Leu,Val,Arg) (Lys-Lys)-Gly-Try-His-Ile-Met-Glu-Ser-Phe-Ala-Val-Leu-Gln-COOH

The incorporation of a non-metabolizable amino acid analog alpha-aminoisobutyric acid into parathyroid gland tissue protein has been demonstrated by Raisz and O'Brian (36). This indicated that in vitro studies of parathyroid hormone biosynthesis could

NH_2Ala-Val-Ser(Glu,Glu,Phe,Ileu,Gly)Asp(Lys,His,His,
Ser,Leu,Leu,Met,Lys)(Glu,Glu,Pro,Pro,Ala,Ala,Lys,Lys,
Lys)(Glu,Glu,Leu,Asp,Ser,Gly,Glu,Val,Val,Asp,His,Lys,
Lys)(Ser,Arg,Gly,Arg,Arg,Asp,Ser,Glu,Pro,Arg,)(Asp,
Ala,Ala,Glu,Gly,Lys,Ser,Asp,Val,Val,Ileu,Leu,Leu,Leu,
Asp,Tyr,Lys)(Glu,Leu,Val,Arg)(Lys-Lys)Gly-Try-His-
Ileu-Met-Glu-Ser-Phe-Ala-Val-Leu-Gln-COOH

FIGURE 1

MODEL STRUCTURE OF THE PARATHYROID HORMONE PROTEIN MOLECULE

probably be achieved in the laboratory.

Polypeptide hormones have provided very attractive model systems for the study of in vitro protein biosynthesis in animal tissues. A considerable amount of work has been done with insulin in this connection. In vitro incorporation of ^{3}H-labeled amino acids into insulin in rat pancreas has been studied (7,25). Substantial uptake of tritiated leucine was found in the acidic ethanol-soluble portion of the pancreatic protein. The incorporation was specific; that is, when rat pancreas fragments were incubated with labeled isoleucine, there was little radioactivity in the isolated B-chain of insulin. The protein synthesis appeared to be energy-dependent. Another investigation (49) showed that a concentration of glucose in the incubation medium was important in achieving a rapid rate of incorporation. Wagle (50) used rat pancreas slices and demonstrated the incorporation of ^{14}C-labeled amino acids into insulin. The newly synthesized insulin was assayed quantitatively by a technique using insulin anti-serum; the resulting activity obtained was equivalent to 300 microunits of standard insulin activity. 2,4-dinitrophenol inhibition of the incorporation of labeled amino acids into insulin has been observed (7,25,49). Bauer and Lazarow (7) employing goosefish islet tissue were able to demonstrate incorporation of ^{14}C-leucine, ^{14}C-Valine, and ^{3}H-leucine in vitro into insulin. The rate of incorporation increased progressively with an increase in incubation time, and decreased in absense of

oxygen.

Sachs (45) has demonstrated the incorporation of ^{35}S-Cysteine into vasopressin by direct infusion of the labeled amino acid into the third ventricle of the dog. The isolated labeled vasopressin from the hypothalamus was associated with neurosecretory particle fraction. In contrast with the infusion of ^{3}H-Leucine, a high specific activity protein fraction was found in the microsomal fraction. Incorporation of ^{35}S-Cysteine and ^{3}H-Tyrosine into vasopressin and tissue proteins has also been observed under proper conditions with intact hypothalamus-neurohypophyseal apparatus in guinea-pigs. The incorporation was time dependent and energy requiring; biosynthesis was inhibited by puromycin (46). In vitro as well as in vivo synthesis of vasopressin has also been observed in hypothalamic infundibular stem and process (46).

Recently an investigation of the in vitro biosynthesis of adrenocorticotropin was reported (1). Upon incubation in Krebs-Ringer bicarbonate buffer, slices of bovine anterior pituitary glands were found to incorporate ^{14}C-labeled amino acids efficiently into adrenocorticotropic hormone and tissue proteins. The isolated hormone was identified by electrophoresis, chromatography, and biological assay, as well as by amino acid analysis of chymotryptic fragments. The biosynthesis was strongly suppressed by: dinitrophenol, cyanide, azide and puromycin. A report (52) on adrenocorticotropin biosynthesis using rat anterior

pituitary tissue indicated that the rate of biosynthesis was linear for the first four hours and was enhanced by adrenalectomy.

An investigation of the in vitro biosynthesis of luteinizing hormone was published in 1965 (51). Hormone protein was obtained by incubating ^{14}C-leucine with isolated pituitary glands of rats, rabbits, and sheep, and was precipitated out using an anti-ovine LH-antiserum. The incorporation of the radioactivity into the hormone was time dependent, and was markedly reduced when the anti-LH-antiserum had been previously absorbed with luteinizing hormone. Castration and immunization of the animals with luteinizing hormone increased the incorporation strongly. Puromycin showed an inhibiting effect and the radioactivity in the protein matched with leucine fraction exclusively on chromatogram.

STATEMENT OF THE PROBLEM

At the present time, no work has yet been published regarding the in vitro biosynthesis of parathyroid hormone. It appeared to us worthwhile to apply approaches used in similar studies with other polypeptides to this problem. The main objective of the present work was the determination of optimum conditions for amino acid incorporation into protein with hypercalcemic properties in slices of bovine parathyroid tissue.

CHAPTER II

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

Frozen bovine parathyroid glands were obtained from the Wilson Laboratories, the Pharmaceutical Division of Wilson Company, Chicago, Illinois. These were dissected free from fat, connective tissue and other adhering structures, then washed with cold Krebs-Ringer bicarbonate buffer (pH 7.4) and cut with a Stadie-Riggs plastic tissue slicer into slices approximately 0.5 to 1.0 mm in thickness. The slices were then transferred into a series of 25 ml Erlenmeyer flasks equipped with disposable center wells in which filter paper strips soaked with hyamine hydroxide were placed to absorb the $^{14}CO_2$ evolved during the incubation process. The slices were incubated in 5.0 ml of Krebs-Ringer buffer with glucose added. Uniformly labeled ^{14}C-L leucine and ^{14}C-labeled algal protein hydrolysate were used as tracers. The incubations were carried out at pH 7.4 in a constant temperature shaker water-bath at 37°C. All incubations were terminated by an addition of one ml of 50% tricholoroacetic acid to each flask.

Tissue slices, after incubation, were separated from the trichloroacetic acid precipitate, and washed with 5% trichloroacetic acid. The washed slices were further cut and extracted in 90% aqueous phenol solution with stirring at room temperature for three to four hours. To the extracts were added five volumes of a solution of 20% acetic acid in acetone. After the addition of

1 M NaCl solution (4ml/liter), the mixture was allowed to stand in cold for one hour. The mixture was filtered and the protein was precipitated from the filtrate by the addition of an equal volume of ether. After standing in a refrigerator overnight, most of the supernantant was decanted and the precipitate was collected by centrifugation, washed twice with ether, and dried.

The tricholoroacetic acid precipitated material from the incubation mixture was washed with 5% trichloroacetic acid, chloroform and then 5% trichloroacetic acid with non-radioactive amino acids added, and then freeze-dried. This dried material and the ether precipitate were combined and stored in a freezer until used.

Portions of the stored protein were extracted with weak acetic acid or water acidified with diluted hydrochloric acid, and then centrifuged. The supernantant was analyzed for radioactivity and protein content. Biological activity of the acid soluable material was measured by determining the hypercalcemic response to the injection of the test material in thyroparathyroidectomized rats. Injection Parathyroid (Lilly) was used as the standard for the bioassay. Radioactivity was measured by liquid scintillation counting (Packard Tri-Carb Liquid Scintillation Spectrometer Model 314X) and total protein was estimated by biuret method.

CHEMICALS

Acetic acid, glacial, Mallinckrodt Chemical Works, #2504

Amino acids mixture, ^{14}C-labeled, New England Nuclear Corp. NEC-445, #272-81-82a, Batch 25, Specific activity=40 mc/m-atom of carbon

Amino acids mixture, ^{14}C-labeled, Tracerlab, C-425, #969-27-1A Specific activity=1.5 mc/mg

1,4-bis-2(5-phenyloxazolyl)-benzene, Packard Instrument Company, Inc., #2306, C-6002030, Fluor Max. 4200 A

Calcein indicator, Fisher Scientific Co. #752833

Calcium carbonate, Mallinckrodt Chemical Works, #4071

Calcium chloride, Mallinckrodt Chemical Works, #4160

Chloroform, Mallinckrodt Chemical Works

Copper sulfate, J.T. Baker Chemical Co.

Dextrose anhydrous, Mallinckrodt Chemical Works, #4912

p-Dioxane, Matheson Coleman & Bell Lab. DX-2095, SG-2347

2,5-Diphenyloxazole, Packard Instrument Company Inc. #2616, C-6002022, Fluor Max. 3800 A

Disodium ethylenediamine tetraacetate, Fisher Scientific Co. S-311

Ether, J.T. Baker Chemical Co.

Ethylene glycol, Matheson Coleman & Bell Lab. #5087

Filter paper, Whatman #1

Glycerin, Mallinckrodt Chemical Works, #5092

Hyamine hydroxide, Nuclear Chicago Corp. #65

Hydrochloric acid, J. T. Baker Chemical Co.

L-leucine, Calbiochem. #4320

^{14}C-U-L-leucine, Tracerlab, C-432, #985-371-5, Specific activity= 220 mc/mM

^{14}C-U-L-leucine, Volk Radiochemical Co. CAA-51, SG-5812, Specific activity=230 mc/mM (1.75 mc/mg)

Magnesium chloride, Mallinckrodt Chemical Works

Magnesium sulfate, Mallinckrodt Chemical Works, #6066

Methanol, absolute, J.T. Baker Chemical Co.

Naphthalene, Matheson Coleman & Bell Lab. #2616

Nembutal sodium, Abbott Lab. #722-1394, List No. 3117

95% 02-5% CO_2, Puritan Compressed Gas Corp. #04J21X

Parathyroid extract, Injection Parathyroid, Eli Lilly Co. 100 U.S.P. units/ml

Phenol, Mallinckrodt Chemical Works, #0028

Potassium chloride, Mallinckrodt Chemical Works, #6858

Potassium phosphate, Mallinckrodt Chemical Works, #7100

Potassium sodium tartrate, Mallinckrodt Chemical Works, #2367

Protein solution, standard, Armour Pharmaceutical Co. #V2106 Assayed Potency=10 mg protein nitrogen/ml

Sodium bicarbonate, Mallinckrodt Chemical Works, #7412

Sodium chloride, J.T. Baker Chemical Co. #3624

Sodium cyanide, J.T. Baker Chemical Co. #1144

Sodium hydroxide, Mallinckrodt Chemical Works, #7708

Sodium hydroxide, 50% solution, J.T. Baker Chemical Co. #3727

Sodium sulfite, Allied Chemical Corp. #2301

Sulfuric acid, J.T. Baker Chemical Co. #9681

Toluene, J.T. Baker Chemical Co.

Trichloroacetic acid, Mallinckrodt Chemical Works, #2924

Yeast protein hydrolysate, Nutritional Biochemicals Corp. #4266

PREPARATION OF INCUBATION MEDIA

Krebs-Ringer bicarbonate buffer or its modifications served as the medium for the incubations. Normal composition of Krebs-Ringer bicarbonate buffer is as follows:

100 parts	0.90% (0.154 M)	NaCl solution
4 parts	1.15% (0.154 M)	KCl solution
3 parts	1.22% (0.110 M)	$CaCl_2$ solution
1 part	2.11% (0.154 M)	KH_2PO_4 solution
1 part	3.82% (0.154 M)	$MgSO_4 \cdot 7H_2O$ solution
21 parts	1.30% (0.154 M)	$NaHCO_3$ solution

Two and one half grams of glucose were added to each liter of buffer. 95% oxygen-5% carbon dioxide gas mixture was bubbled into the medium for fifteen minutes and the pH was adjusted to 7.4 before the medium was used for the incubation.

Modifications of the above mentioned Krebs-Ringer bicarbonate buffer were prepared to serve as a low calcium medium by reducing the calcium ion concentration to one third of its original concentration in the normal buffer solution. The difference in ionic strength from the original buffer was made up without change in total volume and concentration of other components, by the addition of either two parts of 0.22 M (1.29%) NaCl solution

or two parts of 0.11 M (1.05%) $MgCl_2$ solution. The final compositions of the modified buffer solution were as follows:

Modified low-calcium medium A:

100 parts	0.90% (0.154 M)	NaCl solution
21 parts	1.30% (0.154 M)	$NaHCO_3$ solution
4 parts	1.15% (0.154 M)	KCl solution
1 part	2.11% (0.154 M)	KH_2PO_4 solution
1 part	3.82% (0.154 M)	$MgSO_4 \cdot 7H_2O$ solution
1 part	1.22% (0.110 M)	$CaCl_2$ solution
2 parts	1.29% (0.220 M)	NaCl solution

Modified low-calcium medium B:

100 parts	0.90% (0.154 M)	NaCl solution
21 parts	1.30% (0.154 M)	$NaHCO_3$ solution
4 parts	1.15% (0.154 M)	KCl solution
1 part	2.11% (0.154 M)	KH_2PO_4 solution
1 part	3.82% (0.154 M)	$MgSO_4 \cdot 7H_2O$ solution
1 part	1.22% (0.110 M)	$CaCl_2$ solution
2 parts	1.05% (0.110 M)	$MgCl_2$ solution

TISSUE PREPARATION AND INCUBATION

Frozen bovine parathyroid glands obtained from The Wilson Laboratories, Chicago, Illinois, were dissected free from fat, connective and other adhering structures, then washed with cold Krebs-Ringer bicarbonate buffer (pH 7.4) and sliced in the cold into slices approximately 0.5 to 1.0 mm in thickness with a razor blade or a Stadie-Riggs plastic tissue slicer. The slices

were weighed and then transferred into a series of 25 ml Erlenmeyer flasks each equipped with a plastic disposable center well. In each flask was placed 5 mls of the desired incubation medium which had been previously warmed in the bath to the desired incubation temperature. The desired amount of radioactively labeled amino acids mixture was also added to the medium prior to the addition of the tissue slices into the incubation system. A small piece of folded Whatman No. 1 filter paper saturated with hydroxide of hyamine was placed in the center well to absorb the radioactive carbon dioxide evolved during the entire incubation period. The flasks were sealed with rubber stoppers. In the incubations conducted in oxygen, an extra set-up was added to the flasks. This included two hypodermic 22 gauge needles inserted through the rubber stopper into the flask. One of the needles served as the inlet for the gas mixture, and the other as the outlet from the flask. After flushing the flask with 95% oxygen-5% carbon dioxide mixture for four to five minutes, both needles were pulled out, and the flask re-sealed.

All incubations were carried out in a temperature controlled shaker water-bath. The incubation temperature was kept at 37° ±1° centigrade. The duration of the incubation time varied from one hour to eight hours. The amount of tissue slice in each flask ranged from one hundred milligrams to two grams of total dry weight. In the various incubations one to five microcuries of radioactively labeled amino acid mixture or ^{14}C-labeled L-leucine

was placed in each incubation flask. After the desired incubation time period had been reached, the process was terminated by the addition of one ml of 50% trichloroacetic acid injected into each flask through a 5 c.c. hypodermic syringe. The trichloroacetic acid denatured the tissue slices and precipitated the protein present in the medium. Fifteen minutes were allowed for the diffusion of the labeled carbon dioxide gas and the equilibration of the system; then, the flasks were opened. The center well was taken out; and the content in the flask was subjected to the further isolation and extraction.

EXTRACTION AND ISOLATION

Ninety percent aqueous phenol solution was used in this study for the extraction of the biologically active material. Slices of tissue of parathyroid glands, after incubation with radioactively labeled amino acids, were removed from the incubation, and then washed five times with 5% trichloroacetic acid. In an attempt to free the tissue as completely as possible from contamination of free radioactive amino acids, the slices were subjected to two further washings with 5% trichloroacetic acid containing five grams of yeast protein hydrolysate amino acid mixture per 100 ml, followed by centrifugation. The tissue was then blotted with filter paper, washed with chloroform, and cut with scissors into smaller pieces. Ten milliliters of 90% (w/V) aqueous phenol solution were used for extraction of each gram of incubated tissue. The extraction was carried out at room temper-

ature for three to four hours with occasional stirring.

The mixture was centrifuged after the completion of the extraction. Five volumes of a solution of 20% acetic acid in acetone was added to the supernantant, and the residue was subjected to re-extraction for another 30 minutes. After the addition of 1 M sodium chloride solution to the combined extract (0.4 ml to each 100 ml extract), the mixture was allowed to stand in a refrigerator for one hour. The mixture was then filtered and an equal volume of ether was added to the filtrate. The mixture was then allowed to stand in a refrigerator overnight. The precipitate was collected by centrifugation in a clinical centrifuge. After several washings with ether, the precipitate was dried and stored in cold.

The trichloroacetic acid precipitated material from the incubation medium was centrifuged. It was then washed three times with 5% trichloroacetic acid and four times with 5% trichloroacetic acid containing yeast hydrolysate nonradioactive amino acids mixture. After a shaking with chloroform, the protein material was then combined with the above obtained phenol extract, and stored in cold for further use.

These protein materials were further treated with either weak acetic acid or water acidified with diluted hydrochloric acid. Weak acetic acid contained 0.5% w/V glacial acetic acid in water with a pH value around 3; and the acidified water contained of 1% 0.5 N hydrochloric acid. After the extraction with above

mentioned solvents, the insoluble portion was centrifuged down, and the supernantant was collected. Radioactivity of all fractions and the total protein content of the supernantant were analyzed. The acidic aqueous soluble supernantant was stored in vials in a refrigerator for the injection into animals to determine its biological activity.

MEASUREMENT OF RADIOACTIVITY

A Packard Tri-Carb Model-314X liquid scintillation spectrometer was used for all the radioactivity determinations. Samples were counted in twenty-two milliliter low potassium-40 content glass vials with tin-foil lined plastic screw caps. In each vial ten milliliters of the desired scintillation fluid was employed.

Samples assayed included: (1) aqueous protein solutions; (2) protein solutions in hydroxide of hyamine; (3) 5% trichloroacetic acid washings; and (4) filter paper strips containing the absorbed radioactive carbon dioxide. Variations in the characteristics of the different samples required special scintillation fluids for the counting of particular sample preparations. Three different preparations of scintillation fluid were used. These scintillation fluids had the following compositions:

(1) For paper strips with radioactive CO_2:

4 grams of PPO

50 milligrams of POPOP

dissolved in one liter of toluene.

(2) For protein hyamine hydroxide solutions:

4 grams of	PPO
100 milligrams	POPOP

dissolved in one liter of toluene.

(3) For all solutions containing water:

4 grams of	PPO
200 milligrams	POPOP
60 grams	Naphthalene
100 milliliters	Absolute methanol
20 milliliters	Ethylene glycol

dissolved in one liter of p-dioxane.

The instrument was set at the optimal counting HV tap (1000 v) for carbon-14, with discriminator settings of 10-50-100 volts. Relative counting efficiencies for the various sample preparations were as follows: aqueous protein solutions in dioxane, 50-55%; protein in hyamine hydroxide solution in toluene 41-47%; labeled paper strips 27-31%; and trichloroacetic acid washings in dioxane, 51-57%. Tissue after extraction, then washed with chloroform and dried was counted in toluene; the highest efficiency obtained was 19%, and sometimes as low as only 9%.

All samples were measured three times, each for a period of ten minutes. For all the solutions, 100 ul aliquot was used as the sample volume. Solid protein samples were counted with a dry

weight of 100 mg of protein. Tissue was counted as the tissue in each vial; however, all values were converted into the values based on 500 mg of slices incubated originally. Counting efficiencies were determined by means of prepared standards containing known amounts of radioactivity.

DETERMINATION OF BIOLOGICAL ACTIVITY

Male Sprague-Dawley rats weighing from 80 to 100 grams, obtained from Abrams Small Stock Breeders, Chicago, Illinois, were kept in separate cages and fed with minimum amount of Purina rat chow for one or two days before use. Then a total thyroparathyroidectomy was performed on each rat. Three blood samples were taken for serum calcium analysis. The first one was taken immediately prior to the thyroparathyroidectomy. The second blood sample was obtained twenty-four hours after the surgical operation. Test material was then subcutaneously injected in two doses, each 1 ml in volume. All animals were fasted after the injection, but were given water ad libitum. Twenty-four hours after the first injection, the third blood sample was obtained. All serum samples were titrated for their calcium content. Control animals were injected with distilled water slightly acidified with dilute hydrochloric acid or weak acetic acid. Eli Lilly Injection Parathyroid, 100 units U.S.P. was used as the standard. Extracted material from glands without incubation was also tested for endogenous hormonal activity.

THYROPARATHYROIDECTOMY

Rats were anesthetized with Nembutal injected intraperitoneally at a dose of 4 mg per 100 grams of body weight. This dosage will effectively maintain the animal under a proper level of anesthesia for approximately an hour.

The anesthetized animal was placed on an operating board with a magnifying glass mounted in a suitable position. The animal was secured on the board with adhesive tape over its limbs. The neck of the animal was held slightly taut by means of a rubber band placed about the upper incisor teeth. The other end of the rubber band was fastened to a pair of nails imbedded in the board, which were about one inch above the head and one inch apart from each other.

A mid-line incision was made on the neck about one inch long from the cephalic boarder of the sternum to the mandible. Several subcutaneous layers were separated along the mid-line with a pair of forceps until the musculature underneath was exposed. Special care was taken to avoid damaging the submaxially salivary glands and injury to the blood vessels in the area.

Once the muscle layers were exposed, they were separated also with forceps. These muscle flaps were retained by forceps. Parathyroids may be observed as lighter colored spots at the upper edges of the thyroids; however, this identification was usually impossible. Both lobes of the thyroparathyroid apparatus lying beside the trachea were removed by teasing with a pair of forceps.

After removing both glands, a cotton swab was inserted to stop bleeding. Then the wound was closed with wound clips. Animals were returned to their cages for recovery. A check-up after a few hours was necessary to observe if any abnormality of breathing was developing. If so, the animal was discarded. The entire surgical procedure usually required ten to fifteen minutes.

BLOOD SAMPLING

Blood samples were obtained from the rats by tail bleeding. The blood was allowed to stand for about thirty minutes to one hour at room temperature for clotting and retraction. It was then centrifuged from three to five minutes in a clinical centrifuge or a Beckman-Spinco microcentrifuge. Serum supernantant was isolated and stored in refrigeration or for immediate analysis.

DETERMINATION OF TOTAL PROTEIN CONTENT

The biuret method was used for the determination of total protein. The reagent produces a blue-purple copper complex with protein or polypeptide in solution. The color production is proportional to the protein concentration in the solution.

Bovine albumin containing an assayed potency of 10 mg of protein per ml was used as the standard. Dilutions with distilled water were made to provide standards containing 1, 2, 3, 4, and 5 mg of protein.

The biuret reagent was made by dissolving 0.15 g of copper

sulfate and 0.60 g of sodium potassium tartrate into 50 ml of distilled water, then adding with stirring, 30 ml of 2.5 N carbonate-free sodium hydroxide. This was diluted to 100.0 ml.

Twenty-eight grams of anhydrous sodium sulfite dissolved to 100 ml of distilled water provided the other necessary reagent.

The Beckman Ultramicro Analytical System was used for these protein measurements. The following chart shows the preparation of the blank, sample, and the standard tubes.

Reagent	Blank	Standard	Protein
Distilled water	5 ul		
Protein standard		5 ul	
Sample			5 ul
Biuret reagent	200 ul	200 ul	200 ul
Sodium sulfite	50 ul	50 ul	50 ul

Thirty minutes after the addition of the biuret reagent, all tubes were read at 540 mu on a Beckman Spinco colorimeter, with the blank tubes set at zero absorbance.

The total protein content of the samples were calculated from the readings of the standard, of the sample, of the protein concentration of the standard using the equation:

$$\text{Protein Concentration In Sample} = \frac{\text{Absorbance for sample}}{\text{Absorbance for standard}} \times \text{Protein Concentration in Standard}$$

Figure 2 indicates the linear relationship between absorbance at 540 mu and the concentration of total protein. The data for the preparation of this standard curve are given in Table 1.

DETERMINATION OF SERUM CALCIUM

The method used in this study for determining ionizable calcium concentration in serum was reported by Ashby and Roberts (2).

The method is based upon the fact that when the pH is above 12, an iminoacetate derivative of fluorescein named calcein fluoresces under long-wave ultraviolet light only in the presence of ionized calcium. A given amount of EDTA is added to the sample which provides sufficient excess amount of EDTA to complex all the calcium and magnesium presented in the sample. Any iron or copper which would interfere is complexed by the addition of a small amount of cyanide. The mixture is back-titrated with a standard calcium solution, complexing all the excess and replacing all the EDTA in EDTA-Mg complex. Any excess of free calcium in the system will react with calcein and show a bright yellowish green color and fluoresce. The concentration of calcium in the sample can be calculated from concentrations of the standard calcium solution and EDTA, which is proportional to the difference in microliters of standard calcium solution required to titrate the sample, and a water blank. If standard A was run with samples then:

TABLE I

STANDARD CURVE FOR TOTAL PROTEIN CONTENT

PROTEIN CONCENTRATION (mg/ml)	NUMBER OF OBSERVATIONS	ABSORBANCE AT 540 mu
1	12	0.026 ± .008
2	12	0.049 ± .006
3	12	0.077 ± .003
4	12	0.102 ± .009
5	12	0.127 ± .008
10	12	0.233 ± .011

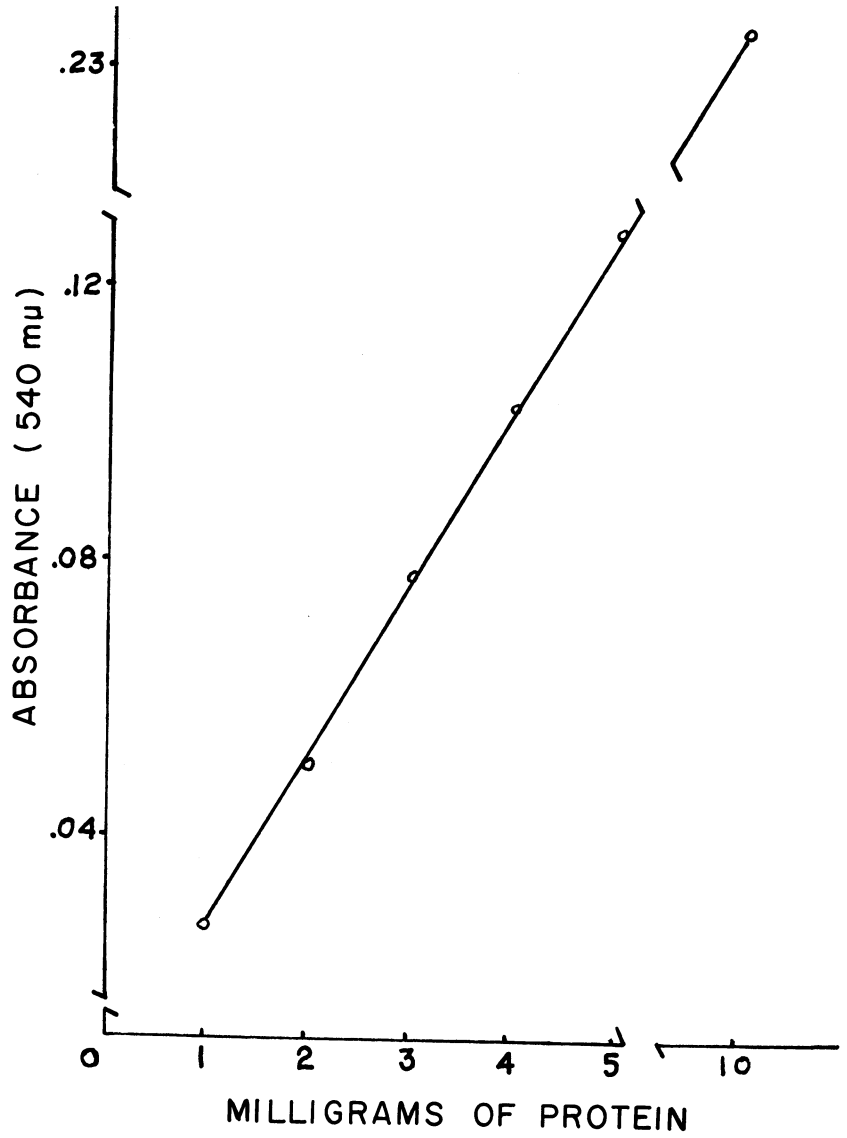

FIGURE 2

STANDARD CURVE FOR TOTAL PROTEIN DETERMINATION

$$\text{mgs\% calcium concentration in sample} = \text{calcium concentration in standard} \times \frac{\text{uls of titrant required to titrate blank - uls of titrant to titrate sample}}{\text{uls of titrant required to titrate blank - uls of titrant to titrate standard A}}$$

A linear relationship between the titrant used and the calcium concentration in sample is shown in figure-3 and the pertinent data appear in Table II.

Procedure: 30 uls of the serum sample was added to a micro titration cup. To each cup was added 150 ul of EDTA, one drop of diluted calcein indicator, one drop of 1% NaCN and one drop of 1.0 N NaOH. Titration was performed with a Beckman microtitrator containing 20.0 mg% standard calcium solution as titrant. A long-wave ultraviolet lamp (Mineralight Model SL-3660) was placed about two inches above the titration cup. The standard calcium solution was added with constant stirring until the green fluorescence which was observed no longer increases in intensity.

Reagents:

Calcein solution concentrated: Dissolved 0.25 g of powdered indicator in 4.0 ml of 1.0 N NaOH. When solution was complete, it was diluted to 100.0 ml with glass distilled water.

Calcein indicator solution: 0.5 ml of the concentrated solution of calcein was diluted to 25.0 ml with glass distilled water.

Ethylenediamine tetraacetatic acid standard, 0.002 M: 0.750 g of EDTA was dissolved in 1000 ml of CO_2-free glass redistilled water.

Calcium standard solution: 0.2497 g of oven-dried calcium carbonate was dissolved in 30 ml of 2N HCl and diluted to 500.0 ml with

TABLE II

CALCIUM STANDARD CURVE

ACTUAL CALCIUM (mg%)	NUMBER OF SAMPLES	MICROLITERS (Titrant)	MICROLITERS (Titrant - 0 mg%)
0	9	30.1 ± 0.3	
6	9	20.6 ± 0.2	9.5
8	9	17.4 ± 0.5	12.7
10	9	14.5 ± 0.3	15.6
12	9	11.4 ± 0.4	18.7
14	9	8.1 ± 0.3	22.0
16	9	5.2 ± 0.4	24.9

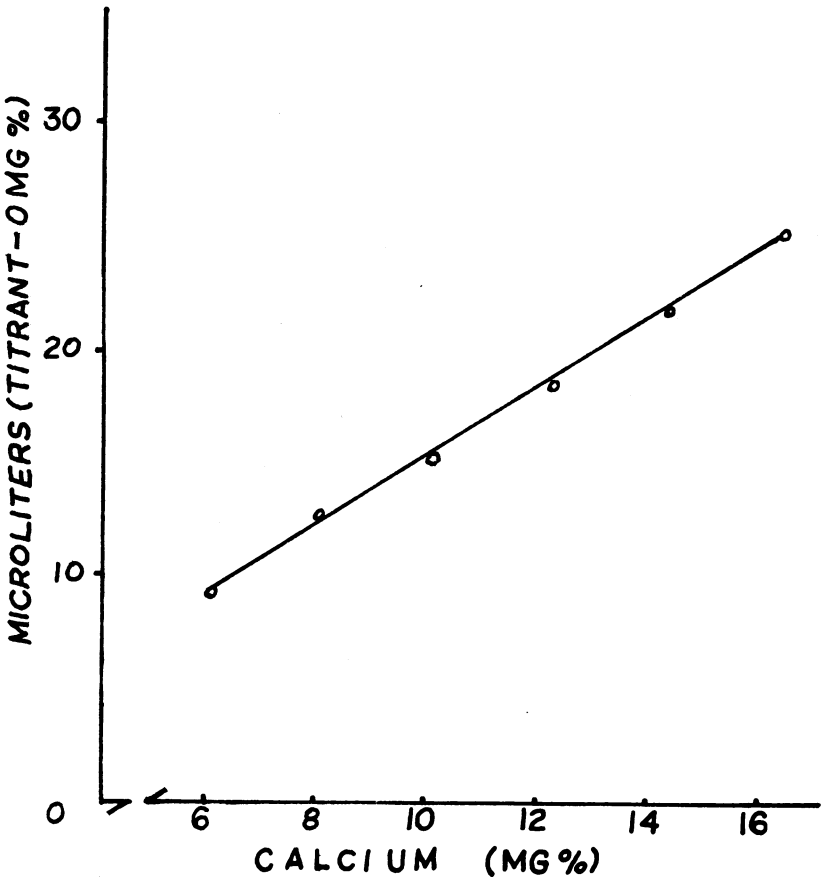

FIGURE 3

STANDARD CURVE FOR CALCIUM DETERMINATION

glass re-distilled water. This gives a standard calcium solution of 20 mg%; working solutions of different concentrations were prepared from this stock solution by dilution.

CHAPTER III

EXPERIMENTAL RESULTS

The experimental portion of this work comprise two parts. These include the incubation studies and the bioassay. In the incubation studies, the incorporation of the radioactive amino acids into biologically active protein or into other structural protein fraction was of paramount concern. Here one was interested in finding out the optimal conditions under which the maximal amount of amino acids would be incorporated into various protein fractions. Attempts were made to improve the protein biosynthesis by varying the length of the incubation time, by changing the quantities of tissue slices incubated with a given amount of radioactive amino acids or by incubating different amount of radioactive amino acids with a fixed amount of tissue. The calcium ion concentration in the medium is important which affects parathyroid hormone release and synthesis. Raisz and O'Brain (36) demonstrated the incorporation of a-aminoisobutyric acid into parathyroid gland tissue was higher in a medium of low calcium content than it was in a medium of high calcium content. Thus, the incorporation of labeled amino acids into protein was studied by varying the calcium ion concentration of the incubation medium. Incubations in oxygen or in air, and with glucose or without glucose were also carried out to study the effect of these parameters.

Carbon-14 labeled leucine and carbon-14 labeled mixture of

algal protein hydrolysate were the radioactive tracers used in this study. The degree of the incorporation was measured by the radioactivity shown in the various fractions of protein isolated. For purposes of comparison, the total protein content and its associated radioactivity are usually reported in terms of 500 mg of tissue incubation.

The second phase of the work comprised chiefly a series of tests for the biological activity of the protein synthesized.

The data presented in this chapter are the summaries of the averaged values of all the results in all the experiments performed.

Effects of the Length of the Incubation Time, and the Calcium Concentration in the Medium in the Radioactive Amino Acids Incorporation into Various Proteins in Frozen Bovine Parathyroid Tissue Slices

Five hundred milligrams of the tissue slices were incubated with two microcuries of thelabeled algal amino acid mixture. The incubation time varied from one, two, four, six to eight hours. Six separate experiments were conducted under the same conditions. In each experiment, two different kind of incubation media were used, which varied in their calcium ion concentration. One medium high in calcium content contained 2.5 mM of calcium while the low calcium medium contained only 0.85 mM of calcium.

Twenty flasks were incubated each time, ten flasks with each medium of different calcium content. All incubations were started

at the same time but terminated at different desired periods. All incubations were run at 37°C and the pH of the media was 7.4.

The effect of the length of time of incubation and of the calcium ion concentration of the medium on the incorporation of radioactive amino acids into the "acid soluble" protein fraction of the tissue are given in Table III. The results show that the incorporation was rapid in both media during the first four hours and then it gradually leveled off during the subsequent two to four hours. The effect of varying the calcium ion concentration was not great, but the difference in incorporation was significant at four hours of incubation, not so much at later hours.

From Table IV, it can be seen that there is also rapid incorporation of labeled amino acids into tissue protein fraction and that this incorporation is still increasing at about eight hours. No real difference in incorporation is observed between the media of different calcium content.

The data given in Table V show the incorporation of labeled amino acids into parathyroid gland tissue.

Effect of the Presence of Magnesium Ion in the Incubation Media on the Incorporation of Radioactive Amino Acids into Protein Fractions and Tissue in Bovine Parathyroid Glands

Three kinds of media were involved in these experiments. These were: (1) The low calcium medium containing 0.85 mM of calcium. (2) The low calcium medium containing in addition 3.4 mM of sodium ion. (3) The low calcium medium containing in addition

TABLE III

THE EFFECT OF INCUBATION TIME ON THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEINS IN PARATHYROID GLANDS AND THE EFFECT OF THE CALCIUM CONCENTRATION IN THE MEDIUM: I) ACIDIC SOLUBLE FRACTION

High calcium medium:

Incubation Time	Number of Incubations	Radioactivity (cpm/500 mg tissue)	Protein (mg/500 mg tissue)
1 hr.	12	200 ± 30	0.00 ± 0.00
2	12	280 ± 30	0.02 ± 0.01
4	12	1000 ± 110	0.07 ± 0.02
6	12	1200 ± 90	0.11 ± 0.02
8	12	1150 ± 200	0.12 ± 0.02

Low calcium medium:

Incubation Time	Number of Incubations	Radioactivity (cpm/500 mg tissue)	Protein (mg/500 mg tissue)
1 hr.	12	270 ± 40	0.04 ± 0.02
2	12	320 ± 30	0.04 ± 0.01
4	12	1350 ± 100	0.15 ± 0.07
6	12	1300 ± 150	0.21 ± 0.04
8	12	1300 ± 125	0.24 ± 0.07

TABLE IV

THE EFFECT OF INCUBATION TIME ON THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEINS IN PARATHYROID GLANDS AND THE EFFECT OF THE CALCIUM CONCENTRATION IN THE MEDIUM: II) TISSUE PROTEIN

High calcium medium:

Incubation Time	Number of Incubations	Radioactivity (cpm/500 mg tissue)	Protein (mg/500 mg tissue)
1 hr.	12	1800 ± 250	7.2 ± 0.9
2	12	2000 ± 600	9.0 ± 0.6
4	12	2500 ± 450	10.2 ± 1.5
6	12	3400 ± 650	9.5 ± 1.0
8	12	5000 ± 1200	11.1 ± 1.2

Low calcium medium:

Incubation Time	Number of Incubations	Radioactivity (cpm/500 mg tissue)	Protein (mg/500 mg tissue)
1 hr.	12	1700 ± 120	6.5 ± 1.0
2	12	2500 ± 150	6.7 ± 1.5
4	12	2250 ± 170	6.0 ± 0.5
6	12	4700 ± 700	12.5 ± 0.8
8	12	5500 ± 950	14.3 ± 1.2

TABLE V

THE EFFECT OF INCUBATION TIME ON THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEINS IN PARATHYROID GLANDS AND THE EFFECT OF THE CALCIUM CONCENTRATION IN THE MEDIUM: III) TISSUE RADIOACTIVITY

High Calcium medium:

Incubation Time	Number of Incubations	Radioactivity (cpm/500 mg tissue)
1 hr.	12	450 ± 90
2	12	600 ± 170
4	12	1000 ± 140
6	12	1700 ± 350
8	12	1400 ± 250

Low calcium medium:

Incubation Time	Number of Incubations	Radioactivity (cpm/500 mg tissue)
1 hr.	12	500 ± 150
2	12	900 ± 250
4	12	2000 ± 450
6	12	3500 ± 600
8	12	5000 ± 1000

1.7 mM magnesium ion. The incubations were carried out at 37°C and at pH 7.4 for four hours. Five hundred milligrams of the tissur slices were used. Three experiments were conducted; four flasks of each incubation medium were used in each experiment.

Additional magnesium ion in the medium showed a significant enhancement of both the incorporation of the amino acids in the tissue protein fraction (Table VI) and protein synthesis (Table VII). Slight increase by magnesium ion was demonstrated with acidic soluble fraction. Due to the fact that magnesium had an enhancing effect on the incorporation into total protein observed here, magnesium ion was added in all further incubations.

Effects of the Quantities of Either the Tissue or the Radioactive Tracers on the Incorporation of Radioactivity into Various Protein Fractions in Bovine Parathyroid Tissue

The effect of varying the amount of tissue on the incorporation of radioactivity into protein fractions was studied by incubating one hundred milligrams to two grams of bovine parathyroid gland tissue slices with two microcuries of the ^{14}C-amino acid mixture in 5 ml of the low calcium and magnesium containing buffer. Then possible effects of differences in radioactivity used was investigated by another series of incubations varying both ^{14}C-amino acid mixture and ^{14}C-L-leucine. From one microcurie to five microcuries were used for these incorporations into parathyroid proteins with 500 mg of tissue slices.

The data is Tables VIII and IX demonstrate that an increase

TABLE VI

THE EFFECT OF MAGNESIUM ION ON THE INCORPORATION OF AMINO ACIDS INTO PROTEIN FRACTIONS IN THE PARATHYROID GLAND TISSUE SLICES IN VITRO: I) INCORPORATION OF RADIOACTIVITY

Incubation Conditions	Number of Incubations	Fractions	Activity (cpm/500 mg tissue)
Low Ca^{++}	12	Acidic soluble	1200 ± 100
Low Ca^{++} + Na^{+}	12	Acidic soluble	1350 ± 150
Low Ca^{++} + Mg^{++}	12	Acidic soluble	1500 ± 100
Low Ca^{++}	12	Tissue protein	2500 ± 200
Low Ca^{++} + Na^{+}	12	Tissue protein	2250 ± 170
Low Ca^{++} + Mg	12	Tissue protein	3700 ± 400
Low Ca^{++}	12	Tissue	2400 ± 150
Low Ca^{++} + Na^{+}	12	Tissue	2000 ± 450
Low Ca^{++} + Mg^{++}	12	Tissue	3100 ± 400

TABLE VII

THE EFFECT OF MAGNESIUM ION ON THE INCORPORATION OF AMINO ACIDS INTO PROTEIN FRACTIONS IN THE PARATHYROID GLAND TISSUE SLICES IN VITRO: II) TOTAL PROTEIN CONTENT

Incubation Conditions	Number of Incubations	Fractions	Protein Content (mg/500 mg tissue)
Low Ca^{++}	12	Acidic soluble	0.12 ± 0.04
Low Ca^{++} + Na^{+}	12	Acidic soluble	0.15 ± 0.007
Low Ca^{++} + Mg^{++}	12	Acidic soluble	0.14 ± 0.04
Low Ca^{++}	12	Tissue protein	5.30 ± 0.6
Low Ca^{++} + Na^{+}	12	Tissue protein	6.00 ± 0.5
Low Ca^{++} + Mg^{++}	12	Tissue protein	9.70 ± 1.0

TABLE VIII

THE EFFECT OF THE CONTENT OF RADIOISOTOPE IN THE MEDIA ON THE INCORPORATION OF RADIOACTIVITY INTO PROTEIN IN PARATHYROID GLANDS: I) ^{14}C-AMINO ACID MIXTURE

Quantity of Isotope	Number of Incubations	Fraction	Activity (cpm/500 mg tissue)
1 uc	12	Acidic-soluble	1450 ± 150
2	12	Acidic-soluble	1500 ± 100
3	12	Acidic-soluble	2100 ± 300
4	12	Acidic-soluble	2000 ± 570
5	12	Acidic-soluble	2500 ± 400
1 uc	12	Tissue protein	2700 ± 200
2	12	Tissue protein	3700 ± 400
3	12	Tissue protein	7200 ± 800
4	12	Tissue protein	11700 ± 1500
5	12	Tissue protein	13000 ± 1500
1 uc	12	Tissue	2500 ± 120
2	12	Tissue	3100 ± 400
3	12	Tissue	4000 ± 500
4	12	Tissue	17000 ± 1800
5	12	Tissue	20000 ± 2400

TABLE IX

THE EFFECT OF THE CONTENT OF RADIOISOTOPE IN THE MEDIA ON THE INCORPORATION OF RADIOACTIVITY INTO PROTEIN IN PARATHYROID GLANDS: II) L-Leucine-^{14}C(U,L)

Quantity of Isotope	Number of Incubations	Fraction	Activity (cpm/500 mg tissue)
1 uc	8	Acidic-soluble	400 ± 150
2	8	Acidic-soluble	650 ± 100
3	8	Acidic-soluble	900 ± 120
4	8	Acidic-soluble	1000 ± 100
5	8	Acidic-soluble	1300 ± 140
1 uc	8	Tissue protein	1500 ± 100
2	8	Tissue protein	1200 ± 300
3	8	Tissue protein	1700 ± 200
4	8	Tissue protein	3000 ± 600
5	8	Tissue protein	4700 ± 450
1 uc	8	Tissue	700 ± 100
2	8	Tissue	800 ± 300
3	8	Tissue	1100 ± 130
4	8	Tissue	2000 ± 450
5	8	Tissue	4500 ± 400

TABLE X

THE EFFECT OF THE CONTENT OF THE RADIOISOTOPE IN THE MEDIA IN THE INCORPORATION OF AMINO ACID INTO PROTEIN IN PARATHYROID GLAND TISSUE: I) ^{14}C-AMINO ACID MIXTURE

Quantity of Isotope	Number of Incubations	Fraction	Protein Content (mgm/500 mg tissue)
1 uc	12	Acidic-soluble	0.14 ± 0.05
2	12	Acidic-soluble	0.14 ± 0.04
3	12	Acidic-soluble	0.10 ± 0.07
4	12	Acidic-soluble	0.07 ± 0.08
5	12	Acidic-soluble	0.12 ± 0.03
1 uc	12	Tissue protein	4.60 ± 0.9
2	12	Tissue protein	6.70 ± 1.0
3	12	Tissue protein	5.70 ± 1.2
4	12	Tissue protein	5.40 ± 0.9
5	12	Tissue protein	6.10 ± 1.1

TABLE XI

THE EFFECT OF THE CONTENT OF THE RADIOISOTOPE IN THE MEDIA IN THE INCORPORATION OF AMINO ACID INTO PROTEIN IN PARATHYROID GLAND TISSUE: II) L-Leucine-^{14}C(U,L)

Quantity of Isotope	Number of Incubations	Fraction	Protein Content (mgm/500 mg tissue)
1 uc	8	Acidic-soluble	0.10 ± 0.01
2	8	Acidic-soluble	0.08 ± 0.01
3	8	Acidic-soluble	0.10 ± 0.01
4	8	Acidic-soluble	0.14 ± 0.02
5	8	Acidic-soluble	0.10 ± 0.05
1 uc	8	Tissue protein	4.00 ± 0.5
2	8	Tissue protein	5.00 ± 0.5
3	8	Tissue protein	5.00 ± 0.7
4	8	Tissue protein	4.60 ± 1.0
5	8	Tissue protein	6.70 ± 1.0

in the quantity of isotope used in the incubation with a constant amount of tissue is accompanied by an increase in the incorporation of the total. This is noted when either a single labeled amino acid or a mixture of labeled amino acids is used. The incorporation is much higher after four hours of incubation time in the tissue and tissue protein fractions than in the acidic-soluble fraction.

The results shown in Tables X and XI show that the quantity of isotope has no effect on extent of protein synthesis observed in the acid soluble fraction with both labeled preparations. It appears, however, that a maximum of incorporation occurs with ^{14}C-amino acid mixture at a level of two microcuries (Table X).

The results of the incubation of two microcuries of ^{14}C-labeled amino acid mixture with varying amounts of bovine parathyroid tissue on the incorporation of radioactivity in protein fractions are shown in Table XII and on the protein content are shown in Table XIII.

The Effects of Glucose and of Air and Oxygen on the Incorporation of Radioactive Amino Acids into Parathyroid Tissue and Its Protein

For the purpose of finding out whether or not glucose in the medium or incubation with oxygen would have a stimulating effect on parathyroid protein biosynthesis or of amino acid incorporation into parathyroid tissue, another series of experiments were conducted. Three parameters were considered here: (1) incubation in oxygen with glucose added to the medium; (2) incubation in air

TABLE XII

THE EFFECT OF THE QUANTITY OF TISSUE INCUBATED ON THE INCORPORATION OF AMINO ACIDS INTO PROTEINS IN PARATHYROID GLAND TISSUE: I) RADIOACTIVITY INCORPORATION

Amount of Tissue	Number of Incubations	Fraction	Activity (cpm/100 mg tissue)
100 mg	8	Acidic-soluble	80 ± 20
500	8	Acidic-soluble	300 ± 70
1000	8	Acidic-soluble	450 ± 150
2000	8	Acidic-soluble	800 ± 250
100 mg	8	Tissue protein	200 ± 40
500	8	Tissue protein	750 ± 180
1000	8	Tissue protein	1200 ± 100
2000	8	Tissue protein	1100 ± 470
100 mg	8	Tissue	500 ± 170
500	8	Tissue	600 ± 400
1000	8	Tissue	400 ± 350
2000	8	Tissue	300 ± 340

TABLE XIII

THE EFFECT OF THE QUANTITY OF TISSUE INCUBATED ON THE INCORPORATION OF AMINO ACIDS INTO PROTEINS IN PARATHYROID GLAND TISSUE: II) TOTAL PROTEIN CONTENT

Amount of Tissue	Number of Incubations	Fraction	Protein Content (mgm/100 mg tissue)
100 mg	8	Acidic-soluble	0.00
500	8	Acidic-soluble	0.03 ± 0.01
1000	8	Acidic-soluble	0.10 ± 0.02
2000	8	Acidic-soluble	0.28 ± 0.10
100 mg	8	Tissue protein	0.90 ± 0.3
500	8	Tissue protein	2.10 ± 0.7
1000	8	Tissue protein	7.50 ± 1.4
2000	8	Tissue protein	19.00 ± 4.5

with glucose added to the medium; and (3) incubation in air with glucose absent from the medium.

Incubation with glucose in the medium and oxygen as the gas phase appeared to comprise an efficient combination for the incorporation of labeled ^{14}C-amino acid mixture into parathyroid gland tissue proteins during a two hour incubation period (Table XIV). When air was used as the atmosphere for the incubation, the presence or absence of glucose in the medium appeared not to have much effect on the incorporation of radioactivity.

The Determination of Biological Activity

The most important part of this investigation was trying to show that a biologically active substance could be synthesized in vitro by incorporating radioactive amino acids into parathyroid gland tissue. The bioassay method was based upon the calcium mobilizing ability of the parathyroid hormone.

Rats after parathyroidectomy or thyroparathyroidectomy show a decline of serum calcium level. Injection of parathyroid hormone or parathyroid extract would be expected to increase the serum calcium level to normal or above normal level.

The administration of the material was in the form of two doses injected subcutaneously twelve hours apart. Rats were thyroparathyroidectomized twenty-four hours prior to the first injection. Animals were fasted during this period. Control rats were injected with water acidified with hydrochloric acid or with weak acetic acid. No evidence of significant change in serum calcium

TABLE XIV

THE EFFECT OF OXYGEN AND GLUCOSE ON THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PARATHYROID GLAND TISSUE PROTEINS: I) RADIOACTIVITY INCORPORATION

Incubation Conditions	Number of Incubations	Fraction	Radioactivity (cpm/500 mg tissue)
O_2 + glucose	12	Acidic-soluble	1700 ± 250
Air + glucose	12	Acidic-soluble	1500 ± 100
Air - glucose	12	Acidic-soluble	1100 ± 150
O_2 + glucose	12	Tissue protein	5200 ± 500
Air + glucose	12	Tissue protein	3700 ± 400
Air - glucose	12	Tissue protein	2900 ± 700
O_2 + glucose	12	Tissue	3900 ± 1100
Air + glucose	12	Tissue	3100 ± 400
Air - glucose	12	Tissue	3400 ± 900

TABLE XV

THE EFFECT OF OXYGEN AND GLUCOSE ON THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PARATHYROID GLAND TISSUE PROTEINS: II) TOTAL PROTEIN CONTENT

Incubation Conditions	Number of Incubations	Fraction	Protein Content (mgm/500 mg tissue)
O_2 + glucose	12	Acidic-soluble	0.12 ± 0.07
Air + glucose	12	Acidic-soluble	0.14 ± 0.05
Air - glucose	12	Acidic-soluble	0.14 ± 0.02
O_2 + glucose	12	Tissue protein	7.00 ± 1.1
Air + glucose	12	Tissue protein	9.70 ± 1.0
Air - glucose	12	Tissue protein	8.10 ± 2.3

level was found upon injection of acidified water alone.

Injection Parathyroid (Eli Lilly) was used as the standard of reference for the estimation of biological activity. The biological activities of control preparations, reference standards and the phenol-extracted, acid soluble fraction isolated from bovine parathyroid gland tissue incubated with labeled amino acids are given in Tables XVI, XVII and XVIII. Control rats were injected with either o.5% acetic acid, water acidified with dilute HCl or 0.01 N HCl. In each case no significant change in serum calcium was observed with these preparations. The administration of 100 U.S.P. units of parathyroid extract resulted in an increase serum calcium level of 3.1 ± 0.6, 2.7 ± 1.0, and 3.5 ± 0.5% respectively in the three series of experiments. Subcutaneous injection of the vehicle present in the commercial extract resulted in no change in the serum calcium level in one series of experiments in which it was biologically tested.

Endogenous hormone was extracted from glandular tissue by the same technique applied to the isolation of radioactively labeled protein from the incubation medium. When this material was tested for hypercalcemic activity, an average serum calcium increase of two milligram percent was obtained at an average dose level of 2.0 mg of protein. Seventy-eight rats were injected with the radioactive phenol extracted, acid soluble protein isolated from the incubation media. When this material was tested, an average serum calcium increase of two milligrams percent was

TABLE XVI

BIOLOGICAL ACTIVITIES OF CONTROL PREPARATIONS, REFERENCE STANDARDS AND THE PHENOL EXTRACTED ACID SOLUBLE FRACTION ISOLATED FROM BOVINE PARATHYROID GLAND TISSUE INCUBATED WITH LABELED AMINO ACIDS: FIRST SERIES

Test	Number of Rats	Material Injected	Average Serum Calcium Change
Control	3	2 ml 0.5% acetic acid	- 0.2 mg%
Standard PTE* (Eli Lilly)	3	100 U.S.P. units	+ 3.1 ± 0.6
Endogenous hormone	3	0.8 mg protein in 2 ml control solution	+ 1.5 ± 0.5
Gland extract	9	0.36 mg protein in 2 ml control solution	+ 1.4 ± 0.4
Gland extract	12	0.56 mg protein in 2 ml control solution	+ 1.0 ± 0.5
Gland extract	11	0.56 mg protein in 2 ml control solution	+ 0.8 ± 0.2

*PTE: Parathyroid Extract

TABLE XVII

BIOLOGICAL ACTIVITIES OF CONTROL PREPARATIONS, REFERENCE STANDARDS AND THE PHENOL EXTRACTED ACID SOLUBLE FRACTION ISOLATED FROM BOVINE PARATHYROID GLAND TISSUE INCUBATED WITH LABELED AMINO ACIDS: SECOND SERIES

Test	Number of Rats	Material Injected	Average Serum Calcium Change
Control	3	2 ml water acidified with 0.5 N HCl	No change mg%
Standard PTE (Eli Lilly)	3	100 U.S.P. units	+ 2.7 ± 1.0
Endogenous hormone	3	1.2 mg protein in 2 ml control solution	+ 2.4 ± 0.5
Gland extract	7	0.8 mg protein in 2 ml control solution	+ 2.4 ± 0.7
Gland extract	9	0.9 mg protein in 2 ml control solution	+ 2.1 ± 0.5
Gland extract	11	0.8 mg protein in 2 ml control solution	+ 2.5 ± 0.5

TABLE XVIII

BIOLOGICAL ACTIVITIES OF CONTROL PREPARATIONS, REFERENCE STANDARDS AND THE PHENOL EXTRACTED ACID SOLUBLE FRACTION ISOLATED FROM BOVINE PARATHYROID GLAND TISSUE INCUBATED WITH LABELED AMINO ACIDS: THIRD SERIES

Test	Number of Rats	Material Injected	Average Serum Calcium Change
Control	3	2 ml 0.01 N HCl	No change mg%
Standard PTE (Eli Lilly)	3	100 U.S.P. units	+ 3.5 ± 0.5
Vehicle	3	2 ml water contained 1.6% glycerin 0.2% phenol	+ 0.1
Endogenous hormone	3	2.2 mg protein in 2 ml control solution	+ 2.1 ± 1.0
Gland extract	5	2.2 mg protein in 2 ml control solution	+ 3.0 ± 1.0
Gland extract	6	2.2 mg protein in 2 ml control solution	+ 3.4 ± 1.4
Gland extract	7	2.2 mg protein in 2 ml control solution	+ 4.1 ± 1.0

obtained at an average dose level of about one milligram of protein.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

From the information provided by previously reported studies on the in vitro biosynthesis of several polypeptide hormone molecules in the last decade (1,7,25,45,46,49,50,51,52), it appeared likely that the biosynthesis of the parathyroid hormone could be demonstrated in vitro. In the experiments described in the literaure, the general experimental approaches and procedures were essentially similar except for the especial techniques which involved the isolation and the purification of a particular hormonal protein.

Roth and Raisz (55) first showed the presence of radioactivity in trichloroacetic acid precipitated fraction obtained after parathyroid glands were cultured for forty-eight hours in a medium containing radioactive amino acids. This work presented evidences that biosynthesis of this hormone could be achieved in vitro in the laboratory by using the conventional techniques of protein biosynthesis.

In the studies presented here, attempts were made to obtain information on the conditions which would lead to parathyroid hormone biosynthesis by incubating amino acids with parathyroid glands tissue in vitro. Carbon-14 labeled amino acids were used as radioactive tracers for the detection of biologically active material which might be synthesized. Several

parameters pertinent to the incubation procedure were studied. These included: the length of the incubation time, the amount of tissue slices incubated, the concentrations of the radioactive amino acids present in the incubation media and the variations of ion concentrations including those of calcium, magnesium and sodium in the media employed. In addition, incubations were carried out in an atmosphere of ninety-five percent oxygen and five percent of carbon dioxide as were as in an air atmosphere. The influence of glucose concentration in the media on the incorporation of labeled amino acids was also studied.

Total protein, presumably including hormone protein and tissue protein other than hormone polypeptide, was precipitated out from the incubation mixture with addition of trichloroacetic acid. The precipitate was extracted with a ninety percent aqueous phenol solution reprecipitated with ether and then re-extracted with dilute acid.

Washing of the precipitate with five percent trichloroacetic acid several times to get rid of the contamination of the free radioactive amino acids was used instead of dialysis because in the first few experiments, dialysis against distilled water for twenty-four hours was suspected to be responsible for the nearly complete loss of the incorporated radioactivity.

The radioactivity present in the various fractions isolated was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer. The radioactivity of the acid-soluble fraction of the

phenol extracted protein was taken as the index of the incorporation of labeled amino acids into the parathyroid hormone polypeptide chain.

A summary of the effects of various conditions of incubations upon the incorporation of carbon labeled amino acids into the parathyroid tissue protein extractable with aqueous phenol is presented in Table XIX.

The uptake of carbon-14 labeled amino acids into the tissue or the incorporation of it into total protein or acid-soluble protein in parathyroid tissue slices was increased by increasing the duration of the incubation period.

The incorporation of labeled amino acids into the acid-soluble fraction reached a maximum in four hours of incubation when the medium was low in calcium content and a maximum in six hours at a high level of calcium in the incubation medium.

The low calcium content in the medium had a stimulating effect on the polypeptide biosynthesis. This was to be expected in view of previous work sited already, on the control of parathyroid formation and secretion by the level of calcium in the blood circulating through the gland.

Modifying the sodium ion concentration in the medium had essentially no effect upon the incorporation of amino acids into protein. However, increasing the magnesium ion concentration of the incubation medium resulted in an increased incorporation. This finding was in accord with the report of Raisz and O'Brian (36) on the influence of magnesium ion on the uptake of

TABLE XIX

A SUMMARY OF THE EFFECTS OF VARIOUS INCUBATION CONDITIONS UPON THE INCORPORATION OF CARBON-14 LABELED AMINO ACIDS INTO NINETY PERCENT PHENOL EXTRACTABLE PARATHYROID TISSUE PROTEIN.

INCUBATION CONDITION	EFFECT UPON INCORPORATION OF RADIOACTIVITY INTO PROTEIN
Increase in time of incubation	Increase
Increase in sodium ion concentration in the medium	Essentially no change
Increase in calcium ion concentration in the medium	Marked decrease
Increase in magnesium ion concentration in the medium	Increase
Increase in the concentration of labeled amino acids in the medium	Increase
Increase in the amount of tissue slices incubated per flask	Increase
Oxygen phase	Increase
Excess glucose in the medium	Increase

a-aminoisobutyric acid by rat parathyroid glands.

The relationships between the quantity of tissue and of radioisotope used on the incorporation of labeled amino acids into protein were considered. Increasing the amount of tissue is accompanied by an increase in the incorporation of labeled amino acids into protein but the specific activity of the protein is lower, the greater the amount of tissue used. When the quantity of labeled amino and mixture used was modified, it was found that increasing the amount of isotope was followed by an increase in the incorporation but at a diminishing rate at the higher concentration. When the high specific acitivity L-leucine was used, a more linear relationship was obtained between quantity of isotope and the amount of incorporation.

The presence of oxygen as the gas phase and of glucose in the medium during incubations enhanced the incorporation of radoactivity of the labeled amino acids into protein. These findings were expected on the basis of the known requirements for protein biosynthesis.

The purpose of the second part of the study was to investigate whether or not the isolated protein was biologically active. The method of Ashby and Roberts (2) was employed to determine the serum ionizable calcium concentrations. Totally thyroparathyroidectomized male Sprague-Dawley rats were used twenty hours after the surgical operation. The change of the serum calcium level determined twenty-four hours after the injection of the test material served as the index of the

biological potency in comparison with that obtained with a commercially available parathyroid extract as reference.

A summary of the biological testing data from all the animal experiments is tabulated in Table XX.

The radioactive protein material, solubilized in dilute acid solution exhibited a hypercalcemic activity when it was tested on the thyroparathyroidectomized rats. The biological activity of this protein material was found to be equivalent to about 60 U.S.P. Units per one milligram of protein.

The endogenous hormone activity indicates the hormone activity previously stored or present in the glands prior to incubation. A considerable fraction of this activity however, was found to be due to the presence of endogenous parathyroid hormone. Only a small amount of activity could be associated with biosynthesis of hormone.

The bovine parathyroid gland tissue used in this study was entirely obtained from the Wilson Laboratories, Chicago, Illinois. These had been obtained at the slaughter house some months previously and had been stored in a freezer. Some of the difficulties encountered here such as the relatively low incorporation values and the low biological activity and yield of the desired product are no doubt due in part to the long storage time. Fresh parathyroid glands were almost impossible to obtain at regular intervals for our use. Since other workers in the field had employed frozen tissue with success in their studies

TABLE XX

SUMMARY OF THE BIOLOGICAL ACTIVITIES OF CONTROL PREPARATIONS, REFERENCE STANDARD AND THE PHENOL EXTRACTED, ACID-SOLUBLE FRACTION ISOLATED FROM BOVINE PARATHYROID GLAND TISSUE SLICES INCUBATED WITH LABELED AMINO ACIDS.

Parameter	Number of Rats	Test Material	Change in Serum Ca^{++} (mgs%)
Control	9	2 ml. of acidified water	no significant change
PTE (Standard)	9	100 U.S.P. units	$+3.1 \pm 0.7$
Vehicle (For PTE)	3	2 ml. of water containing 1.6% glycerin 0.2% phenol	no significant change
Endogenous Hormone Activity Present in the Glands	9	1.4 mg of extracted protein from glands without incubation in 2 ml. of acidified water	$+2.0 \pm 0.65$
Experimental Preparation	78	0.93 mg of phenol extracted radioactive protein in 2 ml. of acidified water	$+2.04 \pm 0.54$

with insulin we thought it worthwhile to proceed with the use of frozen tissue. We now feel however, that any further work along these lines should be done with fresh glandular material.

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Abstract of the thesis entitled "INCORPORATION OF AMINO ACIDS INTO PARATHYROID TISSUE PROTEIN" submitted by Thomas S. Liu in partial fulfillment of the requirements for the degree of Master of Science, June 1967.

Frozen bovine parathyroid glands tissue slices were incubated with carbon-14 labeled amino acids in Krebs-Ringer bicarbonate buffer. A radioactive protein material was obtained by precipitation with 50% trichloroacetic acid. The isolated protein was further extracted with 90% aqueous phenol solution reprecipitated with ether and then extracted with dilute hydrochloric acid solution.

The incorporation of radioactivity during the incubation into the total protein and the acid-soluble protein fraction were observed to be time-dependent. It was also noted that a greater extent of amino acid incorporation occurred with an incubation medium low in calcium content (0.85 mM) than in one with a higher calcium concentration (2.5 mM). The acid-soluble protein fraction showed a maximum incorporation of radioactivity at six hours when the incubations were conducted in a high calcium containing medium and at four hours when the incubation medium contained a much lower calcium concentration.

Modifying the sodium ion concentration of the medium had essentially no effect upon the incorporation of labeled amino acids into protein. However, increasing the concentration of magnesium ion in the incubation medium increased significantly

the amino acid incorporation into protein.

Increasing the concentration of carbon-14 labeled radioactive amino acids present in the incubation system; or increasing the amount of tissue slices incubated, resulted in observed enhancements of the incorporation of radioactivity. The use of an oxygen atmosphere or an increase in the glucose concentration in the incubation medium led to increases in amino acid incorporation into protein as well.

The solubilized radioactive protein material in dilute acid solution exhibited a hypercalcemic activity when it was tested on thyroparathyroidectomized rats. The biological activity of this protein material was found to be equivalent to about 60 U.S.P. units per milligram of protein.

A considerable fraction of this activity however, was found to be due to the presence of endogenous parathyroid hormone. Only a small amount of activity could be associated with biosynthesis of hormone.

APPROVAL SHEET

The thesis submitted by Thomas S. Liu has been read and approved by the undersigned member of the faculty of the Loyola University Stritch School of Medicine who served as director of the research program.

The final copies have also been examined 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.

May 29, 1967
Date

Maurice V. L'Heureux
Signature of Adviser