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# The In Vitro Biosynthesis of Calcitonin

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THE IN VITRO BIOSYNTHESIS

OF CALCITONIN

by

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# LIFE

Nino Sorgente was born in Aquara, Salerno (Italy), on January 8, 1940.

He graduated from the Torquato Tasso Classic Lyceum in Salerno, in 1958. He received a Bachelor of Arts degree from the University of Illinois, Urbana, in June 1963. In June 1966, he received a Master of Science degree in Biology from DePaul University, Chicago, Illinois.

In September 1966, Nino Sorgente began his graduate studies in the Department of Biochemistry and Biophysics, Loyola University Stritch School of Medicine under the guidance and counsel of Dr. Maurice V. L'Heureux. From September 1967 to June 1969 he served as a graduate teaching assistant in the Department. He has been a Trainee in a National Institute of Health Training Grant in Biochemistry since September 1966.

Nino Sorgente is the co-author of the following papers:

Salmon, M., Winn, H. E. and Sorgente, N. (1968). Sound Production and Associated Behavior in Triggerfishes. Pacific Science 22, 11.

Cortelyou, J. R., McWhinnie, D. J. and Sorgente, N. (1967). Some Responses of Iguanid Lizards to Mammalian Parathyroid Extract.

Am. Zoologists <u>7</u>, 719.

Nino Sorgente is a member of the American Association for the Advancement of Science and the American Chemical Society and is an Associate Member of the Society of the Sigma Xi.

## ACKNOWLEDGEMENTS

## TABLE OF CONTENTS

# TABLE OF CONTENTS CONTINUED

# LIST OF TABLES

# LIST OF TABLES CONTINUED

# LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

Calcium plays a critical role in a variety of essential processes such as excitation, contraction, blood clotting, enzymatic reactions and as a calcium phosphate complex it serves as the supporting component of the vertebrate system. McLean and Hastings (1935) have described the calcium concentration in body fluids as "one of nature's physiological constants". The interrelationships among the various calcium compartments and the controls operating to maintain the proper balance among them are poorly understood.

Bone represents by far the largest calcium compartment of the vertebrate system and it would be expected that any change in its state would be readily reflected in a change in the other compartments, especially blood. Then, the physiological regulation of blood calcium would be mediated mainly by those factors that influence the formation and resorption of bone, namely parathyroid hormone, calcitonin, and vitamin D. But there are other factors which have been implicated in the control of bone metabolism and plasma calcium. Thyroxine, corticoids, estrogens and growth hormone affect bone resorption, blood and urine calcium and phosphate (Nordin, 1966). The titers of these hormones do not change with experimentally induced changes in plasma calcium. It is not clear whether their action is a direct or an indirect

one or whether they are of importance in the overall physiological maintenance of calcium homeostasis.

The importance of the parathyroid glands in the mediation of calcium homeostasis was recognized by MacCallum and Voegtlin (1908) who showed that the removal of the parathyroid glands caused a marked fall in serum calcium, and that the tetany associated with the hypocalcemia could be relieved by administration of calcium salts. An active parathyroid extract able to relieve tetany and restore to normal, the calcium and phosphate levels in the blood was prepared by Collip and coworkers who also developed a method for its bioassay (Collip, 1925; Collip et al. 1925; Collip and Clark, 1925a, b).

Albright and Reifenstein (1948) considered the primary effect of parathyroid hormone to be on the phosphate dissolved in the body fluids. The hormone would affect the phosphate in such a way as to make it more readily excreted by the kidneys with a resulting decrease in serum phosphate level. Resorption of the hydroxyapatite of bone would, therefore, increase resulting in an elevated serum calcium. According to this hypothesis the administration of parathyroid hormone results in a rise in serum calcium because of a primary effect on phosphate excretion.

In the second hypothesis developed by McLean and Urist (1955),

bone was considered as the primary target of parathyroid hormone action with an indirect effect on the tubular reabsorption of phosphate. A negative feedback mechanism was proposed to regulate the hormone secretion. A low blood calcium titer would evoke an increased parathyroid hormone release, which would in turn effect calcium mobilization from bone to raise the serum calcium to the normal level; a high blood calcium titer would reduce the release of parathyroid hormone and the consequent bone resorption.

Rasmussen (1961) pointed out that a mechanism of blood calcium regulation based only on the parathyroid glands and their effect on bone would lead to wide fluctuations in blood calcium because of the slow and extended response of bone to parathyroid hormone by increasing tubular reabsorption of calcium and distal tubular secretion of phosphate. Rasmussen believed that the kidney may be responsible for the rapid control of small fluctuations in plasma calcium. Sanderson et al. (1960) observed that in intact dogs blood calcium would return to the initial level within a few hours after it had been raised by the administration of calcium or lowered by the administration of EDTA. In parathyroidectomized animals, however, it had not returned to the initial levels in 24 hours.

During perfusion experiments designed to test the hypothesis of

McLean and Urist (1955), Copp and Davidson (1961) and Copp et al. (1961, 1962) obtained results similar to those of Sanderson. The feedback hypothesis could explain the difference in the response to hypocalcemia, consequent to EDTA administration, between the normal and parathyroidectomized animals. Hypocalcemia would stimulate parathyroid hormone synthesis and release, which in turn would increase calcium mobilization from bone, and the blood calcium would return to its initial level; in parathyroidectomized animals the absence of the parathyroid glands results in the observed lack of calcium return to its initial level. The time difference for calcium to return to its initial level observed between the normal and parathyroidectomized animals after induced hypercalcemia by administration of calcium chloride cannot be explained by the simple hypothesis that high levels of calcium suppress parathyroid hormone secretion. If this were so, the response should be similar in both the normal animal, where parathyroid hormone output has been inhibited, and in the parathyroidectomized animal, which lacks parathyroid hormone. Using the dog as the experimental animal, Copp et al. (1962) showed that during hypercalcemia the parathyroid glands produce a hypocalcemic factor, which he named calcitonin.

Hirsch, Gautier and Munson (1963) and MacIntyre, Foster and Kumar (1965) confirmed the existence of a hypocalcemic factor, but

found that it was secreted by the thyroid glands. Hirsch et al. (1963) called the thyroid factor, thyrocalcitonin. Foster, MacIntyre and Pearse (1964) and Pearse (1965) suggested that thyrocalcitonin was synthesized by the parafollicular cells of the thyroid, and Pearse (1966) introduced the term C cells (C for calcitonin) for these cells. Immuno-fluorescence studies (Bussolati, Carvalheira and Pearse, 1967) have demonstrated the presence of calcitonin in the C cells of the thyroid glands of the guinea-pig. The C cells of the thyroid glands are large, have an oval nucleus and contain spherical granules in their cytoplasm (Nonidez, 1932). Matsuzawa and Kurosumi (1967) in a study of the morphology of the C cells demonstrated that the granules are released by hypercalcemic stimulation.

In a study of the embryology of the C cells, Carvalheira and Pearse (1967) established that these cells originate from the last pair of pharyngeal pouches and are present in all vertebrates except the cyclostomes. In bony fishes, elasmobranchs, amphibia, reptiles and birds, these cells remain separate from the thyroid glands and form the ultimobranchial bodies. It appeared that, at least, in some birds and reptiles some of these cells migrate to the thyroids as evidenced by extraction of calcitonin from both the ultimobranchial bodies and the thyroid. Copp et al. (1967) were able to extract calcitonin from the

ultimobranchial bodies of chickens and Moseley et al. (1968) extracted

calcitonin from the ultimobranchial bodies of the chicken, pigeon and

the African skink (Mabuya striato). They found that in the chicken and

skink most of the calcitonin was found in the ultimobranchial bodies,

whereas in the pigeon it was found in equal amounts in both the

ultimobranchial bodies and thyroid glands. Clark (1968) identified

calcitonin in the ultimobranchial bodies of turtles (Crysemis pictà and

Pseudemys scripta).

Calcitonin seems to exist in at least four forms, designated alpha,

beta, gamma and delta; the four calcitonins differ in their elution

patterns on Sephadex gels (Gudmundsson, 1967) and the C-terminal

proline of beta calcitonin does not have an amide group. The $\alpha$-

calcitonin from porcine thyroid glands has been purified and its amino

acid sequence determined (Potts et al. 1968; Neher et al. 1968). The

sequence has been confirmed by the chemical synthesis of the poly-

peptide (Rittel et al. 1968). The polypeptide has 32 amino acids and a

molecular weight of 3585 (Figure 1). The pure peptide has an activity

of 200-250 Medical Research Council units/mg dry weight (Kahnt et al.

1968). One MRC unit is defined in terms of a Standard Preparation,

10 milliunits of which produce a 10% fall in plasma calcium when

administered intravenously in a 150 gm rat (Kumar et al. 1965).

$$\overbrace{\text{Cys-Ser-Asn-Leu-Ser-Thr-Cys}}^{\text{S — S}}\text{-Val-Leu-}$$

Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-
 1    2    3    4    5    6    7    8    9

Ser-Ala-Tyr-Trp-Arg-Asn-Leu-Asn-
10   11   12   13   14   15   16   17

Asn-Phe-His-Arg-Phe-Ser-Gly-Met-
18   19   20   21   22   23   24   25

Gly-Phe-Gly-Pro-Glu-Thr-Pro-C$\overset{\displaystyle O}{\underset{NH_2}{\parallel}}$
26   27   28   29   30   31   32
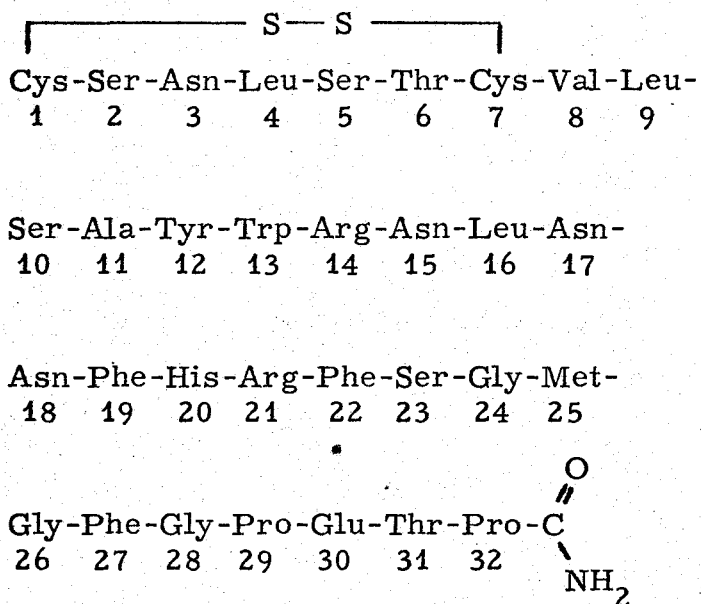
FIGURE 1.   AMINO ACID SEQUENCE

OF PORCINE ALPHA-CALCITONIN

(Potts et al. 1968).

The calcitonin peptide has several distinctive structural features (Potts et al. 1968). The single tyrosine and tryptophane residues are adjacent; the number of charged amino acids is quite low. There is a 1-7 intrachain disulfide bridge, providing a 23-membered ring at the amino-terminus. The carboxyl-terminal amino acid is prolinamide. The terminal disulfide group and the alpha-carboxyl amide are analogous structural features found in oxytocin and vasopressin. What portions of calcitonin are necessary for biological and immunological activity are not yet known; however, methionine (residue 25) may be alkylated or oxidized without loss of biological activity (Brewer et al. 1968). The serine, glycine and proline constitute 35% of the amino acid residues in the molecule; isoleucine is absent (Potts et al. 1967).

There seem to be some species differences with respect to the chemistry and biological activity of calcitonin. Tashjian and Munson (1965) found that antiserum to porcine calcitonin produced in rabbits neutralized pig and calf calcitonin but had no effect on the activity of calcitonin from rats, monkeys or man. Likewise, the difficulty in demonstrating hypocalcemic activity of porcine calcitonin in man may be due to differences in the calcitonin of the two species; in fact human calcitonin from C cell tumors has been isolated and the amino acid sequence determined (Neher et al. 1968) and it was found that even

though the disulfide bridge between position 1 and 7, and the C-terminal proline amide are the same as in porcine alpha-calcitonin 18 amino acid residues are different throughout the molecule. Arginine and tryptophane are absent, lysine and isoleucine are found in position 18 and 27 respectively, and methionine changes its place from position 25 to 8. Pigeon calcitonin seems to be different from porcine calcitonin, since it has a different elution pattern than porcine calcitonin on Sephadex (Moseley et al. 1968). More evidence for species differences comes from the report that two porcine and one avian (chicken) calcitonin preparations, which had equal activities in the rat, were found to differ when tested in the mouse. One porcine and the avian preparation were ten and five times, respectively, more potent than the other porcine preparation (Parson and Reynolds, 1968).

Calcitonin acts on both bone and kidney. Kenny (1964) and Hirsch Voelkel and Munson (1964) showed that calcitonin decreases serum phosphate and that the administration of calcitonin was followed by a phosphaturia (Kenny and Heiskell, 1965; Milhaud, Moukhtar, Cherion and Perault, 1966; Robinson, Martin and MacIntyre, 1966). The renal effect, however, requires doses 10-100 times higher than those necessary to produce hypocalcemia (Russell and Fleish, 1967). The hypophosphatemia produced by calcitonin is not entirely due to an in-

creased excretion of phosphate, but also to increased phosphate de-

position in bone; in fact, hypophosphatemia is still observed in nephrec-

tomized rats (Robinson, Martin, Matthews and MacIntyre, 1967). The

exact nature of the renal effect is not known, and it may be secondary

to increased parathyroid hormone secretion (Stahl et al. 1966).

Several investigators have attempted to clarify the relationship

between calcitonin and parathyroid hormone. Hirsch et al. (1964)

reported that parathyroidectomy did not affect the hypocalcemic or the

hypophosphatemic response to calcitonin. Gitelman et al. (1965) con-

firmed Hirsch's findings. Gudmundsson et al. (1966) observed that after

parathyroidectomy, rats maintained on a normal diet did not exhibit the

hypocalcemic effect of calcitonin but did exhibit the hypophosphatemic

response. When the parathyroidectomized rats were maintained on a

high calcium, low phosphate diet both the hypocalcemic and hypophos-

phatemic effects of calcitonin were observed. Milhaud et al. (1966),

however, obtained results which showed that parathyroidectomy in rats

maintained on a normal diet abolished only the hypophosphatemic effect.

Stahl and Kenny (1967) have reported that thyroparathyroidectomy in the

dog abolished the hypophosphatemic response of calcitonin, and calcitonin

administration in the thyroparathyroidectomized dogs caused a relative

hyperphosphatemia. On the other hand, the hyperphosphaturic effect in

the rat is not abolished by thyroparathyroidectomy (Milhaud et al. 1966)

or parathyroidectomy (Robinson, Martin and MacIntyre, 1966).

The hypocalcemic effect of calcitonin is due to its inhibitory

action on calcium mobilization from bone. Friedman and Raisz (1965)

studied the effect of calcitonin on isolated embryonic rat bones which

had been pre-labeled in vivo with $^{45}$Ca. These investigators found that

the addition of calcitonin to the culture medium decreased the release

of $^{45}$Ca from the bone; when both parathyroid hormone and calcitonin

were added to the medium the effect was more pronounced. Histological

examination showed that calcitonin diminished the fibroblastic and osteo-

clastic proliferation stimulated by parathyroid hormone. A similar, but

less marked, change was observed in the culture to which parathyroid

hormone was not added. Aliapoulios et al. (1966) demonstrated an

inhibition of resorption in calvariae from 5-day old mice when calcitonin

was added to the incubation medium. Calcitonin does not act as an

inhibitor of parathyroid hormone. Reynolds et al. (1967) found that the

changes in the osteocytes and osteoblasts brought about by parathyroid

hormone or vitamin A could not be prevented by calcitonin, but calci-

tonin would inhibit the increase in osteoclasts. No changes were ob-

served in hydroxyproline and hexosamine levels of mouse calvariae

when calcitonin was added to the culture medium. In parathyroidecto-

mized rats, Foster et al. (1966) noted a drop is osteoblasts after pro-
longed treatment with calcitonin.

The evidence for an effect of calcitonin on bone formation is un-
clear and contradictory. Raisz et al. (1967) observed no increase in
the deposition of calcium from the medium into bone when calcitonin
was added to the incubation medium of embryonic cultures of radii and
ulnae. Gaillard (1967) observed an increase in osteoblasts in cultures
treated with calcitonin, and Gaillard and Thesingh (1967) observed a
net increase in the length and width of in vitro cultivated radius rudi-
ments from 15-day old mouse embryos when calcitonin was added to
the cultivating medium. These investigators also cultivated frontal
bone from 18-day old chick embryos together with thyroid tissue of 8-
day old chick embryos and observed an increase in the growth of the
cultured bones. These observations indicate that the thyroid tissue of
an 8-day old embryo releases some factor (probably calcitonin) that in-
duces an increase in the width of bone as compared to controls. In
contrast to Gaillard's results, Baylink, Morey and Rich (1969) using
histological methods concluded that calcitonin decreases both the forma-
tion and resorption of bone. Goldhaber et al. (1968) reached the same
conclusion from investigations which showed that calcitonin reduces the
incorporation of tritiated proline into bone in culture systems. Nisbet

and Nordin (1967), on the other hand, found that calcitonin did not change the uptake of proline-C-14 suggesting that bone formation is not affected.

Studies on the metabolism of bone are of very little help in establishing whether bone formation is affected by calcitonin. Bexter et al. (1968) reported that calcitonin increased the in vitro synthesis of glucosaminoglycan, indicating an increase in bone formation. Johnston and Deiss (1966) incubating calvariae from young adult rats showed that calcitonin decreased glucose utilization and lactate production. Insoluble hydroxyproline was also decreased; hexosamine was reduced but not significantly. Klein and Talmage (1967) demonstrated that calcitonin reduces the serum hydroxyproline and the wash hydroxyproline of peritaneal lavage. Martin, Robinson and MacIntyre (1966) and Aer (1968) found that calcitonin reduces urinary hydroxyproline excretion.

The increase in citric acid following the administration of parathyroid hormone has been well documented. Nisbet and Nordin (1967) in an in vitro system in which calvariae were incubated, studied the relation between calcitonin and citric acid. They found that less citric acid accumulated in the medium when parathyroid hormone and calcitonin were added to the cultures than when parathyroid hormone alone was added. The differences observed in the incubation medium were not

reflected in the calvariae themselves.

The mode of action of calcitonin on bone is unknown. It does not act by inhibiting parathyroid hormone, since it acts in the absence of the parathyroid glands; it does not have any effect on ribonucleic acid (Gudmundsson et al. 1966; Tashjian, 1965). The hypothesis that it may increase the rate of the de novo synthesis of some enzyme does not have any experimental support.

Four possibilities have been considered which need not be exclusive of each other. First, the hormone both in vitro and in vivo has been shown to reduce the number of osteoblasts (Friedman and Raisz, 1965; Reynolds et al. 1967; Matrajt et al. 1967). Such changes in the cellular makeup of bone could account for the hypocalcemic effect of the hormone. No data is available on the time such changes occur after hormone administration; the studies available have been done after the animals had been chronically treated with calcitonin or after bone cultures had been incubated for days in a medium containing calcitonin. The hypocalcemic effect, however, is manifest 15 minutes after the hormone is administered. It is not known whether the cellular changes occur within such short a period of time or whether more subtle functional shifts occur at the molecular level and precede the morphological changes.

A second hypothesis has been advanced by Wells and Lloyd (1968b) who found that imidazole produces a drop in blood calcium and antagonizes the hypercalcemic effect of parathyroid extract (Wells and Lloyd, 1968). Imidazole activates adenosine 3', 5'-phosphodiasterase, the enzyme which degrades cyclic 3', 5'-adenosine monophosphate. Previously Wells and Lloyd (1967) showed that theophylline, which inhibits adenosine 3', 5'-phosphodiasterase, produced hypercalcemia. Chase and Aurbach (1967, 1968) showed that parathyroid hormone stimulates the urinary excretion of cyclic adenylate which is dependent upon a direct action of the hormone on the kidneys. Wells and Lloyd (1968b) proposed that the level of cyclic AMP in bone cells mediates the rate of calcium mobilization and, secondarily, the level of serum calcium. Elevations of cyclic AMP level brought about by parathyroid hormone, theophylline or other drugs, increase the mobilization of calcium; a decrease in cyclic AMP, brought about by calcitonin, or imidazole, would decrease calcium mobilization. Substantiation of the hypothesis proposed by Wells and Lloyd can be found in the work of Chase and Aurbach (1968). These investigators have reported that parathyroid hormone increases adenyl cyclase activity in bone 3 to 5 fold; calcitonin, however, does not seem to affect cyclase activity.

A third hypothesis involves the regulation of pH at the cellular

level. It has long been known that parathyroid hormone increases acid production. Recently Nisbet and Nordin (1967) observed that citric acid levels are reduced in bone culture media when calcitonin is present, and the reduction is more marked when resorption is stimulated by parathyroid hormone. Calcitonin may increase the pH at the cellular level and decrease calcium mobilization.

Finally, Doty, Schofield and Robinson (1968) observed that acid phosphatase and ATPase are affected by calcitonin and parathyroid hormone in an opposite manner. Parathyroid extract increased the acid phosphatase of metaphyseal bone and the ATPase of osteoclasts, whereas calcitonin decreased the levels of these enzymes. These enzymes may be involved in the mechanism of action of calcitonin; acid phosphatase has been implicated as an important factor in the calcification process (Cartier, 1951). How these enzymes are related to bone formation and resorption is unknown.

Aliapoulios et al. (1966) have shown that extracts of human thyroid glands contain hypocalcemic activity and Barrett et al. (1968) has purified human calcitonin and found that it was stable and soluble at neutral pH, whereas porcine calcitonin is not. In man calcitonin-like activity has been detected also in the thymus and parathyroid glands (MacIntyre, 1968). Abnormal calcitonin states are suspected to be involved in

medullary carcinoma of the thyroid gland, pseudoparathyroidism and
hyperparathyroidism. Increased calcium-lowering activity in either
blood or tumor tissue or both have been found. Removal of the tumor
results in decreased levels of circulating calcium lowering activity
(Milhaud et al. 1968; Cunliffe et al. 1968; Melvin and Tashjian, 1968).
The hypocalcemia associated with pseudohypoparathyroidism is believed
to be caused by excessive secretion of calcitonin. Aliapoulios et al.
(1966) and Tashjian et al. (1966) have shown that thyroid tissue from
patients with pseudoparathyroidism contains approximately 100 times
more calcitonin-like activity than normal thyroid tissue. Tetany and
diffuse non-toxic goiter was found to be associated with increased
calcitonin levels and thyroidectomy resulted in disappearance of the
syndrome (Mazzuoli et al. 1966).

The therapeutic uses of calcitonin have not yet been explored in
great detail. A serious hindrance to studies on the possible clinical
application of calcitonin is the fact that normal man responds poorly to
porcine calcitonin, at the present the main source of the hormone
(Foster et al. 1967; Milhaud et al. 1965; Bell et al. 1966). Calcitonin
elicits a weak hypocalcemic response in osteoporotic patients (Foster
et al. 1968). In a patient with Paget's disease the administration of
calcitonin was found to relieve the pain (Bijvoet and Jansen, 1964).

The secretion of calcitonin in response to hypercalcemic states has been well documented (Talmage et al. 1965; Toverod et al. 1966; Hirsch and Munson, 1966).  In fact it was the result of perfusion studies with hypercalcemic blood that led Copp to postulate the presence of a hypocalcemic factor (Copp et al. 1962).  There has been some question as to whether the hormone is secreted continuously or only in response to hypercalcemia.  Talmage et al. (1965) postulated that calcitonin secretion occurred at normal plasma calcium levels.  It has now been demonstrated that secretion of calcitonin occurs at normal and sub-normal calcium levels (Deftos, Lee and Potts, 1968).  It has also been established that the plasma calcium controls the levels of circulating calcitonin (Care et al. 1969).

## STATEMENT OF THE PROBLEM

A study of the in vitro biosynthesis of calcitonin would be of value in attempting to define those conditions which in the organism control the synthesis and release of the hormone.  There have been a number of studies of the in vitro biosynthesis of polypeptide hormones. · Bauer and Lazarow (1961) and Mallory et al. (1964) have reported the incor-poration of radioactive amino acids into insulin using goosefish islet

tissue and rat pancreas, respectively.   More recently Steiner and Oyer

(1967) and Steiner et al. (1967) using rat pancreas and islet cell tumor

tissue demonstrated the incorporation of labeled amino acids into insulin

and a precursor molecule.   Takabatake and Sachs (1964) reported the in

vitro biosynthesis of vasopressin by hypothalamic tissue.   Wool et al.

(1961) and Adiga et al. (1965) using various labeled amino acids demon-

strated the in vitro biosynthesis of ACTH in anterior pituitary tissue.

Wakabayashi and Tamaoki (1967) have reported the synthesis of LH by

isolated anterior pituitary glands and have studied the effect of androgens

on the synthesis.

By using the general experimental approach used for the bio-

synthesis of these polypeptide hormones we propose to synthesize cal-

citonin in an ovine thyroid slice system.   Glycine-C-14 will be used as

the tracer amino acid.   Calcitonin titers in the blood are thought to be

controlled by the levels of calcium; high levels of calcium inducing an

increase and low levels inducing a decrease in the circulating calcitonin.

In perfusion studies of the thyroid glands (Care et al. 1968; Lee et al.

1969) it has been shown that in vivo an increase in the calcium concen-

tration of the perfusing medium resulted in increased circulating hypo-

calcemic activity.

Since in vivo the increased hypocalcemic activity, obtained in the

high calcium perfusion studies, may have been the result of synthesis, release or both, we will study the effect of calcium on the synthesis and the effect of calcium on the release of the hormone in the in vitro synthesizing system to establish the role of these ions on calcitonin biosynthesis and release. Since it has been reported that magnesium affects the synthesis of parathyroid hormone (Buckle and Care, 1967), we will also study the effect of magnesium on the synthesis of calcitonin in the ovine thyroid slices.

# CHAPTER II

## MATERIALS AND METHODS

### GENERAL EXPERIMENTAL PROCEDURE

In the first part of this work our main purpose was to develop a system for the incorporation of glycine-C-14 into proteins of ovine thyroid slices, and more specifically the isolation, from the slices of calcitonin containing a radioactive amino acid. For this purpose ovine thyroid glands were obtained from the slaughterhouse as soon as possible after the animal was killed -- usually 10-20 minutes. The glands were placed in cold Krebs-Ringer bicarbonate buffer* (pH 7.4) and were brought to the laboratory where they 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 - 1.0 mm in thickness. The slices were then transferred into a series of 50 ml Erlenmeyer flasks (approximately 2.0 gm of slices per flask) containing 10 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) to which was added

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*The phrase "Krebs-Ringer bicarbonate buffer" whenever used later in this dissertation means a Krebs-Ringer bicarbonate buffer to which was added glucose (320 mg/100 ml) and all the amino acids of calcitonin (20 mg/liter) with the exception of the one used as a tracer.

glucose (320 mg/100 ml) and all the amino acids of calcitonin (20 mg/ liter) but the tracer. To each flask 2.5 $\mu$Ci of the tracer amino acid were added. The incubation was carried out at pH 7.4 in a constant shaker water bath at 37$^o$ C for 4 hours. Flasks which contained, in addition to the above, 1.0 ml of 50% trichloroacetic acid served as controls. The incubation of the experimental flasks was terminated by the addition of 1.0 ml of trichloroacetic acid to each flask.

The tissue slices after incubation were cut into small pieces with scissors and washed 10 times with cold acetone, 6 times with cold chloroform and again 10 times with cold acetone. The defatted tissue was then dried at room temperature and stored at 4$^o$ C until it was used.

The defatted tissue was then extracted with 10 volumes of 8.0 $\underline{M}$ urea - 0.1 $\underline{N}$ cysteine in 0.2 $\underline{N}$ HCl for one hour at room temperature. The mixture was then squeezed through cheese cloth and washed with a volume of doubly-distilled water equal to that of the extraction solution. The filtrate and washings were combined and dialyzed against 0.1 $\underline{M}$ acetate buffer (pH 4.6) for 24 hours at 4$^o$ C. To the dialysate was then added 3.0 $\underline{M}$ NaCl to a final concentration of 1.0 $\underline{M}$. After standing for 72 hours at 4$^o$ C the precipitate was collected by centrifugation at 4$^o$ C at 1400 RCF for 3 hours. The inactive precipitate was dis-

carded and to the supernatant was added solid NaCl to a final concentration of 2.0 $\underline{M}$. After 72 hours at $4^{\circ}$ C the active precipitate was collected by centrifugation at $4^{\circ}$ C at 1400 RCF for 3 hours. The precipitate was then dried by lyophilization. The dried material was then purified on Sephadex columns.

Radioactivity and biological activity were measured at each step during the isolation. Biological activity was estimated by the hypocalcemic response to the injection of the test material in rats. Radioactivity was measured by liquid scintillation counting (Beckman LS 250 Liquid Scintillation Spectrometer) and total protein was determined by a modification of the Lowry method.

Once we were able to show that calcitonin is synthesized in vitro we studied the effect of high concentrations of calcium on the synthesis and the release of the hormone. The effect of high concentrations of magnesium on the synthesis of calcitonin was also studied. In this part of our investigation, the slices were incubated in normal, high calcium, or high magnesium media. To assess the extent of synthesis the slices were extracted and calcitonin isolated through the second sodium chloride precipitation step. The lyophilized material was dissolved in 0.1 $\underline{M}$ acetic acid and injected intravenously into rats. The fall in serum calcium at 50 minutes was taken as the index of hypo-

calcemic activity. The difference between the hypocalcemic activity of glands incubated in normal and high calcium or high magnesium was taken as representing the extent of synthesis. To measure the amount of released hormone at the end of the incubation the slices were removed and the media was injected into rats for bioassay; the fall in serum calcium 50 minutes after the administration of the calcitonin preparation was taken as the index of hypocalcemic activity released into the medium.

## CHEMICALS

Acetic Acid, Glacial, J. T. Baker Chemical Company, No. 9507.

Acetone, Mallinckrodt Chemical Works, AR No. 2440.

Amino Acids, L form, Mann Research Laboratories, Inc., (M.A.)
   Kit 00100-721.

Albumin, Crystalline, Pentex, Inc., Lot No. 16.

Blue Dextran 2000, Pharmacia Fine Chemicals, Inc., No. 4474.

Calcein Indicator, Fisher Scientific Company, No. C-566.

Calcium Carbonate, Mallinckrodt Chemical Works, AR No. 4071.

Calcium Chloride $\cdot$ 2 $H_2O$, Mallinckrodt Chemical Works, AR No. 4160.

Chloroform, Mallinckrodt Chemical Works, AR No. 4440.

Copper Sulfate $\cdot$ 5 $H_2O$, Mallinckrodt Chemical Works, AR No. 4844.

L-(+)-Cysteine Hydrochloride, Eastman Organic Chemicals, No. 2367.

Dextrose, Anhydrous, Mallinckrodt Chemical Works, AR No. 4912.

p-Dioxane, (stabilized), Mallinckrodt Chemical Works, AR No. 4937.

Disodium Ethylenediamine Tetracetate, Versenes, Inc.

Folin-Ciocalteu Phenol Reagent, Harleco.

Formic Acid 88%, Mallinckrodt Chemical Works, AR No. 2592.

$^{14}$C-Glycine (UL), Nuclear-Chicago, Batch No. 14, S.A. 108 mc/mM,

    Tracerlab, Lot No. 78-81-2, S.A. 70 mc/mM.

Hydrochloric Acid, J. T. Baker Chemical Company, No. 9535.

Magnesium Sulfate $\cdot$ 7 $H_2O$, Mallinckrodt Chemical Works, AR No. 6056.

Naphthalene, Matheson, Coleman and Bell, NX5.

Pentobarbital Sodium, Abbott Laboratories, No. 3117.

Potassium Phosphate Monobasic, Mallinckrodt Chemical Works, AR No.

    7100.

Potassium Sodium Tartrate, Mallinckrodt Chemical Works, AR No. 2367.

Sephadex G-100, Pharmacia Fine Chemicals, Inc., No. 6164.

Sephadex G-50, Superfine, Pharmacia Fine Chemicals, Inc., No. 5739.

Sodium Acetate, Anhydrous, J. T. Baker Chemical Company, No. 3742.

Sodium Bicarbonate, Mallinckrodt Chemical Works, AR No. 1412.

Sodium Chloride, Mallinckrodt Chemical Works, AR No. 7581.

Sodium Cyanide, J. T. Baker Chemical Company, No. 1144.

Sodium Hydroxide, Mallinckrodt Chemical Works, AR No. 7708.

Trichloroacetic Acid, Mallinckrodt Chemical Works, AR No. 2928.

Urea, Mallinckrodt Chemical Works, AR No. 8648.

## PREPARATION OF INCUBATION MEDIA

Krebs-Ringer bicarbonate buffer or a modification thereof served as the medium for the incubation. Normal composition of the Krebs-Ringer bicarbonate buffer is as follows:

| | | | | | |
|---|---|---|---|---|---|
| 100 part(s) | 0.90% | (0.154 $\underline{M}$) | • | NaCl solution | (0.118 $\underline{M}$)[*] |
| 4 " | 1.15 | " | | KCl " | (0.0047 $\underline{M}$)[*] |
| 3 " | 1.22 | (0.110 $\underline{M}$) | | $CaCl_2$ " | (0.0025 $\underline{M}$)[*] |
| 1 " | 2.11 | (0.154 $\underline{M}$) | | $KH_2SO_4$ " | (0.0012 $\underline{M}$)[*] |
| 1 " | 3.82 | " | | $MgSO_4 \cdot 7H_2O$ solution | (0.0012 $\underline{M}$)[*] |
| 21 " | 1.30 | " | | $NaHCO_3$ " | (0.0248 $\underline{M}$)[*] |

[*]Final concentration

Ionic strength = 0.1600

($Ca^{++}$ = 11.51 mg/100 ml)

($Mg^{++}$ = 3.7 mg/100 ml)

Twenty milligrams of each of the amino acids of calcitonin (with the exception of the tracer) and 3.2 grams of glucose were added to

each liter of buffer. An oxygen-carbon dioxide (95%:5%) mixture was bubbled into the medium for fifteen minutes and the pH was adjusted to 7.4 before it was used for incubation.

Modifications of the above mentioned Krebs-Ringer bicarbonate buffer were prepared to serve as high calcium and high magnesium media by increasing the calcium and magnesium ion concentration in such a way that calcium ion plus magnesium ion concentration were approximately equal. The final compositions of the modified buffer solution were as follows:

High-calcium medium

| 100 part(s) | 0.90% | (0.154 $\underline{M}$) | NaCl solution | (0.1180 $\underline{M}$)[*] |
| 12 " | 1.22 | (0.110 $\underline{M}$) | $CaCl_2$ " | (0.0101 $\underline{M}$)[*] |
| 4 " | 1.15 | (0.154 $\underline{M}$) | KCl " | (0.0047 $\underline{M}$)[*] |
| 1 " | 2.11 | " | $KH_2SO_4$ " | (0.0012 $\underline{M}$)[*] |
| 1 " | 3.82 | " | $MgSO_4 \cdot 7H_2O$ solution | (0.0012 $\underline{M}$)[*] |
| 21 " | 1.30 | " | $NaHCO_3$ " | (0.0248 $\underline{M}$)[*] |

[*] Final concentration

Ionic strength = 0.1838

($Ca^{++}$ = 45.6 mg/100 ml)

($Mg^{++}$ = 3.7 mg/100 ml)

High-magnesium medium

| 100 part(s) | 0.90% | (0.154 $\underline{M}$) | NaCl solution | (0.1180 $\underline{M}$)[*] |
|---|---|---|---|---|
| 3 " | 1.22 | (0.110 $\underline{M}$) | CaCl$_2$ " | (0.0025 $\underline{M}$)[*] |
| 4 " | 1.15 | (0.154 $\underline{M}$) | KCl " | (0.0047 $\underline{M}$)[*] |
| 1 " | 2.11 | " | KH$_2$PO$_4$ " | (0.0012 $\underline{M}$)[*] |
| 8 " | 3.82 | " | MgSO$_4$ · 7H$_2$O solution | (0.0089 $\underline{M}$)[*] |
| 21 " | 1.30 | (0.154 $\underline{M}$) | NaHCO$_3$ " | (0.0248 $\underline{M}$)[*] |

[*]Final concentration

Ionic strength = 0.1838

(Ca$^{++}$ = 11.51 mg%)

(Mg$^{++}$ = 29.78 mg%)

## TISSUE PREPARATION AND INCUBATION

Ovine thyroid glands were obtained from the slaughterhouse (Chicago Lamb Packing Company, 3907 Halsted Street, Chicago, Illinois) between nine and ten o'clock in the morning and usually 10-20 minutes after sacrifice the glands with all adhering tissues were placed in cold Krebs-Ringer bicarbonate buffer (pH 7.4) and brought to the laboratory where they were dissected free from fat, connective and other adhering tissue and sliced in the cold into slices approximately 0.5 - 1.0 mm in thickness with a Stadie-Riggs tissue slicer. The elapsed time between

sacrifice and incubation was usually four hours. The slices were weighed and about two grams were placed in each of a series of 50 ml Erlenmeyer flasks. Each flask contained 10 ml of Krebs-Ringer bicarbonate buffer and 2.5 $\mu$Ci of the labeled glycine. Some flasks contained, in addition to the above, 1.0 ml of 50% trichloroacetic acid and served as controls. The gas phase for all incubations was air, and the incubations were carried out in a hood.

All incubations were carried out in a temperature controlled shaker water bath at $37^{\circ}$ C for 4 hours.

Incubations were terminated by the addition to the flasks of 1.0 ml of 50% TCA or by placing the slices into cold acetone.

To determine the effect of high calcium and high magnesium ions on synthesis and release of hormones, the incubations were carried out in buffer containing normal (11.4 mg $Ca^{++}$ / 100 ml; 3.7 mg $Mg^{++}$ / 100 ml), high calcium (45.6 mg $Ca^{++}$ / 100 ml; 3.7 mg $Mg^{++}$ / 100 ml) and high magnesium (4.51 mg $Ca^{++}$ / 100 ml; 29.78 mg $Mg^{++}$ / 100 ml) concentrations. After incubation, aliquots of the high calcium medium were assayed for calcitonin activity to determine the release of the hormone from the slices. The slices were extracted for calcitonin through the second sodium chloride fractionation step. The material obtained was lyophilized, dissolved in 0.1 $\underline{M}$ acetic acid buffer (pH 4.6) and assayed

for hypocalcemic activity.

## EXTRACTION, ISOLATION AND PURIFICATION OF CALCITONIN

When the incubations were terminated, the slices were minced with scissors and washed ten times with cold acetone, six times with cold chloroform and again ten times with cold acetone. The resulting lipid-free tissue was dried at room temperature (Rasmussen, 1959) and stored in a desiccator, unless immediately used. The acetone dried material was weighed and extracted with ten volumes of 8 $\underline{M}$ urea - 0.1 $\underline{N}$ cysteine in 0.2 $\underline{N}$ HCl (Rasmussen, 1964). The isolation and purification of the extract follows the procedure of Gudmundsson, MacIntyre and Soliman (1966). The mixture was stirred and allowed to stand for one hour at room temperature. It was then filtered through cheese cloth and the residue washed with doubly distilled water (10 ml/gm dry powder). The washings were combined with the filtrate and were dialyzed against 0.1 $\underline{M}$ sodium acetate buffer (pH 4.6) for 24 hours at $4^{\circ}$ C. The dialyzate was made up to 1.0 $\underline{M}$ with respect to sodium chloride with 3.0 $\underline{M}$ NaCl and allowed to stand at $4^{\circ}$ C. After 72 hours the precipitate (inactive) was removed by centrifugation (1400 R.C.F.) for 3 hours at $4^{\circ}$ C. Solid sodium chloride was added to the supernatant to a final concentration of 2.0 $\underline{M}$. After standing for a further 72

hours at $4^{o}$ C the precipitate was collected by centrifugation (1400

R.C.F.) for 3 hours at $4^{o}$ C. The precipitate was dried by lyophiliza-

tion.

The dried precipitate was dissolved in 0.1 $\underline{M}$ formic acid (18 mg/

ml) and applied to a 2.5 x 45 cm column of Sephadex G-100 and eluted

with the same solvent. The transmission of the effluent at 280 mμ was

monitored with a Uvicord Absorptiometer and Recorder (LKB Instruments,

Inc., Rockville, Maryland) and fractions were collected on a Gilson

Fraction Collector V$^{15}$ (Gilson Medical Electronics, Middleton,

Wisconsin). Every fourth fraction was assayed for hypocalcemic

activity. When one fraction containing hypocalcemic activity was found,

the fractions preceding and following that fraction were assayed for

hypocalcemic activity until one fraction on either side was found not to

contain biological activity. The fractions containing hypocalcemic

activity were pooled, lyophilized and stored at $4^{o}$ C until used. The

dried material was dissolved in 1.0 $\underline{M}$ formic acid and applied to a

0.9 x 45 cm column of Sephadex G-50 superfine and eluted with a

solution of 0.1 $\underline{M}$ formic acid - 1.0 $\underline{M}$ urea - 0.2 $\underline{M}$ sodium chloride.

For biological activity the dried material was appropriately dissolved in

0.1 $\underline{M}$ acetic acid and assayed. The fractions from the Sephadex G-50

column were scanned for hypocalcemic activity (vide supra); the

fractions containing hypocalcemic activity were pooled, lyophilized and biological activity determined. A diagram of the fractionation and purification procedure is shown in Figure 2.

To assess the purity of the fractions containing hypocalcemic activity, an aliquot from each fraction was chromatographed on thin layer cellulose sheets (Eastman Chromatogram Sheet 6065, cellulose with fluorescein indicator). For chromatography 50 $\mu$l of each fraction was applied to a sheet and developed with the upper phase of a butanol: acetic acid: water (4:1:5) system for 2.5 hours. The combined fractions were also chromatographed. Two dimensional chromatography was done on the combined fractions on cellulose thin layer sheets. 25 $\mu$l of the combined fractions were applied on one corner of the sheet and developed in one direction with a butanol: acetic acid: water system and in the other direction with a propanol: water (70:30 v/v) system. The migration of the sample applied was determined with a UV lamp.

To characterize further the biological active peptide 100 $\mu$l (4 $\mu$g protein) of the combined fractions containing hypocalcemic activity from the Sephadex G-50 column were subjected to hydrolysis with 6.0 $\underline{N}$ HCl for 24 hours at $110^{o}$ C. The hydrolyzate was evaporated to dryness, redissolved in water and again evaporated to dryness; the dry material was dissolved in 0.01 $\underline{N}$ HCl and analyzed for amino acids on

MINCED THYROID SLICES

Extraction
(10 x with cold acetone
  6 x with cold chloroform
 10 x with cold acetone)

RESIDUE (dry at room temperature)

Extraction
10 volumes 8.0 $\underline{M}$ urea - 0.1 $\underline{N}$
cysteine - 0.2 $\underline{N}$ HCl
1 hour at room temperature
Filter through cheese cloth

FILTRATE                                    RESIDUE

+                                           wash with 10
                                            volumes water

WASHINGS

Dialyze against                             Residue
0.1 $\underline{N}$ acetate buffer (pH 4.6)
24 hours $4^{\circ}$ C

DIALYSATE

Add 3.0 $\underline{M}$ NaCl
to make a 1.0 $\underline{M}$
Allow to stand 72 hr $4^{\circ}$ C
Centrifuge 3 hr 1400 RCF

Precipitate                    SUPERNATANT
(Inactive)

Figure 2.  Flow Diagram for Fractionation and Purification of Calcitonin

Figure 2, continued

Precipitate
(Inactive)                    SUPERNATANT

                        Add solid NaCl to make 2.0 $\underline{M}$
                        Allow to stand 72 hr 4° C
                        Centrifuge 3 hr 1400 RCF

PRECIPITATE
  (active)                                    Supernatant

      Lyophilize
      Dissolve in 0.1 $\underline{M}$ formic acid
      Apply to Sephadex G-100
      Elute with 0.1 $\underline{M}$ formic acid

              ACTIVE FRACTIONS

                  Lyophilize.   Dissolve in 1.0 $\underline{M}$ formic acid
                  Apply to Sephadex G-50 superfine column.
                  Elute with 0.1 $\underline{M}$ formic acid - 1.0 $\underline{M}$ urea -
                  0.2 $\underline{N}$ HCl

ACTIVE FRACTIONS

    Thin layer chromatography
    Amino acid analysis

a TSM Amino Acid Autoanalyzer.


## DETERMINATION OF SERUM CALCIUM

A method for determining ionizable calcium in serum by Ashby
and Roberts was modified for use in this work. Blood samples were
obtained from rats by tail bleeding. The tail was placed in warm
water for about one minute, a quarter of one-half inch of the tail was
cut with a sharp blade and the blood collected. The blood was placed
in the refrigerator for about one hour before the clot was separated
from the serum by centrifugation for two minutes in a Beckman Spinco
Microfuge. Thirty microliter aliquots of serum were added to micro-
titrator cups; to each cup were added 150 microliters of 0.002 $\underline{M}$
EDTA, one drop (about 60 microliters) of properly diluted calcein
indicator, one drop of 1% sodium cyanide solution, and one drop of 1.0
$\underline{N}$ sodium hydroxide. Titrations were performed with a Beckman
Spinco Microtitrator filled with a 20.0 mg% standard calcium solution.
A long wave ultraviolet lamp (Mineralight, Model SL 3660) was placed
about 2 inches above the titration cup. The standard calcium solution
was added with continuous stirring until a green fluorescence appeared.
The calcium concentration of the sample is directly proportional to the
difference in microliters of standard calcium solution required to

titrate the sample and to titrate a water blank.    A 12.0 mg% standard solution was titrated with each group of samples, and the calcium concentrations were calculated as follows:

$$mg\% \text{ calcium} = 12.0 \text{ mg}\% \times \frac{\text{microliters for unknown}}{\text{microliters for 12.0 mg}\% \text{ standard}}$$

microliters for unknown  =  microliters of titrant required to titrate

blank minus microliters of titrant required

to titrate unknown sample

microliters for 12.0 mg%  =  microliters of titrant required to titrate
standard

blank minus microliters of titrant

required to titrate 12.0 mg% standard

Figure 3 indicates the linear relationship between the microliters of titrant used and the concentration of calcium and the data for this standard curve appears in Table I.
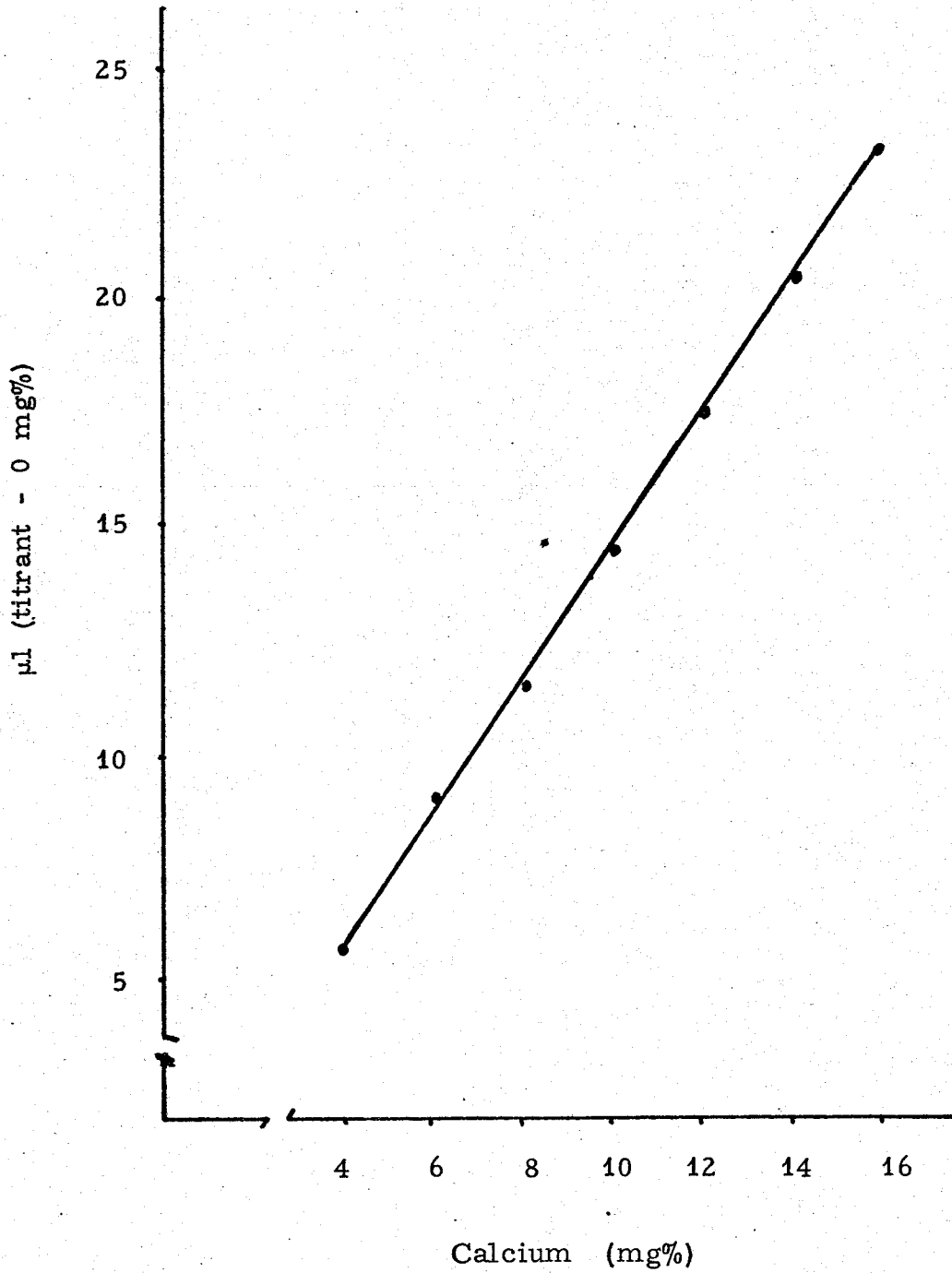
FIGURE 3.   STANDARD CURVE FOR CALCIUM DETERMINATION

## TABLE I

Standard Curve Data for Calcium Determination

| Calcium mg% | No. of Samples | Microliters of Titrant | Microliters (Titrant - 0 mg%) |
|---|---|---|---|
| 0  Blank | 6 | 29.31 ± 0.13 | ----- |
| 4 | 6 | 23.62 ± 0.26 | 5.69 |
| 6 | 5 | 20.29 ± 0.13 | 9.02 |
| 8 | 6 | 17.66 ± 0.19 | 11.65 |
| 10 | 6 | 14.69 ± 0.08 | 14.62 |
| 12 | 6 | 11.59 ± 0.13 | 17.72 |
| 14 | 4 | 8.77 ± 0.15 | 20.54 |
| 16 | 5 | 5.67 ± 0.13 | 23.64 |

Solutions

<u>Calcein solution-concentrated</u>: 0.25 gm of powdered indicator was dissolved in 40 ml of 1.0 <u>N</u> NaOH. The solution was diluted to 1000 ml with doubly distilled $H_2O$.

<u>Calcein Indicator solution</u>: 0.5 ml of the concentrated calcein solution was diluted to 25 ml with doubly distilled water.

<u>Calcium standard solution</u> (20 mg%): 0.2497 gm of oven dried calcium carbonate was dissolved in 30 ml of 2 <u>N</u> HCl and diluted to 500 ml with doubly distilled water. Working solutions were prepared from this stock solution.

<u>Ethylenediaminotetraacetic acid</u>: 187.5 mg of $Na_2EDTA$ was dissolved in $CO_2$-free $H_2O$ to a total volume of 500 ml.

## PROTEIN ANALYSIS

Protein was determined according to the method of Lowry (1951) as modified by Oyama and Eagle (1956). One milliliter of reagent "C" was placed in each of 9 x 75 mm test tube. To each tube was added 200 µl of sample (water for the blanks), the contents were mixed and allowed to stand for 10 minutes. Then 0.1 ml of Folin-Ciocalteu reagent was added to each tube with mixing. The tubes were allowed to stand for 30 minutes and the transmittance read at 750 mµ on a

Beckman DU spectrophotometer. A standard curve was prepared

following the same procedure using bovine serum albumin as standard.

The data for the calibration curve are given in Table II and the

standard curve appears in Figure 4.

## Calculations

The transmittance is converted to optical density and the density

of the reagent blank is subtracted from that of the experimental and

standard tubes. The concentration of the unknown is calculated using

the formula:

$$\frac{OD_u}{OD_s} \times \text{(concentration of standard)} \times 5 = \text{protein/ml of unknown sample}$$

## Solutions

Reagent A: 20 gm of $Na_2CO_3$, 4 gm of NaOH and 200 mg of

$KNaC_4H_4O_6 \cdot 4H_2O$ were dissolved in one liter of doubly distilled water.

Reagent B: 5 gm of $CuSO_4 \cdot 5H_2O$ were dissolved in one liter of

doubly distilled water.

Folin-Ciocalteu Reagent: Folin-Ciocalteu Reagent was obtained

commercially from Fisher Scientific Company and diluted with doubly

distilled $H_2O$. The dilution (usually 5:7) giving the highest optical

## TABLE II

Standard Curve Data for Protein Determination

| Albumin (μg) | No. of Determinations | OD$_{750}$ |
|:---:|:---:|:---:|
| 5 | 6 | 0.0873 |
| 10 | 6 | 0.1634 |
| 20 | 6 | 0.1634 |
| 30 | 5 | 0.4036 |
| 40 | 6 | 0.5393 |
| 50 | 5 | 0.6025 |
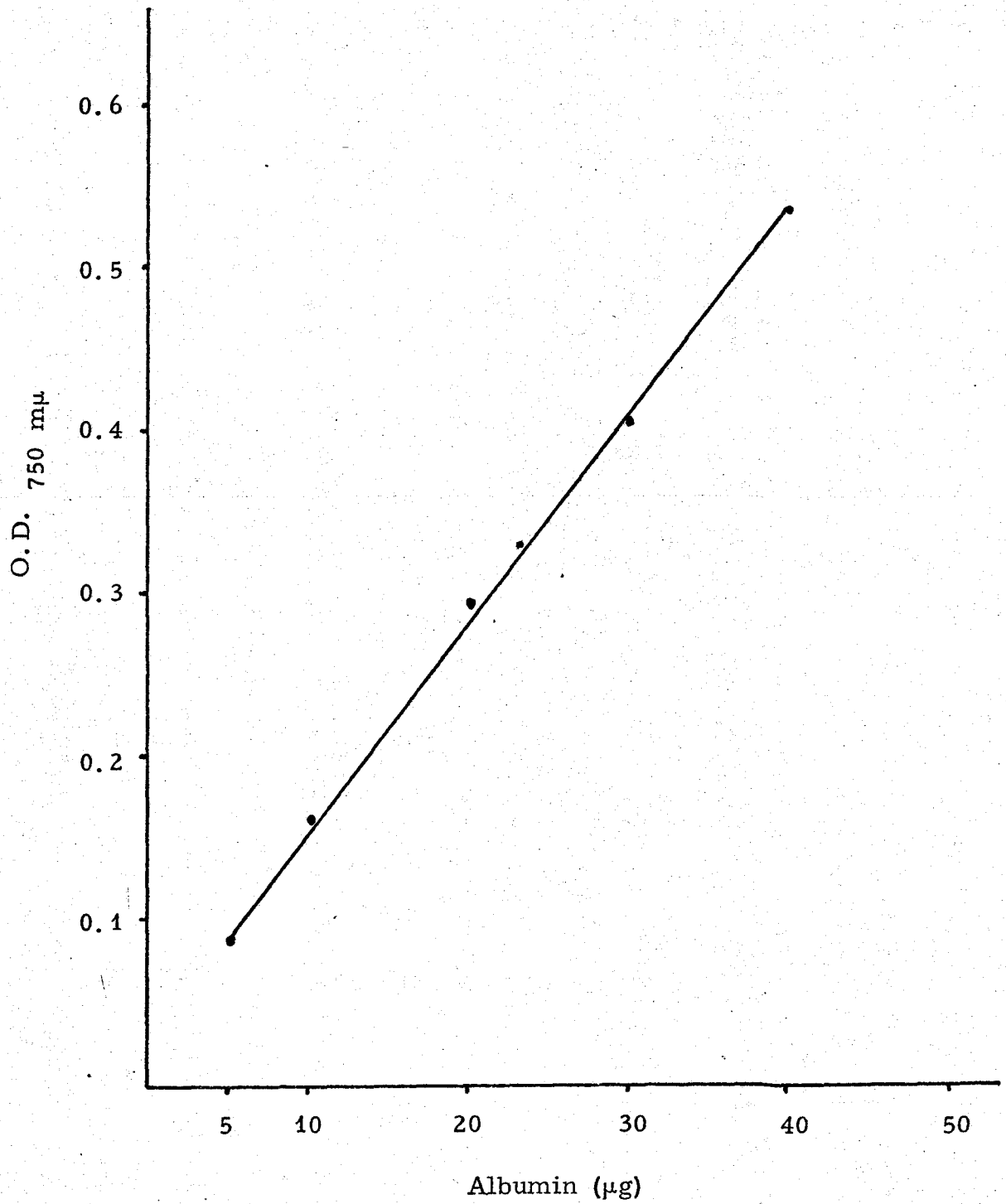
FIGURE 4. STANDARD CURVE FOR PROTEIN DETERMINATION

density that was stable for two hours was selected.

Reagent C: Fifty parts of reagent A and one part of reagent B were mixed fresh on the day of use.

## Measurement of Radioactivity

A Beckman LS-250 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. Ten milliliters of the desired scintillation fluid were placed in each vial. To the scintillation fluid was added an appropriate amount of the sample and the sample was counted five times for five minutes each time.

The scintillation fluid was prepared by dissolving 4.0 gm of PPO and 100.0 gm of naphthalene in p-dioxane and made up to the volume of one liter.

## DETERMINATION OF BIOLOGICAL ACTIVITY

Male Holtzman rats weighing from 100 to 110 grams (Holtzman Company, Madison, Wisconsin) were kept in the laboratory and fed standard rat chow until they reached a weight of 140 to 170 grams at which time they were used for determination of biological activity of calcitonin preparations.

The animals were anesthetized with sodium pentobarbital injected intraperitoneally at a dose of 5 mg per 100 grams of body weight. A blood sample was collected from the tail. The animal was placed on an operating board, an incision was made along the thigh to expose the femoral vein. 250 µl of calcitonin preparation, or vehicle, was injected into the vein using a Hamilton syringe of appropriate size, except for the experiments to determine the effect of calcium on calcitonin release from the thyroid slices when 150 µl of incubation medium or buffer was injected into the vein. The incision was then closed with wound clips. After fifty minutes, while the animal was still under anesthesia, another blood sample was collected.

The blood was allowed to remain in the refrigerator for about one hour for clot formation. It was then centrifuged for two minutes to separate the serum. The serum was analyzed for calcium and the difference between the calcium at zero time and fifty minutes was used as an index of the effect of the material administered.

## CHAPTER III

## EXPERIMENTAL RESULTS

The experimental portion of this work comprises two parts. These include the biosynthesis and purification of calcitonin, and the bioassay of the synthesized material. In the biosynthesis and purification studies, a system for the incorporation of glycine-C-14 into ovine thyroid slices was developed. The slices were then subjected to isolation and purification procedures to obtain a highly purified calcitonin preparation. The medium used for the incubation was Krebs-Ringer bicarbonate buffer, pH 7.4, to which was added glucose and the amino acids of calcitonin to stimulate protein synthesis and calcitonin synthesis in particular.

The second part of this work comprised the study of the effects of high calcium and high magnesium concentrations on the synthesis and release of calcitonin from the thyroid slices. This was done in an attempt to define the conditions which control the synthesis and release of the hormone.

The Effect of the Addition of Glucose and Amino Acids of Calcitonin to the Incubation Medium on the Incorporation of Glycine-C-14 in Thyroid Proteins.

One gram of ovine thyroid slices was incubated with 1.25 µc of

glycine-C-14 (UL) in 25 ml Erlenmeyer flasks containing 5.0 ml of buffer. The incubations were carried out at $37^{\circ}$ C for 4 hours in a constant temperature shaking water bath. The gas phase was air. The incubations were terminated with the addition of one milliliter of 50% trichloroacetic acid. The slices were homogenized in distilled water and 100 μl of the homogenate was counted in a liquid scintillation mixture containing 6% Cab-O-Sil (Packard Instruments Company, Downers Grove, Illinois). The protein in the homogenate was determined and the radioactivity expressed in disintegrations per minute per milligram protein.

The data on the effect of glucose and the amino acids of calcitonin are presented in Table III. It is readily evident that the radioactivity represents true incorporation since the slices in the "killed" flasks show negligible radioactivity. It is also evident that glucose markedly stimulates the incorporation of glycine-C-14 into the thyroid tissue, the increase above the basal level is approximately 200%. The addition of the calcitonin amino acids alone has no significant effect on the isotope incorporation. The addition of both glucose and amino acids increases incorporation with respect to the basal level (102%). However, a decrease in the incorporation of the radioactive label is observed when compared to the medium with glucose alone.

TABLE III

Effect of Glucose and Calcitonin Amino Acids on the Incorporation of Glycine-C-14

in the Proteins of Ovine Thyroid Slices

| Medium | No. of Incubations | DPM/100 mg Tissue | DPM/mg Protein | % Change[***] |
|---|---|---|---|---|
| Killed[*] | 8 | $8963 \pm 90$ | $84 \pm 8$ | ----- |
| Basal[**] | 8 | $30275 \pm 300$ | $3283 \pm 33$ | ----- |
| Basal + glucose | 8 | $79527 \pm 795$ | $10207 \pm 102$ | + 211 |
| Basal + amino acids | 8 | $30484 \pm 305$ | $3804 \pm 380$ | + 16 |
| Basal + glucose + amino acids | 7 | $60957 \pm 610$ | $6623 \pm 662$ | + 102 |

[*] Killed refers to flasks to which 1.0 ml of 50% TCA was added to the medium at the start of incubation.

[**] Basal refers to those flasks containing only Krebs-Ringer bicarbonate buffer with no additions.

[***] % Change is the change in DPM/mg protein over the basal level.

In all subsequent incubations we used the medium to which glucose and the amino acids of calcitonin were added. We believe that the decrease in incorporation observed with this medium was due to a decrease in the incorporation of label into proteins requiring other amino acids besides those of calcitonin. The lack of these amino acids depressed the synthesis of those proteins requiring other amino acids in addition to those added, whereas the synthesis of those proteins and polypeptides made up only of the amino acids of calcitonin would be accelerated. When glucose alone was added the synthesis of many small peptides would result with very few complete proteins formed, since the only source of essential amino acids would be the pool of amino acids present in the slices and from the breakdown of other proteins.

The Synthesis of Calcitonin in Krebs-Ringer Bicarbonate Buffer Containing Physiological Concentrations of Calcium.

To determine whether calcitonin was being synthesized at physiological concentrations of calcium, ovine thyroid slices were incubated in Krebs-Ringer bicarbonate buffer containing 11.5 mg calcium per 100 ml of buffer. 18 to 20 grams of slices were found necessary to extract an amount of calcitonin sufficient for the assay of hypocalcemic activity.

For this experiment, 20 grams of slices were incubated in Krebs-Ringer bicarbonate buffer in 2.0 gram quantities in 50 ml Erlenmeyer flasks containing 10 ml of buffer. The incubations were carried out in an atmosphere of air at $37^{\circ}$ C for 4 hours and were terminated with the addition of 1.0 ml of 50% trichloroacetic acid to each flask.

The incubated glands and 20 grams of unincubated glands were extracted and calcitonin was isolated through the second sodium chloride precipitation step. The active precipitate was lyophilized, dissolved in 0.1 $\underline{M}$ acetic acid and the hypocalcemic activity assayed in 140-170 gram Holtzman rats. The rats were fasted 18 hours before assay. Each rat received intravenously 250 µl of the calcitonin preparation which contained 125 µg of lyophilized material. The calcitonin preparations were analyzed for protein content and the change in serum calcium was expressed as $\Delta$ Ca per 10 µg protein. Comparison of the decrease in serum calcium (Table IV) obtained with the calcitonin preparation obtained from incubated and unincubated glands shows that only a small difference statistically not significant (0.11 mg% Ca) exists. This finding is in accord with in vivo observations which indicate that at physiological serum calcium concentration only small amounts of calcitonin are present in the circulation.

## TABLE IV

Hypocalcemic Activity of Calcitonin Preparations Isolated from Unincubated
Ovine Thyroid Glands and from Thyroid Slices Incubated in Krebs-Ringer
Bicarbonate Buffer Containing Physiological Concentrations of Calcium.

| | UNINCUBATED GLANDS | | | |
|---|---|---|---|---|
| Animal No. | Ca mg% | | $\Delta$ Ca mg% | $\Delta$ Ca/10 μg mg%/Protein |
| | $t_0$ | $t_{50}$ | | |
| 1 | 11.46 | 11.27 | − 0.19 | − 0.22 |
| 2 | 11.52 | 10.44 | − 1.08 | − 1.23 |
| 3 | 11.78 | 11.47 | − 0.31 | − 0.36 |
| 4 | 11.44 | 10.73 | − 0.71 | − 0.81 |
| 5 | 11.86 | 11.31 | − 0.56 | − 0.64 |
| Mean $\pm$ s.e. | | | | − 0.65 $\pm$ 0.17 |

| | INCUBATED GLANDS | | | |
|---|---|---|---|---|
| Animal No. | Ca mg% | | $\Delta$ Ca mg% | $\Delta$ Ca/10 μg mg%/Protein |
| | $t_0$ | $t_{50}$ | | |
| 1 | 12.04 | 11.30 | − 0.74 | − 0.87 |
| 2 | 11.45 | 11.22 | − 0.23 | − 0.27 |
| 3 | 11.97 | 11.36 | − 0.61 | − 0.71 |
| 4 | 12.20 | 11.54 | − 0.66 | − 0.78 |
| 5 | 12.12 | 11.10 | − 1.02 | − 1.19 |
| Mean $\pm$ s.e. | | | | − 0.76 $\pm$ 0.15 |

The Effect of High Calcium Concentrations on the Synthesis of Calcitonin

by Ovine Thyroid Slices.

Since calcitonin titers in the circulation seem to be controlled by the serum calcium concentration, and since at physiological concentrations of calcium no net synthesis was observed, it became apparent that if a significant amount of synthesis was to be observed, the calcium concentration in the incubation media would have to be increased. Krebs-Ringer bicarbonate buffer was prepared such that the calcium ion concentration was 45.6 mgs per 100 ml of buffer. To eliminate any variation which may have existed in the content of calcitonin of the glands due to the source and age of the animals, food ingested and season of sacrifice, incubations in buffer containing physiological and high concentrations of calcium were carried on at the same time.

After incubation the glands were pooled and calcitonin isolated through the second sodium chloride precipitation step. The precipitate was lyophilized, dissolved in 0.1 $\underline{M}$ acetate buffer and its hypocalcemic activity assayed in 140-170 gram rats.

When the amount of calcitonin is determined, the preparation obtained from glands incubated in Krebs-Ringer bicarbonate buffer containing high concentrations of calcium (45.6 mg%) show higher hypocalcemic activity than those preparations obtained from glands incubated in Krebs-

Ringer buffer containing physiological concentrations of calcium (11.5 mg%) (Tables V and VI).

The net effect has in each case been calculated by adding to the effect of the administered preparation the effect of the vehicle alone that of 0.1 $\underline{M}$ acetic acid. As shown in Table VII, the vehicle alone raised the serum calcium by an average of 0.55 mg. The decrease in serum calcium is greater for the three preparations obtained from the glands incubated in buffer containing high calcium concentrations than for those incubated in buffer containing physiological calcium concentrations. The fall in serum calcium is 4.9%, 5.0% and 6.2% for the three preparations respectively.

A comparison of the hypocalcemic activity of the calcitonin preparations obtained from slices incubated in buffer containing physiological concentrations of calcium with preparations obtained from glands incubated in buffer containing high concentrations of calcium shows that the difference of the means of preparations 2 and 3 is statistically significant at the 5% level or less, as evaluated by a Student's "t" Test. The significance of the difference of the means of preparation 1 is questionable and lies between the 10 and 5% level.

These findings support the $\underline{in}$ $\underline{vivo}$ studies which show that hypercalcemia increases the circulating calcitonin activity.

## TABLE V

Hypocalcemic Activity of Calcitonin Isolated from Ovine
Thyroid Slices Incubated in Krebs-Ringer Bicarbonate
Buffer Containing Physiological Calcium Concentrations

| Preparation | Animal No. | Ca mg% | | Ca/10 µg mg%/Protein | Net Change | % Change |
|---|---|---|---|---|---|---|
| | | $t_0$ | $t_{50}$ | | | |
| 1 - | 1 | 11.52 | 11.04 | - 0.45 | - 1.00 | 8.9 |
| | 2 | 12.13 | 11.89 | - 0.23 | - 0.78 | 6.5 |
| | 3 | 11.88 | 10.82 | - 1.00 | - 1.55 | 13.1 |
| | 4 | 12.13 | 11.92 | - 0.20 | - 0.75 | 6.3 |
| Mean $\overset{+}{-}$ s.e. | | | | | - 1.02 $\overset{+}{-}$ 0.18 | 8.7 |
| 2 - | 1 | 9.97 | 8.25 | - 1.43 | - 1.88 | 20.0 |
| | 2 | 9.76 | 8.63 | - 0.93 | - 1.48 | 15.2 |
| | 3 | 10.87 | 9.98 | - 0.73 | - 1.28 | 11.9 |
| Mean $\overset{+}{-}$ s.e. | | | | | - 1.55 $\overset{+}{-}$ 0.18 | 15.7 |
| 3 - | 1 | 12.04 | 11.30 | - 0.87 | - 1.43 | 11.8 |
| | 2 | 11.45 | 11.22 | - 0.27 | - 0.83 | 7.2 |
| | 3 | 11.97 | 11.36 | - 0.71 | - 1.26 | 10.6 |
| | 4 | 12.20 | 11.54 | - 0.78 | - 1.33 | 11.0 |
| | 5 | 12.11 | 11.10 | - 1.19 | - 1.74 | 14.5 |
| Mean $\overset{+}{-}$ s.e. | | | | | - 1.31 $\overset{+}{-}$ 0.15 | 11.0 |

## TABLE VI

Hypocalcemic Activity of Calcitonin Isolated from Ovine
Thyroid Slices Incubated in Krebs-Ringer Bicarbonate
Buffer (Containg) High Concentrations of Calcium.

| Preparation | Animal No. | Ca mg% | | Ca/10 µg mg%/Protein | Net Change | % Change |
|---|---|---|---|---|---|---|
| | | $t_0$ | $t_{50}$ | | | |
| 1 - | 1 | 10.37 | 9.97 | - 0.35 | - 0.90 | 8.8 |
| | 2 | 10.65 | 9.74 | - 0.82 | - 1.37 | 12.0 |
| | 3 | 12.73 | 10.19 | - 2.25 | - 2.80 | 22.1 |
| | 4 | 12.01 | 10.06 | - 0.84 | - 1.39 | 11.6 |
| Mean $\pm$ s.e. | | | | | - 1.61 $\pm$ 0.41 | 13.6 |
| 2 - | 1 | 11.12 | 9.38 | - 2.22 | - 2.77 | 25.0 |
| | 2 | 11.06 | 9.61 | - 1.22 | - 1.77 | 16.1 |
| | 3 | 10.89 | 8.86 | - 1.72 | - 2.27 | 20.9 |
| Mean $\pm$ s.e. | | | | | - 2.25 $\pm$ 0.29 | 20.7 |
| 3 - | 1 | 11.47 | 10.54 | - 1.10 | - 1.65 | 14.5 |
| | 2 | 11.78 | 10.74 | - 1.22 | - 1.77 | 15.1 |
| | 3 | 12.76 | 10.91 | - 2.17 | - 2.72 | 21.4 |
| | 4 | 11.52 | 10.24 | - 1.50 | - 2.05 | 17.9 |
| | 5 | 12.38 | 11.03 | - 1.56 | - 2.11 | 17.9 |
| Mean $\pm$ s.e. | | | | | - 2.06 $\pm$ 0.18 | 17.2 |

## TABLE VII

The Effect of the Vehicle (0.1 M Acetic Acid) for the

Calcitonin Preparations on the Serum Calcium of Rats

| Animal No. | Ca mg% | | $\Delta$ Ca mg% $(t_0 - t_{50})$ |
|:---:|:---:|:---:|:---:|
| | $t_0$ | $t_{50}$ | |
| 1 | 12.60 | 12.94 | + 0.34 |
| 2 | 12.31 | 13.26 | + 0.95 |
| 3 | 12.53 | 13.12 | + 0.59 |
| 4 | 11.56 | 11.93 | + 0.37 |
| 5 | 10.78 | 11.27 | + 0.49 |
| Mean $\pm$ s.e. | | | + 0.55 $\pm$ 0.11 |

### The Effect of High Magnesium Concentrations on the Synthesis of Calcitonin by Ovine Thyroid Slices.

This experiment was designed to determine whether the stimulation of calcitonin synthesis, observed when the glands were incubated in Krebs-Ringer bicarbonate buffer containing high concentrations of calcium, was due to a specific or a non-specific effect of calcium. For this purpose the Krebs-Ringer bicarbonate buffer was prepared such that the level of magnesium was increased eight times, and at the same time the sum of the magnesium and calcium ions was approximately equal to the sum of the calcium and magnesium ions in the Krebs-Ringer bicarbonate buffer containing high concentrations of calcium. Magnesium was chosen because its serum level is affected by parathyroid hormone the same as is calcium and in some cases it seems to be able to substitute for calcium; in fact, administration of magnesium is followed by an increased elimination of skeletal-bound radioactive calcium (Clark and Geoffray, 1958); furthermore, in vitro (Schachter and Rosen, 1959; Hendrix et al. 1963) and in vivo studies (Alcock and MacIntyre, 1962) suggest that magnesium and calcium act as competitors in intestinal absorption.

The procedures for incubations, isolation and assay of hypercalcemic activity were the same as those used for the demonstration of

the effect of calcium. In both preparations (Tables VIII and IX) there is a small increase in hypocalcemic activity, but the difference of the means is not significant. Preparations incubated on the same day in Krebs-Ringer bicarbonate buffer containing physiological, and high concentrations of magnesium are compared in Table X. It is evident that magnesium has very little effect on the synthesis of calcitonin; the small increase in hypocalcemic activity may be due to the substitution of magnesium for calcium in processes not involved with calcitonin synthesis, thus freeing more calcium to exert its effect on the biosynthesis of calcitonin. The data is summarized in Table XI.

## Effect of High Calcium Concentrations on the Release of Calcitonin from Ovine Thyroid Slices.

The in vivo studies do not distinguish whether the increase in circulating calcitonin is a result of increased synthesis, enhanced release or both. To determine whether calcium affected the release of calcitonin, two gram quantities of ovine thyroid slices were incubated in 50 ml Erlenmeyer flasks containing Krebs-Ringer bicarbonate buffer with either physiological or high concentrations of calcium. The incubations were carried on in an atmosphere of air at $37^{\circ}$ C for 4 hours and were terminated by removing the tissue from the flasks. The medium from

## TABLE VIII

Hypocalcemic Activity of Calcitonin Preparations Isolated
from Slices Incubated in Krebs-Ringer Bicarbonate Buffer
Containing Physiological Concentrations of Magnesium.

| Preparation | Animal No. | Ca mg% | | $\Delta$ Ca/10 µg mg%/Protein | Net Change | % Change |
|:---:|:---:|:---:|:---:|:---:|:---:|:---:|
| | | $t_0$ | $t_{50}$ | | | |
| 1 | 1 | 10.75 | 10.44 | − 0.41 | − 0.96 | 9.0 |
| | 2 | 10.94 | 10.61 | − 0.49 | − 1.04 | 9.6 |
| | 3 | 11.10 | 9.95 | − 1.65 | − 2.21 | 19.9 |
| Mean $\overset{+}{-}$ s.e. | | | | | − 1.40 $\overset{+}{-}$ 0.42 | |
| 2 | 1 | 12.04 | 11.30 | − 0.87 | − 1.42 | 11.8 |
| | 2 | 11.45 | 11.22 | − 0.27 | − 0.82 | 7.2 |
| | 3 | 11.97 | 11.36 | − 0.71 | − 1.26 | 10.6 |
| | 4 | 12.20 | 11.54 | − 0.78 | − 1.33 | 11.0 |
| | 5 | 12.11 | 11.10 | − 1.19 | − 1.74 | 14.5 |
| Mean $\overset{+}{-}$ s.e. | | | | | − 1.31 $\overset{+}{-}$ 0.15 | |

## TABLE IX

Hypocalcemic Activity of Calcitonin Preparations Isolated from
Ovine Thyroid Slices Incubated in Krebs-Ringer Bicarbonate
Buffer Containing High Concentrations of Magnesium.

| Preparation | Animal No. | Ca mg% | | $\Delta$Ca/10 µg mg%/Protein | Net Change | % Change |
|---|---|---|---|---|---|---|
| | | $t_0$ | $t_{50}$ | | | |
| 1 - | 1 | 11.06 | 9.82 | - 1.64 | - 2.19 | - 19.9 |
| | 2 | 10.93 | 10.34 | - 0.77 | - 1.32 | - 12.2 |
| | 3 | 11.13 | 10.15 | - 1.31 | - 1.86 | - 16.8 |
| Mean $\pm$ s.e. | | | | - 1.78 $\pm$ 0.25 | | |
| 2 - | 1 | 11.95 | 11.06 | - 1.12 | - 1.67 | - 14.9 |
| | 2 | 11.59 | 11.04 | - 0.70 | - 1.25 | - 10.8 |
| | 3 | 12.84 | 11.26 | - 2.00 | - 2.56 | - 20.0 |
| | 4 | 11.56 | 11.03 | - 0.67 | - 1.22 | - 10.6 |
| | 5 | 11.34 | 11.69 | - 0.82 | - 1.37 | - 11.7 |
| Mean $\pm$ s.e. | | | | - 1.61 $\pm$ 0.24 | | |

## TABLE X

Hypocalcemic Activity of Calcitonin Preparations Isolated from

Slices Incubated in Krebs-Ringer Bicarbonate Buffer Containing

Physiological or High Concentrations of Calcium and Magnesium

| Animal No. | Net $\Delta$Ca mg% | | |
|:---:|:---:|:---:|:---:|
| | Physiological | High Magnesium | High Calcium |
| 1 | - 1.43 | - 1.78 | - 1.66 |
| 2 | - 0.83 | - 1.25 | - 1.78 |
| 3 | - 1.27 | - 2.57 | - 2.73 |
| 4 | - 1.34 | - 1.23 | - 2.06 |
| 5 | - 1.75 | - 1.38 | - 2.12 |
| Mean $\pm$ s.e. | - 1.31 $\pm$ 0.15 | - 1.62 $\pm$ 0.25 | - 2.06 $\pm$ 0.18 |
| P* | | N.S. | 0.01 - 0.005 |

*Probability of the significance of the difference determined by use of

a Student's "t" Test.

## TABLE XI

Summary of the Data on the Effect of Calcium

and Magnesium on the Synthesis of Calcitonin[*]

| | Net $\Delta$ Ca mg% (mean) / 10 $\mu$g Protein | | | p[**] |
|---|---|---|---|---|
| **Normal** | | **High Ca$^{++}$ or Mg$^{++}$** | | |
| 1 - N | $- 1.02 \pm 0.18$ | 1 - HC | $- 1.61 \pm 0.41$ | 0.1 - 0.05 |
| 2 - N | $- 1.55 \pm 0.18$ | 2 - HC | $- 2.25 \pm 0.29$ | 0.05 |
| 3 - N | $- 1.31 \pm 0.15$ | 3 - HC | $- 2.06 \pm 0.18$ | 0.01 - 0.005 |
| 1 - N | $- 1.40 \pm 0.42$ | 1 - HM | $- 1.78 \pm 0.25$ | N.S. |
| 2 - N | $- 1.31 \pm 0.15$ | 2 - HM | $- 1.62 \pm 0.24$ | N.S. |

* Explanation of Symbols.

  N - Preparations obtained from slices incubated in Krebs-Ringer
     bicarbonate buffer containing physiological concentrations of
     calcium and magnesium.

HC - Preparations obtained from slices incubated in buffer containing
     high concentrations of calcium.

HM - Preparations obtained from slices incubated in buffer containing
     high concentrations of magnesium.

**Probability of the significance of the difference of the means as deter-
mined by the use of a Student's "t" Test.

each flask was assayed for hypocalcemic activity in two rats. Each rat

received 150 µl of incubation medium. The control rats received 150 µl

of normal or high calcium Krebs-Ringer bicarbonate buffer. The

results are presented in Tables XII, XIII and XIV.

The administration of normal buffer, which contained 676 µg of

calcium, increased the blood calcium by 5.7%. The administration of

the high calcium buffer, which contained 2.7 mg of calcium increased

the blood calcium by 4.5%. The difference observed between the two

groups is due to the response of the animal to the level of hypercalcemia

induced by the administration of the two buffers. The high calcium

buffer produced a higher original hypercalcemia than when the normal

buffer was administered and consequently induced the production of

larger amounts of calcitonin, which in turn decreased the blood cal-

cium. Another factor was probably involved; the hypercalcemia de-

pressed parathyroid function, which in turn decreased calcium mobiliza-

tion from bone. The combined effects resulted in a lesser plasma

calcium increase observed for those animals receiving higher doses of

calcium.

The administration of the incubation medium containing physiological

concentrations of calcium (Table XII) decreased the plasma calcium by

0.76 mg. However, since calcium was injected with the medium it

## TABLE XII

Effect of Krebs-Ringer Bicarbonate Buffer Containing Physiological
and High Concentrations of Calcium on the Plasma Calcium of Rats.

| Animal No. | Physiological Buffer Ca mg% $t_0$ | $t_{50}$ | $\Delta$ Ca mg% $(t_{50} - t_0)$ |
|---|---|---|---|
| 1 | 11.20 | 11.03 | - 0.17 |
| 2 | 11.06 | 11.69 | + 0.63 |
| 3 | 10.71 | 11.64 | + 0.93 |
| 4 | 10.56 | 11.43 | + 0.87 |
| 5 | 10.51 | 11.35 | + 0.84 |
| Mean $\pm$ s.e. | | | + 0.62 $\pm$ 0.12 |

| Animal No. | High Calcium Buffer Ca mg% $t_0$ | $t_{50}$ | $\Delta$ Ca mg% $(t_{50} - t_0)$ |
|---|---|---|---|
| 1 | 10.32 | 11.30 | + 0.98 |
| 2 | 11.24 | 11.50 | + 0.26 |
| 3 | 10.93 | 11.74 | + 0.78 |
| 4 | 10.38 | 10.86 | + 0.48 |
| 5 | 11.28 | 11.24 | - 0.04 |
| Mean $\pm$ s.e. | | | + 0.49 $\pm$ 0.10 |

# TABLE XIII

### Serum Calcium Changes Induced by the Administration of Incubation Media Containing Physiological Concentrations of Calcium.

| Flask No. | Ca mg% | | $\Delta$ Ca mg% | Net Effect[*] | % Change[**] |
|---|---|---|---|---|---|
| | $t_0$ | $t_{50}$ | 25 μg Protein | | |
| 1 | 10.83 | 9.95 | − 0.59 | − 1.21 | 11.2 |
| | 10.01 | 9.28 | − 0.49 | − 1.11 | 11.1 |
| 2 | 10.98 | 9.64 | − 1.25 | − 1.87 | 17.0 |
| | 9.67 | 9.44 | − 0.21 | − 0.83 | 8.5 |
| 3 | 10.29 | 9.15 | − 0.93 | − 1.55 | 15.1 |
| | 9.33 | 8.95 | − 0.31 | − 0.93 | 9.9 |
| 4 | 11.09 | 10.11 | − 0.74 | − 1.36 | 12.2 |
| | 10.52 | 9.12 | − 1.05 | − 1.67 | 15.8 |
| 5 | 10.90 | 9.76 | − 0.99 | − 1.61 | 14.7 |
| Mean $\pm$ s.e. | | | | − 1.34 $\pm$ 0.08 | 12.8 |

[*]Obtained by adding the mean of the change of the blood calcium of the control to the change of the blood calcium of each flask.

[**]Per cent change from plasma calcium values at $t_0$.

- 64 -

TABLE XIV

**Serum Calcium Changes Induced by the Administration of Incubation**

**Media Containing High Concentrations of Calcium.**

| Flask No. | Ca mg% | | $\Delta$ Ca mg% | Net Effect[*] | % Change[**] |
|---|---|---|---|---|---|
| | $t_0$ | $t_{50}$ | 25 μg Protein | | |
| 1 | 10.39 | 9.51 | − 0.82 | − 1.31 | 12.7 |
| | 10.70 | 9.24 | − 1.36 | − 1.85 | 17.3 |
| 2 | 11.70 | 9.93 | − 1.53 | − 2.02 | 17.3 |
| | 10.33 | 9.52 | − 0.70 | − 1.19 | 11.5 |
| 3 | 10.32 | 9.09 | − 1.00 | − 1.49 | 14.4 |
| | 10.51 | 9.32 | − 0.91 | − 1.40 | 13.3 |
| 4 | 11.08 | 9.21 | − 1.75 | − 2.24 | 20.2 |
| | 11.19 | 10.11 | − 1.01 | − 1.50 | 13.4 |
| 5 | 11.60 | 10.10 | − 1.34 | − 1.83 | 15.7 |
| Mean $\pm$ s.e. | | | | − 1.65 $\pm$ 0.08 | 15.2 |

| | Physiological | High Calcium | p[***] |
|---|---|---|---|
| | 1.34 $\pm$ 0.08 | 1.65 $\pm$ 0.08 | 0.05 − 0.01 |

[*]Obtained by adding the mean of the change of the blood calcium of the control (Table VI) to the change of the blood calcium for each flask.

[**]Per cent change from plasma calcium values at $t_0$.

[***]Probability of the significance of the difference determined by the use of a Student's "t" statistic.

should be taken into account. By adding the change brought about by

the vehicle (Table XII) we obtained the net change in plasma calcium,

1.45 mg or 13.6%. The administration of the incubation media contain-

ing high calcium concentrations (Table XIV) decreased the serum cal-

cium by 1.17 mg. The net change is 1.68 mg or 15.4%. When the net

change obtained by administering normal and high calcium incubation

media are compared, we find that the difference of the means is statis-

tically significant, indicating that the release of calcitonin is under the

direct influence of calcium.

## Isolation and Purification of Calcitonin

To determine whether the hypocalcemic activity that was observed

was due to calcitonin and not to some other unknown factor, calcitonin

was isolated and purified. 200 grams of fresh glands were incubated

in 50 ml Erlenmeyer flasks. Each flask contained 2.0 grams of thyroid

slices, 10 ml of Krebs-Ringer bicarbonate buffer containing high concen-

trations of calcium and 2.5 $\mu$Ci of glycine-C-14 (UL). The incubations

were carried on in an atmosphere of air at $37^{\circ}$ C for four hours. After

the incubations were terminated with trichloroacetic acid, the slices

were minced and defatted. The defatted material was extracted with

urea-cysteine-HCl. The extract was dialyzed against 0.1 $\underline{M}$ acetate

buffer for 24 hours at $4^o$ C. The dialyzate was made 1.0 $\underline{M}$ with 3.0 $\underline{M}$ NaCl and allowed to stand 72 hours at $4^o$ C. The inactive precipitated protein was collected by centrifugation and discarded. To the supernatant was added solid NaCl to make a 2.0 $\underline{M}$ solution and allowed to stand 72 hours at $4^o$ C. The active precipitate was collected by centrifugation and lyophilized.

The lyophilized material weighed 120 mg. It was dissolved as completely as possible in 6.5 ml of 0.1 $\underline{M}$ formic acid and the mixture centrifuged to remove suspended material. 4 ml of the supernatant was applied to a 40 x 2.5 cm column of Sephadex G-100 and eluted with 0.1 $\underline{M}$ formic acid. Figure 5 shows the elution pattern, the radioactivity and the protein content of each fraction as measured by the Lowry method (1956). The first 60 ml, the void volume, were discarded. The absorption at 280 m$\mu$ gave only one well-defined peak; however, when each fraction was analyzed for protein content by the Lowry method three protein peaks became evident. The hypocalcemic activity was confined entirely to the second protein peak. Radioactivity was high in the fractions containing hypocalcemic activity with 18,000 DPM/mg protein in fraction 6 and falling to 8,000 DPM/mg protein in fraction 10.

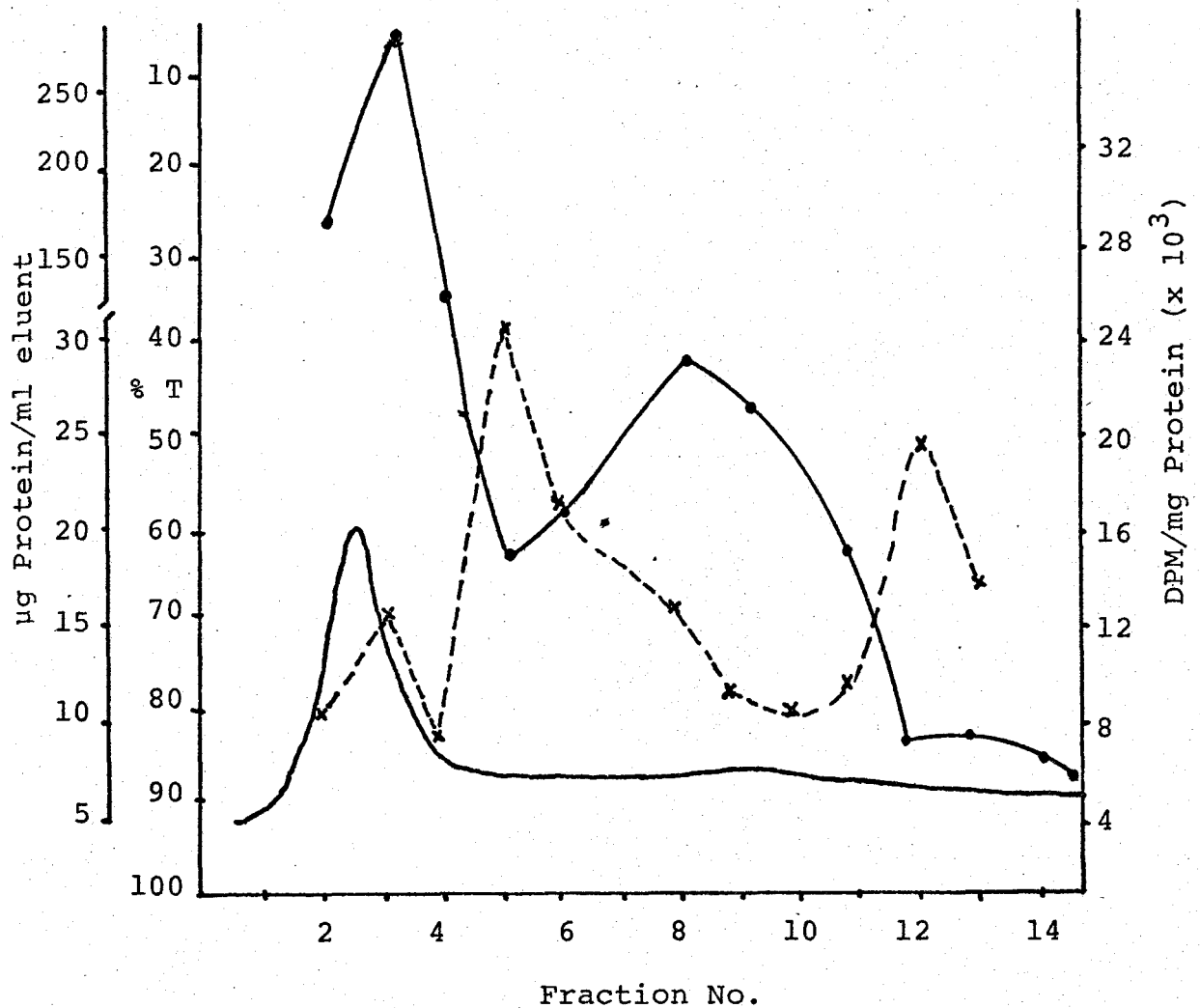The fractions containing hypocalcemic activity were lyophilized

FIGURE 5. Protein content ( ●━━━● ), radioactivity ( x ----- x ) and elution pattern ( ━━━ ) of NaCl precipitated calcitonin preparation on a column of Sephadex G-100. Flow rate: .34.5 ml per hour; 4.0 ml fractions collected. Hypocalcemic activity indicated by horizontal bar ( ⊢━━⊣ ).

and 1.3 mg of solid material was obtained. 600 μg of this material, containing 456 μg of protein, were dissolved in 0.1 $\underline{M}$ acetic acid and used for the assay of hypocalcemic activity. This solution was diluted 1:50 with 0.1 $\underline{M}$ acetic acid and 250 μl of the diluted solution, containing 0.56 μg protein, was injected into each rat. The hypocalcemic activity of this material is shown in Table XV. The average change in serum calcium is - 0.82 mg. When we add the change resulting from the vehicle alone, which is + 0.56 mg we obtain a net effect of - 1.38 mg or an 11% drop in serum calcium. From the definition of a unit, 0.56 μg of protein is equivalent to 11 milliunits and 1.0 mg of protein is equivalent to 19.5 MRC units.

700 μg of the material obtained by lyophilizing the fractions from Sephadex G-100 containing hypocalcemic activity were dissolved in 350 μl of 1.0 $\underline{M}$ formic acid and applied to a 45 x 0.9 cm column of Sephadex G-50 superfine; it was then eluted with 0.1 $\underline{M}$ formic acid - 1.0 $\underline{M}$ urea - 0.2 $\underline{M}$ NaCl. The elution pattern is shown in Figure 6. The hypocalcemic activity is confined to the main protein peak; radio-activity was associated mainly with the fractions containing the hypo-calcemic activity. The active fractions were pooled and lyophilized. The lyophilized material was dissolved in 0.1 $\underline{M}$ acetic acid containing 0.1% albumin and assayed for hypocalcemic activity. The albumin was

## TABLE XV

### Hypocalcemic Activity of Lyophilized Fractions

### from the Sephadex G-100 Column.

| Animal No. | Ca mg% | | $\Delta$Ca mg% | Net Effect[*] $\Delta$Ca mg/0.56 µg Protein |
|---|---|---|---|---|
| | $t_0$ | $t_{50}$ | | |
| 1 | 12.76 | 11.69 | - 1.07 | - 1.62 |
| 2 | 12.67 | 12.16 | - 0.51 | - 1.06 |
| 3 | 12.29 | 11.51 | - 0.78 | - 1.33 |
| 4 | 12.01 | 12.21 | - 0.80 | - 1.35 |
| 5 | 11.94 | 11.00 | - 0.94 | - 1.49 |
| Mean $\pm$ s.e. | | | | - 1.37 $\pm$ 0.09 |

[*]Obtained by adding the mean of the change of the serum calcium of the controls (Table VII) to the change of the serum calcium of each animal that received the experimental preparation.
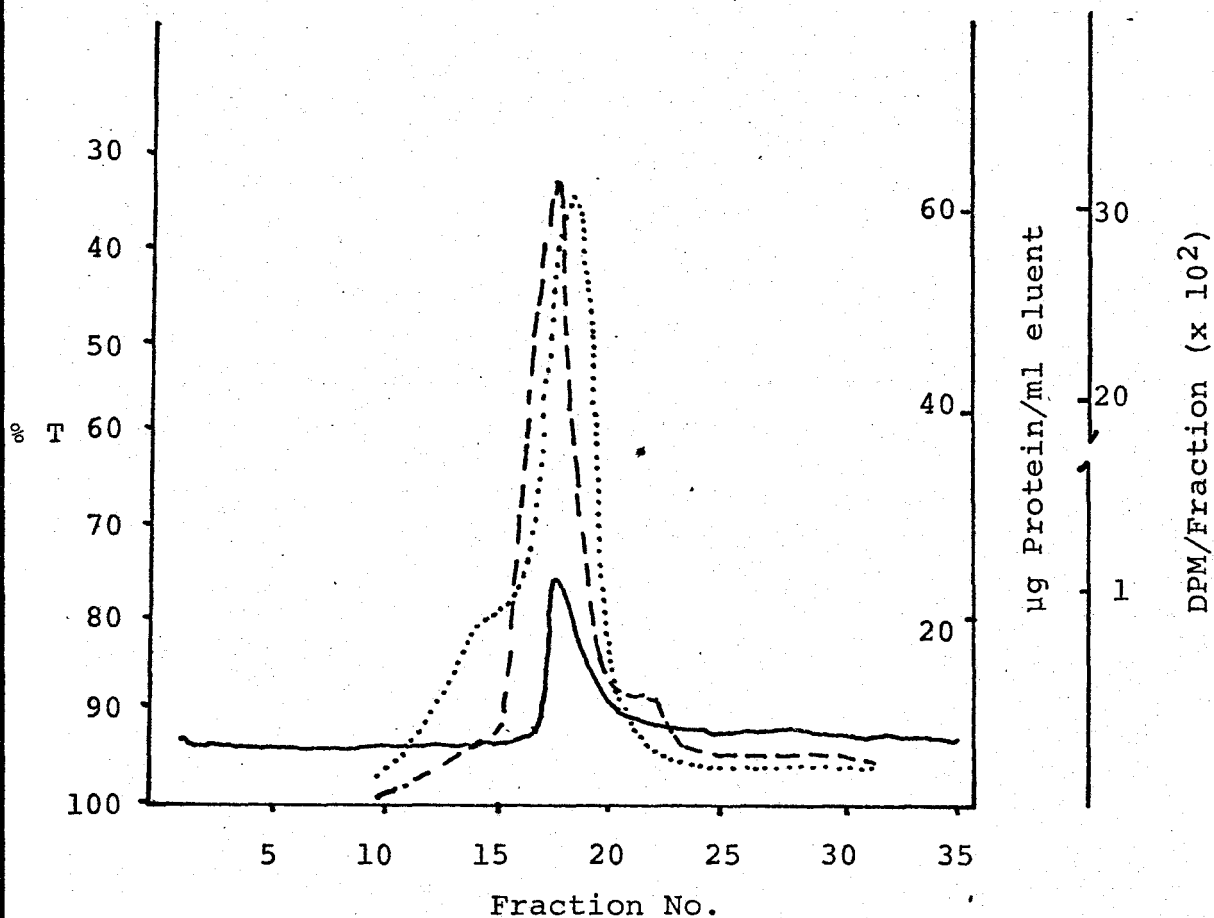
FIGURE 6. Elution pattern of hypocalcemic activity from a Sephadex G-50 superfine column. Flow rate: 6 ml per hour; 1.0 ml fractions were collected. The hypocalcemic activity was eluted as a single peak at fractions 16, 17 and 18.  ⊢————⊣  Hypocalcemic activity. 2x expansion of the % T scale. Protein content ( o ----- o ). Radioactivity ( ..... ).

added to counteract the tendency of calcitonin to become adsorbed to

glass surface of the container (Parsons, 1967). 140-170 gram rats

were injected with 200 μl of the calcitonin solution into the femoral

vein. The control animals received 200 μl of the lyophilized eluent

used to elute calcitonin from the Sephadex G-50 column. The lyophilized

eluent was dissolved in 0.1 $\underline{M}$ acetic acid and administered to the

animals. The hypocalcemic activity is shown in Table XVI. Since

0.18 μg of protein produces an 11.7% fall in calcium, it can be calcu-

lated that the potency of the purified calcitonin is 66.5 MRC units per

milligram protein.


## Characterization of the Purified Calcitonin

To assess the purity of the calcitonin obtained the individual

fractions (16, 17 and 18) containing hypocalcemic activity were chromato-

graphed on a thin layer Eastman Chromatogram Sheet 6065 (cellulose

with fluorescent indicator) and developed with the upper phase of a

butanol: acetic acid: water (4:1:5) system for 2.5 hours. The protein

spots under short ultraviolet light (254 mμ) are visible as dark purple

spots on a yellow fluorescent background. Only one spot for each

fraction was visible (Figure 7), and it appears that the protein in each

fraction is homogenous. Since the spots of all three fractions had the

## TABLE XVI

### Hypocalcemic Activity of Lyophilized Fractions from Sephadex G-50 Superfine

| Animal No. | $Ca^{++}$ mg% ($t_0$) Control | $Ca^{++}$ mg% ($t_0$) Exper. | $Ca^{++}$ mg% ($t_{50}$) Control | $Ca^{++}$ mg% ($t_{50}$) Exper. | $\triangle$ Ca mg% Control | $\triangle$ Ca mg% Exper. | Net Effect* per 0.18 μg Protein | % Change |
|---|---|---|---|---|---|---|---|---|
| 1 | 10.86 | 10.93 | 11.16 | 10.13 | + 0.42 | - 0.80 | - 1.26 | 11.5 |
| 2 | 10.57 | 10.55 | 11.26 | 9.93 | + 0.69 | - 0.62 | - 1.39 | 13.1 |
| 3 | 10.94 | 10.77 | 11.52 | 10.20 | + 0.58 | - 0.57 | - 1.21 | 11.1 |
| 4 | 10.76 | 10.85 | 11.18 | 10.09 | + 0.30 | - 0.76 | - 1.16 | 10.4 |
| 5 | 10.66 | 11.52 | 11.64 | 10.85 | + 0.98 | - 0.67 | - 1.35 | 12.5 |
| Mean ± s.e. | | | | | + 0.59 | - 0.68 | - 1.27 | 11.7 |

*Obtained by adding the mean of the change of the blood of the calcium of the control animals to the change of the blood calcium of each experimental animal.

Solvent Front

16.7 cm

Origin

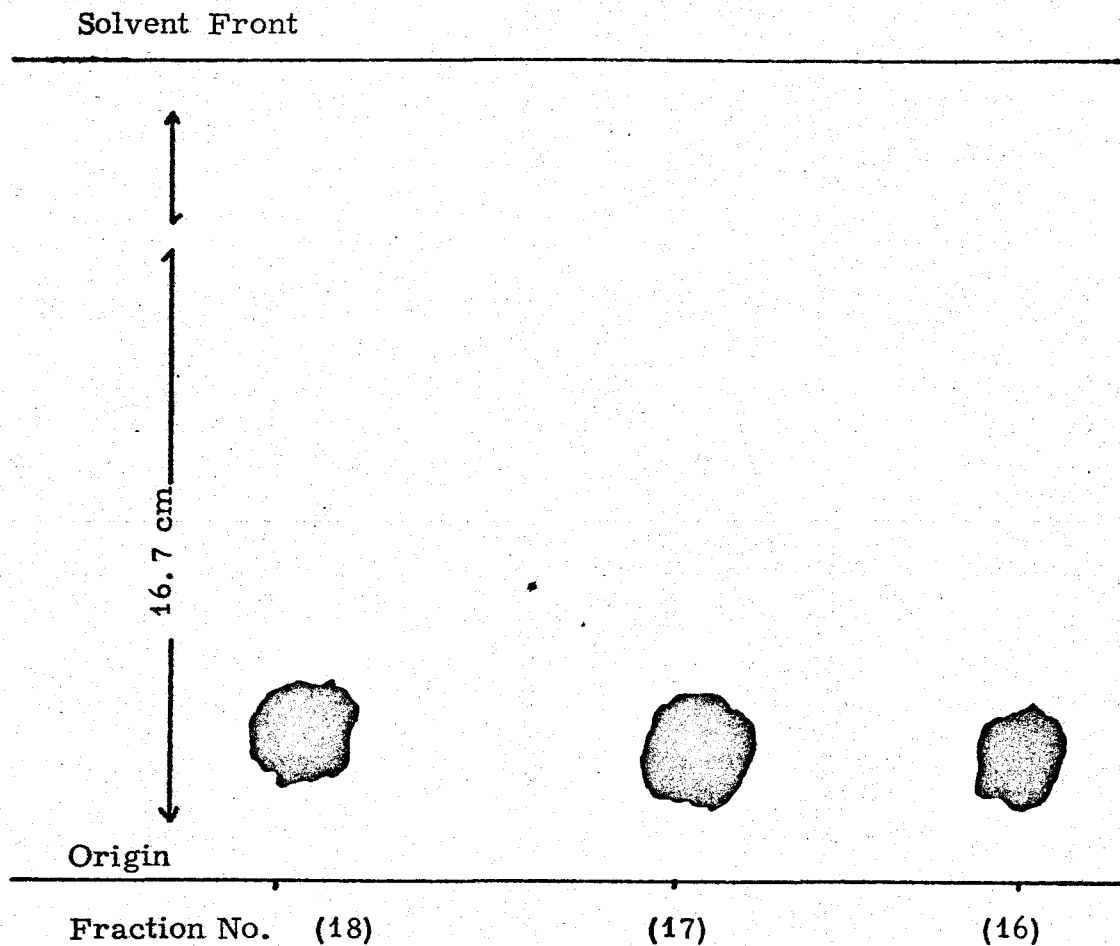Fraction No.    (18)                    (17)                    (16)

FIGURE 7.   Reproduction of thin layer chromatogram of fractions

containing hypocalcemic activity eluted from Sephadex G-50 superfine.

The chromatogram, on Eastman cellulose sheet with fluorescein indicator

was developed in a butanol: acetic acid: water (4:1:5) system for 2.5

hours.

same $R_f$ value (0.116) the protein in all three fractions is presumably

the same.

An aliquot of the combined fractions was subjected to two-dimen-

sional chromatography. The chromatogram was developed in one

direction with the upper phase of a butanol: acetic acid: water system

and in the other direction with a propanol: water (70:30) system. Since

only one spot was visible on the two-dimensional chromatogram (Figure

8), it appears that the preparation was homogenous.

To determine with certainty whether the protein was calcitonin or

not and whether it was contaminated by other protein material 3.0 µg

protein was hydrolyzed for 24 hours at $110^O$ C in 6.0 $\underline{N}$ HCl. The

hydrolysate was evaporated to dryness in a flash evaporator, and twice

redissolved in water and dried again. After the last drying it was

dissolved in 600 µl of 0.01 $\underline{N}$ HCl and analyzed for amino acids on a

TSM Amino Acid Autoanalyzer. Figure 9 shows the pattern of eluted

amino acids. The absence of lysine and isoleucine are strong indica-

tions that the peptide is calcitonin. The glycine peak was collected and

evaporated, almost to dryness, and diluted to 10 ml with water. A 100

µl aliquot was measured for radioactivity and found to contain radio-

activity.
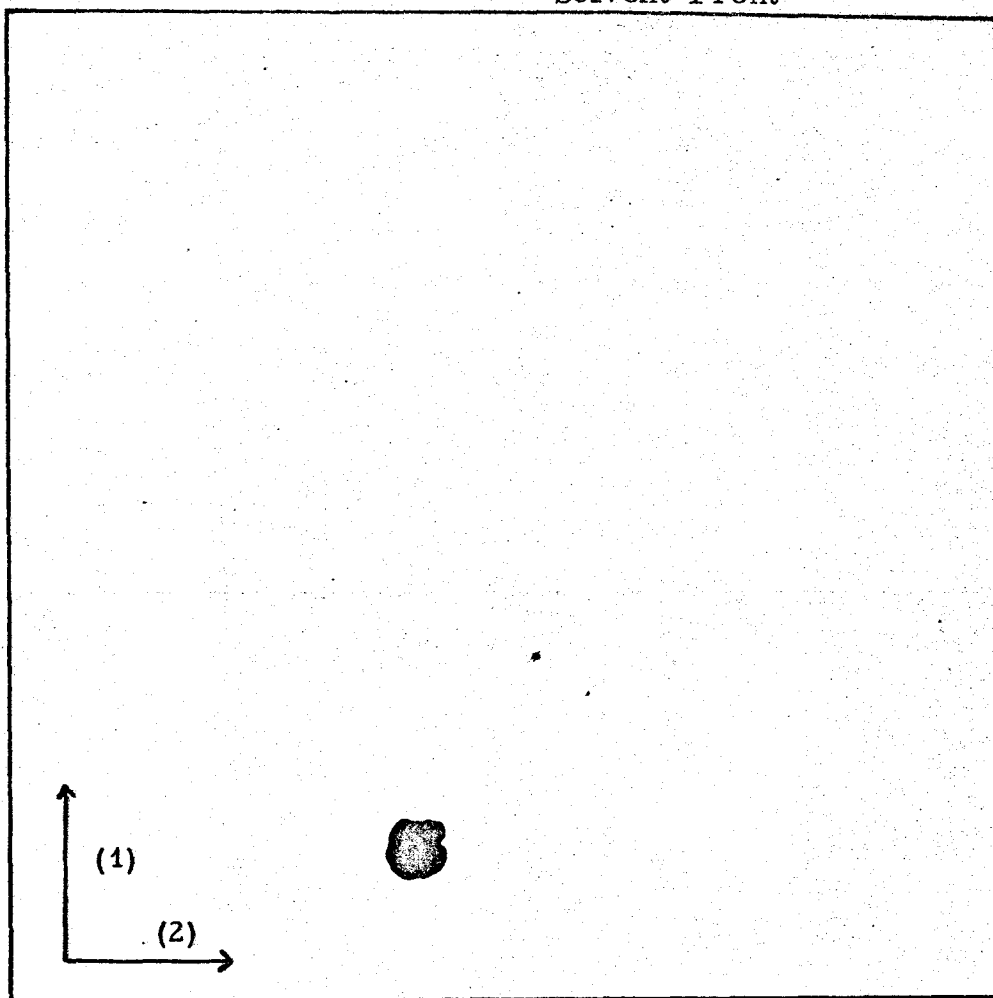
FIGURE 8. Reproduction of two-dimensional chromatogram of lyophilized hypocalcemic active fractions eluted from Sephadex G-50 superfine. The chromatogram on Eastman cellulose sheet was developed in direction 1 with a butanol: acetic acid: water (4:1:5) system and in direction 2 with a propanol: water (70:30) system.
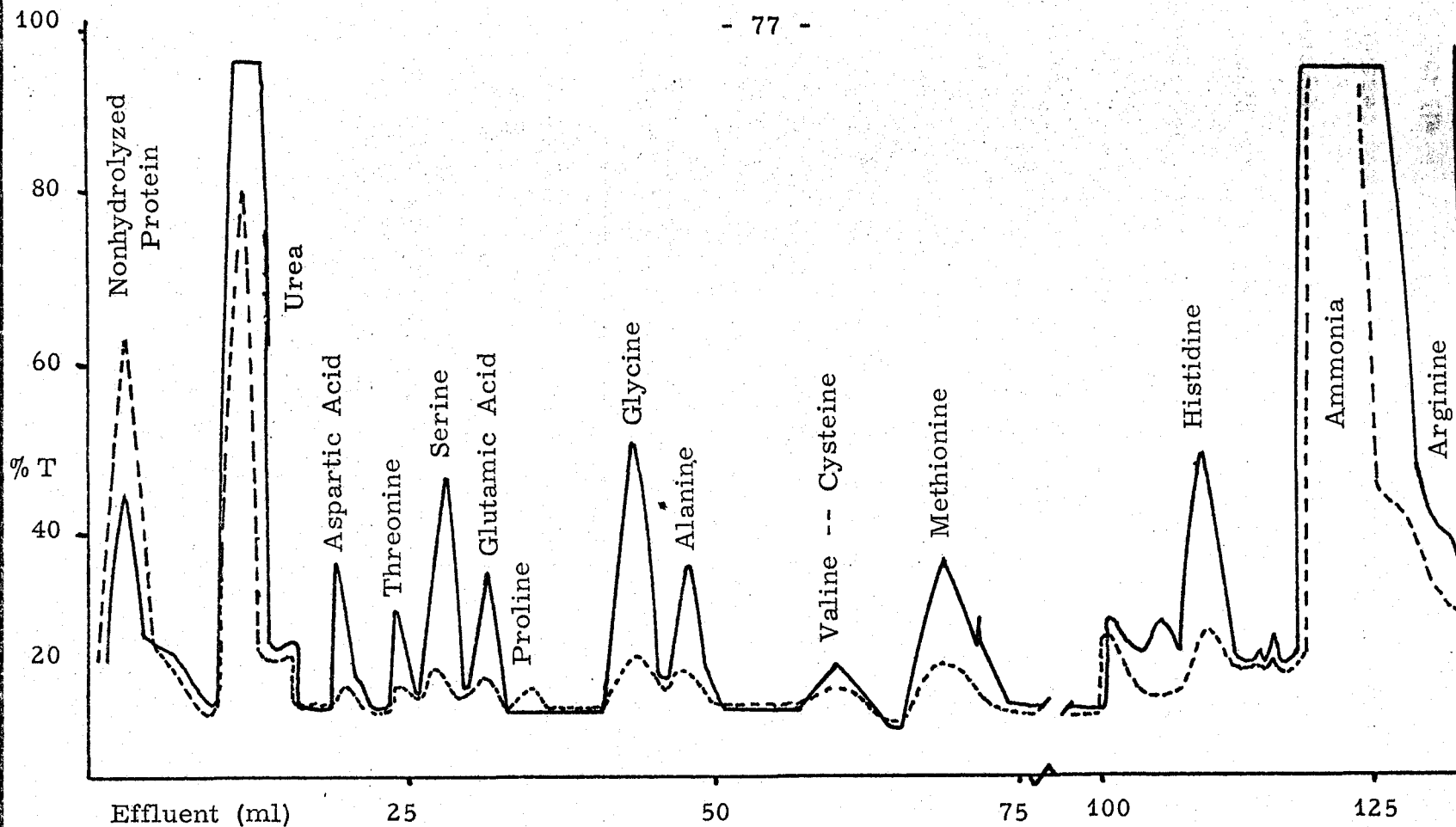
FIGURE 9. Record of the % T of ninhydrin color emerging from the TSM Amino Acid Analyzer during the amino acid analysis of the 24 hours hydrolysis of calcitonin.

## CHAPTER IV

## DISCUSSION

From information provided by previously reported studies on the in vitro biosynthesis of several polypeptide hormones (Adiga et al. 1965; Bauer and Lazarow, 1961; Mallory et al. 1964; Sacks, 1963; Steiner and Oyer, 1967) it appeared likely that the biosynthesis of calcitonin could be demonstrated in vitro. To our knowledge, no attempts have been made to demonstrate the synthesis of calcitonin.

In this study the composition of the incubation medium necessary to give high incorporation of glycine-C-14 into the protein fraction of the thyroid tissue was defined. It was found that the addition of glucose (320 mg/100 ml) to Krebs-Ringer bicarbonate buffer (pH 7.4) increased incorporation markedly (200%). Glucose acts as the energy source for biosynthesis. The addition of the 15 amino acids of calcitonin to the Krebs-Ringer buffer had very little effect on the incorporation of label in proteins. In this instance, as was the case when no additions were made, the lack of an energy source would limit biosynthesis and, thus, the observed glycine-C-14 incorporation. The addition of both glucose and the 15 amino acids of calcitonin increased incorporation of glycine-C-14 by 100%, less than when glucose alone was added. The reason for this inhibitory effect of the amino acids is not clear. In all the

incubations the buffer which contained both glucose and amino acids was used. The inhibitory effect of the amino acids is possibly due to the suppression of synthesis of thyroid proteins in favor of the synthesis of those peptides that contain only the exogenously added amino acids for the de novo synthesis of proteins would have to come from the limited pool of free amino acids already existing in the tissue slices, from breakdown of proteins and from gluconeogenesis, with the result that many peptides and proteins would be synthesized. When exogenous amino acids were added the system would use these preferentially resulting in the preferential synthesis of those proteins and peptides made up of the exogenous amino acids, and since calcitonin is one of these peptides its synthesis would be preferentially stimulated.

To demonstrate the synthesis of calcitonin, ovine thyroid glands were incubated in Krebs-Ringer bicarbonate buffer for 4 hours at $37^\circ$ C. After incubation the slices were defatted and extracted with 10 volumes of 8.0 $\underline{M}$ urea - 0.1 $\underline{M}$ cysteine in 0.2 $\underline{N}$ HCl. The urea-cysteine-HCl system has been used with success in the extraction of calcitonin by Potts et al. (1967). For the isolation of calcitonin from the extract, the method of Gudmundsson et al. (1966) was used. Calcitonin was isolated through the second sodium chloride precipitation step. The precipitate was lyophilized, dissolved in 0.1 $\underline{M}$ acetic acid and administered intra-

venously to 140-170 gram rats. When the hypocalcemic activity of incubated and non-incubated glands was compared, no difference was observed. Since calcitonin titers in the circulation seem to be controlled by the blood calcium concentrations, the calcium concentration in the media was increased in an attempt to stimulate calcitonin synthesis. A four-fold increase in calcium markedly stimulates calcitonin synthesis. In vivo studies by Gittes et al. (1968) have shown that the extractable content of calcitonin in the thyroid gland is markedly reduced after hypercalcemic stimulation. Care et al. (1968), on the other hand, observed no difference in the calcitonin content of the thyroids from pigs subjected to hypercalcemia and from normal pigs. Our results are in disagreement with the findings of Gittes and Care. The reason for the conflicting results is due to the different methods used. Both Gittes and Care made their measurements in vivo, where measurements of the calcitonin content of the thyroid glands are complicated by the fact that during the time of perfusion with high calcium synthesis may occur, but it may be masked by the continuous secretion and destruction of the hormone at the target organ.

In an effort to determine whether the stimulation of calcitonin synthesis was a specific effect of calcium or whether magnesium could substitute for calcium, the concentration of magnesium was increased in

such a way that the ionic strength of the **high** calcium and high magnesium media were the same. Magnesium was **chosen** because about 60% of the total body magnesium is located on the **surface** of bone, incapable of penetration into the lattice interior. It **exchanges** for calcium in bone on a mole-for-mole basis (Blaxter, 1956). **It has** also been shown that the levels of serum and urinary magnesium **are** controlled by parathyroid hormone in a manner similar to that of **calcium** (Bethune et al. 1968). The results of this study show that magnesium **did** not significantly affect the synthesis of calcitonin, therefore, **the** mechanism by which calcium stimulates the synthesis of calcitonin is not affected by magnesium. The molecular basis for the calcium activation **of** the synthesis of this particular peptide is completely unknown. It **may** increase the uptake of amino acids by the cells; in the parathyroid it **has** been shown to decrease the uptake of the amino acid analog α-aminoisobutyric acid (Raisz and O'Brien, 1963). Further studies would **be** needed to establish how calcium brings about its effect.

When calcitonin is injected into rats **it** produces hypocalcemia which starts a few minutes after administration of the hormone and reaches a maximum at about one hour **and** returns to normal within three hours (Kumar et al. 1965; Hirsch et al. 1964). It has been demonstrated both in vivo (Milhaud et al. 1965b; Martin et al. 1966) and

in vitro (Friedman and Raisz, 1965; Aliapoulios et al. 1966) that calcitonin produces its hypocalcemic response by inhibiting bone resorption. MacIntyre (1967) has shown that the hormone is biologically active on isolated, perfused cat tibia proving that the effect is a direct one. The mechanism that controls the titer of the circulating hormone seems to be similar to that controlling parathyroid hormone. Secretion of parathyroid hormone has been shown to be controlled by blood calcium concentration; hypocalcemia enhancing secretion and hypercalcemia abolishing secretion (Sherwood et al. 1968). Calcitonin has also been shown to be dependent upon blood calcium. Work by Care et al. (1967) indicated that the secretion of calcitonin was controlled by a negative feedback which operated through the blood calcium concentration. Care et al. (1968) measured the secretion of calcitonin in pigs in vivo and were able to demonstrate that there was a linear relationship between calcitonin in the venous blood and the concentration of calcium in the perfusing medium over a range of calcium of 6-10 meq/liter. Below 6 meq/liter of calcium they were not able to detect calcitonin. Lee et al. (1969) have shown that calcitonin is secreted at normal blood calcium concentrations and even below. The manner by which calcium controls the secretion rate of calcitonin is unclear. It could act directly on the secretion granules of the C cells or its action may be mediated by an un-

known messenger. Gittes and Irvin (1965) suggested that a parathyroid factor may have been responsible for release of calcitonin; subsequent studies, however, failed to confirm their hypothesis (Foster, 1968; Toverud et al. 1966). Control of calcitonin secretion by the pituitary gland has been investigated and there is no evidence of such a control (Milhaud and Mauktar, 1965). Recently Avioli et al. (1968) have found that calcitonin is released by glucagon. The results obtained in this investigation support the hypothesis that calcium exerts a direct effect on the release of calcitonin from the thyroid gland; in fact, an increase in the concentration of calcium in the incubation media led to an increase in the release of the hormone. From the data obtained, it can be calculated that the glands incubated in Krebs-Ringer buffer containing a high calcium concentration, 500 MRC milliunits, of calcitonin were released per gram of glands per 4 hours. The glands incubated in buffer containing physiological concentrations of calcium released 442 MRC milliunits calcitonin per gram per 4 hours.

Since the hypocalcemic effect observed upon administration of the calcitonin preparations may have been the result of some unknown impurity, the hormone was purified. For purification the method of Gudmundsson et al. (1966) was used. The lyophilized material, obtained by precipitation in 2.0 $\underline{M}$ NaCl, was dissolved in 0.1 $\underline{M}$ formic acid

and applied to a Sephadex G-100 column. The fractions containing hypocalcemic activity were combined and lyophilized. The lyophilized material was found to have very high biological activity; 0.56 μg protein lowered the serum calcium of rats by 11% which corresponds to 11 MRC milliunits activity by definition; it can be calculated that the activity of this fraction was 19.5 MRC units per mg protein. 700 μg of the lyophilizate was dissolved in 0.35 ml of 1.0 M formic acid and applied to a 45 x 0.9 mm column of Sephadex G-50 superfine. The hypocalcemic activity was found in fractions 16, 17 and 18. These three fractions comprised the main protein peak. These fractions were combined and lyophilized and the hypocalcemic activity assayed. It was found that 0.18 μg of protein decreased the serum calcium of rats by 12%, which is equivalent to 12 MRC milliunits activity or 66.7 MRC units per milligram protein. The activity of chemically synthesized calcitonin is much higher, about 200 MRC per milligram. The lower activity of our preparation is possibly due to denaturation which occurred during isolation and purification, or to species differences.

The protein in all three fractions was homogenous on thin layer chromatography and the $R_f$ value was the same, indicating that the protein was identical in the three fractions. An aliquot of the pooled fractions was found to be homogenous on two-dimensional thin layer

chromatography. Amino acid analysis of an aliquot of the combined fractions was found to lack both lysine and isoleucine and since porcine calcitonin lacks these two amino acids, it is further proof that calcitonin was actually isolated and purified, and that the hypocalcemic activity observed was due to calcitonin and not to some other unknown agent. However, the possibility that ovine calcitonin has an amino acid composition different than porcine calcitonin should be considered, since human calcitonin has been found to have many amino acid residues which are not found in the porcine hormone (Neher et al. 1968).

The possibility that calcitonin can be synthesized in an in vitro system opens the way for further studies. The hormone with a radioactive label can be used to trace the locus of action at the cellular level at the target organs. The mechanism and the site of synthesis can be studied by the use of protein synthesis inhibitors and cellular fractionation. Since the hormone is present in granules in the C cells it would be of interest to study the locus of synthesis and the transport of the hormone to the granules. Another aspect of calcitonin synthesis amenable to study with the system developed in this investigation is whether the hormone is synthesized as a 32 amino acid polypeptide or whether it has a precursor molecule, and if so, when and how it is transformed into the biologically active polypeptide which is found in the thyroid glands.

# CHAPTER V

## SUMMARY AND CONCLUSIONS

Recently the in vitro biosynthesis of polypeptide hormones has been reported. In this investigation was presented a method for the biosynthesis of calcitonin in ovine thyroid slices. Ovine thyroid tissue is contaminated with the inferior parathyroid tissue, whereas the superior parathyroids are anatomically separated. We did not attempt to separate the parathyroid tissue, since the calcitonin synthesizing cells are "adventitious" to the thyroid and in some species to the parathyroids and thymus (MacIntyre, 1968); furthermore, since it is now well established that there is only one calcitonin it was thought that if the sheep parathyroid tissue contained C cells the calcitonin synthesized by these cells would not interfere with the purpose of our work. The biosynthesis of the hormone opens the way for studies on the mechanism and control of synthesis and release of the hormone.

In this study it was attempted to establish the role played by calcium in the regulation of synthesis and release of calcitonin. It has been shown that in vivo calcitonin increases in the serum of animals in response to a hypercalcemic stimulus. In vivo studies, however, cannot discern whether the hypercalcemia increases synthesis, release or both. The results presented in this investigation show that calcium increases

both the synthesis and release of the hormone. Other factors may exist, and recently Avioli et al. (1968) has found evidence that glucagon may release calcitonin from the thyroid glands. If this is true the effect of glucagon is probably not related to the calcium effect, since calcium can act in vitro, and the effect is a specific property of the calcium ion. In fact, magnesium cannot substitute for calcium. This may have been anticipated since calcitonin does not affect magnesium levels in the blood (Gitelman et al. 1965).

The purified hormone was found to have very high specific activity, approximately 67 MRC units per milligram protein. The hormone was found to be pure and homogenous by thin layer chromatography and amino acid analysis.

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# ABSTRACT

Abstract of the dissertation entitled "THE IN VITRO BIOSYNTHESIS

OF CALCITONIN" submitted by Nino Sorgente in partial fulfillment of

the requirements for the degree of Doctor of Philosophy, June, 1969.


Slices of ovine thyroid glands were incubated (4 hr., $37^{\circ}$ C) with

glycine-C-14 in Krebs-Ringer bicarbonate buffer (pH 7.4) containing

glucose (320 mg/100 ml) and the amino acids of calcitonin (20 mg/l).

Calcitonin was isolated by sodium chloride precipitation from the

incubated slices, and the hypocalcemic activity assayed in 140-170 gram

rats fasted for 18 hours. The hypocalcemic activity was expressed as

the change in serum calcium between time 0 and 50 minutes per 10 or

25 μg protein of the calcitonin preparation.

The hypocalcemic activity of calcitonin isolated from glands

incubated in buffer containing physiological concentrations of calcium

(11.5 mg%) was not significantly different from the hypocalcemic activity

of calcitonin isolated from unincubated glands. However, when the

hypocalcemic activity of calcitonin isolated from slices incubated in

buffer containing high concentrations of calcium (45.6 mg%) was com-

pared to the hypocalcemic activity of calcitonin isolated from slices

incubated in buffer containing physiological concentrations of calcium, it

was found that the hypocalcemic activity increased 50%. An increase

in the magnesium concentration of the buffer from 3.7 mg% to 29.8 mg% had no significant effect on the hypocalcemic activity of the calcitonin preparations.

To determine whether calcium had an effect on the release of calcitonin from the glands, at the end of the incubation the media was assayed for hypocalcemic activity. A significant increase in hypocalcemic activity was found in the incubation media containing high concentrations of calcium as compared to the media containing physiological concentrations of calcium.

The calcitonin from incubated slices was isolated by sodium chloride precipitation and purified by passage of the isolated material on columns of Sephadex G-100 and Sephadex G-50 superfine. The purified material was homogeneous on thin layer chromatography and contained very high hypocalcemic activity (66.5 MRC units per milligram protein). The purified material was subjected to amino acid analysis, and it was found to contain all amino acids of porcine calcitonin, except leucine, phenylalanine and tyrosine. The glycine peak was collected and was found to contain radioactivity.

In this work, a method for the biosynthesis of calcitonin in ovine thyroid slices was developed. Using this method it has been shown that both the synthesis and release of calcitonin are under the control of calcium ions. Magnesium ions cannot substitute for calcium to stimulate the synthesis of calcitonin.

APPROVAL SHEET

The dissertation submitted by Nino Sorgente has been read and
approved by a committee from the faculty of the Graduate School.

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

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

_May 26, 1969_                          _Maurice V. L'Heureux_

Date                                    Signature of Advisor