PITUITARY STIMULATION OF PARATHYROID HORMONE SECRETION

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PITUITARY STIMULATION OF PARATHYROID

HORMONE SECRETION

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NOMENCLATURE

PTH	-	parathyroid hormone
iPTH	-	immunoreactive parathyroid hormone
c-iPTH	-	immunoreactive parathyroid hormone detected by GP-5 antibody
n-iPTH	-	immunoreactive parathyroid hormone detected by R-4 antibody
RIA	-	radioimmunoassay
HBSS	-	Hanks Balanced Salt Solution
BSA	-	bovine serum albumin
TSH	-	thyroid stimulating hormone
GH	-	growth hormone
LH	-	luteinizing hormone
ADH	-	antidiuretic hormone
MSH	-	melanocyte stimulating hormone
IU	-	international unit

CHAPTER I

INTRODUCTION

"The living being is stable" (10). This stability is dependent upon many regulatory mechanisms which are activated when the normal steady state is disturbed. The six propositions regarding factors maintaining the steady state in the body (Appendix A) made by Cannon (22) in 1925 form the assumptions upon which the work in this dissertation is based.

The earliest mechanism demonstrated for the control of parathyroid hormone (PTH) secretion was the blood calcium concentration (50). A critical experiment using bioassay was performed by Patt and Luckhardt (74) in 1942 which firmly established the stimulatory effect of hypocalcemia on PTH secretion. McLean (70) in 1957 formalized this concept of a parathyroid secretion regulatory mechanism. He postulated that the hypercalcemic hormone (PTH) secretion was controlled by a negative feedback of the serum calcium directly on the parathyroid. This concept was later verified in the cow by radioimmunoassay (RIA) in 1966 (76) (90). Rather than linear as the previous work implies, the PTH secretory response to ionized calcium was found to be inversely related in a sigmoid manner (18) (67) (68). This work depicts the secretory response of PTH as inverse and linear between 7.5 and 10.5 mg calcium/100 ml plasma. At concentrations greater than 10.5 mg/100 ml the PTH secretory rate is non-suppressible. At calcium concentrations less than 7.5 mg/

100 ml, the rate appears to be insenitive to further stimulation by calcium reduction.

A negative feedback mechanism for regulation of PTH secretion by magnesium was proposed by MacIntyre (63) in 1963. He placed the primary site of action of PTH, as it relates to magnesium, at the kidney. Since the calcium/magnesium ratio in bone is about 50/1, he felt bone would be a primary site of action for calcium but not magnesium. PTH, however, is thought to equally affect calcium and magnesium retention by the kidney. This hypothesis implies that calcium and magnesium homeostasis are interdependent and that calcium homeostasis will not be maintained if the magnesium concentration is markedly abnormal. Such interdependency has been recorded (45) (51). This negative feedback has been demonstrated "in vivo" (20) (27) and "in vitro" (88) (98) within the normal plasma magnesium range by RIA. There remains some question as to whether the inverse effect of calcium on PTH secretion is equipotent, on a molar basis, to magnesium (98). Extreme hypomagnesemia, however, appears to inhibit PTH secretion, even when accompanied by hypocalcemia (5) (6) (92) (96). Whether the response of the parathyroid gland to magnesium concentration represents a meaningful physiological regulatory mechanism or a pathological one is still in question (65) (104).

Vitamin D has been shown to affect parathyroid gland activity. Capen (24) (25) (26) demonstrated that an increase in the amount of Vitamin D fed to cows resulted in a reduction of parathyroid prosecretory granules and other structural and ultra-structural changes that were interpreted as indicative of a reduced secretory rate. The exposure of parathyroid glands "in situ" to 24,25 dehydroxycholecalciferol

(DHCC) has been shown to reduce PTH output (15). This work indicates a direct inhibition of the PTH secretion. The more physiologically active 1,25 DHCC has also been shown to inhibit PTH secretion (30). This relationship may contribute to the reciprocal control of 1,25 DHCC metabolism in the kidney.

Epinephrine has been suggested as a secretagogue for PTH. Experiments in the cow demonstrated an increase in peripheral plasma concentration of immunoreactive PTH following injection of epinephrine (39). "In vitro" work has yielded contradictory results. Measurement of canine parathyroid adenyl cyclase failed to show stimulation with epinephrine (34). Incubation of gland slices with epinephrine, however, increased the release of hormone into the media (103). Although the significance of epinephrine in physiological regulation of PTH secretion is questionable, there does seem to be a possible correlation in certain pathological conditions (1) (59) (60).

Calcitonin has been observed to increase PTH secretion. This may be an indirect effect resulting from a lowered plasma calcium concentration or directly by promoting a transient calcium efflux from parathyroid cells (65). This transient efflux may be sufficient to lower cytosol calcium ion content and thereby activate PTH secretion. The physiological significance of a direct calcitonin regulation of PTH secretion is questionable.

The anterior pituitary has been implicated by indirect evidence in the regulation of PTH secretion. The possibility of an anterior pituitary-parathyroid axis will comprise the main portion of this dissertation. A review of some of the more pertinent literature is made in Chapter II.

In summary, the calcium ion concentration in plasma is generally accepted as the predominate regulator of the PTH secretory rate. Magnesium and Vitamin D may play a lesser role in regulating parathyroid activity under normal, non-pathological conditions. Regulation in pathological states may also include epinephrine and calcitonin.

CHAPTER II

LITERATURE REVIEW

Indirect evidence has accumulated in the literature which suggests a direct functional relationship between the pituitary and the parathyroid glands. The evidence can be organized into three major categories: pituitary lesions, hypophysectomies, and anterior pituitary extract injections.

Observations of pituitary lesions and concomitant abnormalities of bone, plasma calcium, and parathyroid tissue have been recorded. Several cases of pituitary adenomas involving basophilic cells have been reported in humans which also exhibited osteitis fibrosa cystica (36) (83). Marked enlargement of human pituitaries associated with acromegaly have also exhibited an increase in serum calcium (85). A case of basophilic pituitary adenoma has been reported with adenomatous hyperplasia of parathyroid glands and osteitis fibrosa (36). In 1936, Houssay (55) reviewed 24 cases of hyperpituitarism and found 14 instances of osteoporsis and decalcification of bone. He found these 14 cases to have 3 parathyroid adenomas, 3 enlarged parathyroids, 1 atrophied parathyroid, and 6 normal appearing parathyroids.

Experimental hypophysectomy has yielded interesting and conflicting results concerning serum calcium concentrations and parathyroid tissue responses. A number of hypophysectomy experiments on toads resulted in a significant decrease of serum calcium concentrations

(28) (53) (86) (87). Similar work on pigeons also yielded a reduction in serum calcium (78). Hypophysectomy in pigeons followed by injections of PTH were found to return the serum calcium to normal (78). A lack of serum calcium response to hypophysectomy was noted in dogs (42) (64) (69) (93) and in rats (7) (101). (Of interest, and perhaps significance, is that the negative results regarding serum calcium response usually, if not always, were in experiments where the measurements were not made for seven or more days after the hypophysectomy.) Hypophysectomy in dogs, rabbits, monkeys, and rats has, however, been reported to result in atrophy and degeneration of the parathyroid tissue (13) (55) (57) (92) (102). In 1972, Salzer (80) reported a 13.5 percent reduction in rat parathyroid cell nuclear size and alterations in secretory granules following hypophysectomy . Negative results were reported, however, in the dog (11). Removal of the pituitary has also been observed to decrease the rat's ability to counter a challenge to its serum calcium concentrations (80) (81) (82).

Since extirpation of the pituitary in toads can result in serum calcium and parathyroid tissue perturbation, replacement therapy provided another avenue of inquiry. Shapiro (85) (86) found that while hypophysectomy reduced the serum calcium to 7.4 mg/100 ml, injection of an anterior pituitary extract then increased the serum calcium to 8.9 mg/100 ml.

Anterior pituitary extracts have also been reported to increase serum calcium concentrations in intact animals. Work on dogs (8) (37) (43) (64), rats (8), guinea pigs (37) (38), and cats (85) demonstrated that an extract of the hypohysis can increase serum calcium concentrations above normal. Hypertrophy of parathyroid glands in rats and

rabbits (8) and hypertrophy and hyperplasia of the glands in rabbits (53) has been reported following anterior pituitary extract injections. Parathyroidectomy followed by injections of the same extracts used in many of the positive experiments above resulted in no change in serum calcium concentration in dogs and rats (64) (79).

Of the known pituitary hormones, three have been implicated in plasma calcium anomalies by indirect evidence. Growth Hormone (GH) has been considered a possible secretagogue of PTH (61). Hyperparathyroidism has also been occasionally associated with acromegaly (97). Thyrotropin (TSH) has been implicated because of an occasional concomitant occurrence of pseudohypoparathyroidism and thyrotropin deficiency (105). Similarly, a prolactin deficiency has been reported in some patients with pseudohypoparathyroidism (29). A study on mud puppies has also implicated prolactin (72).

In 1934, Hertz and Kranes (52) produced an extract which appeared to stimulate parathyroid activity sufficiently for them to theorize about the possibility of a separate "parathyreotropic" action originating in the anterior pituitary. Houssay (55) in 1936, reviewed the literature and formalized the concept of a "parathyrotropic hormone" in the anterior pituitary. The indirect evidence has also led to a negative interpretation (21). An "hypophyseal parathyrotropic hormone" was suggested by Salzer (80) in 1972 which would serve to meet endogenous calcium requirements of organisms under special circumstances such as lactation.

The major purpose of this dissertation was to investigate the possibility of a direct pituitary-parathyroid relationship. The work was divided into four main segments: 1) measure the acute effect of

hypophysectomy on the serum calcium of rats, 2) measure the effect of injecting a pituitary extract on the serum calcium of cows, 3) establish a perfusion system which would allow a direct, minute to minute assessment of effect of suspected secretagogues on parathyroid glands, and 4) assess the direct response of perfused parathyroid gland to various pituitary extracts and hormones.

CHAPTER III

METHODOLOGY

The investigation described in this dissertation used three experimental approaches: hypophysectomy, pituitary extract injections, and "in vitro" parathyroid gland perfusion. The two major responses observed were plasma calcium and immunoreactive parathyroid hormone (iPTH) concentration changes.

Hypophysectomy

In this operation, male Wistar strain rats weighing 220 grams ($\pm$ 20 grams) were used. The pituitary was removed from eight animals (anesthesized with ether) by suction using an 18 gauge needle inserted through the auditory canal into the sella tursica (58). A sham operation was performed on 12 similar rats for control purposes. Immediately after the operation, each animal was marked for identification and injected with 1.0 cc "Liquamycin" intra-peritoneally.

Two control animals were reanesthesized immediately after their sham operation and an 8 cc sample of blood removed from the abdominal aorta. Two drops of heparin (1000 U/ml) from a 20 gauge needle were added to the sample to retard coagulation. The samples were immediately centrifuged, the plasma removed and frozen at -70°C. Samples were taken from control (sham operated) and hypophysectomized animals at 24, 36, and 60 hours post operative and treated in a similar manner. Post

mortem examination was performed on all animals to verify presence or total absence of the pituitary gland. Plasma was analyzed for calcium concentration.

Pituitary Extract Injection

In this procedure three calves were given a local anesthetic (2.5 mls of zylocaine) subcutaneously in the neck over the jugular vein. A percutaneous catheter was inserted about eight inches into the jugular vein and sutured to the skin. This externalized catheter was kept patent by placing 0.5 ml of a weak heparin solution (2 U/ml) in the tube. The calves were allowed to adjust to their new situation for one hour. Blood samples (10 cc) were taken every 15 minutes from the indwelling catheter for one hour following the adjustment period.

An anterior pituitary extract (Preparation A) was injected via the catheter after these four control blood samples were drawn. The single injection of Preparation A was given as a saline solution at a dosage of 1.0 mg/67 kg body weight. This dosage was dissolved in 4 cc of 0.9 percent saline and the catheter was then flushed with an additional 4 cc of saline. (See Appendix B for a description of Preparation A.)

A 10 cc blood sample was drawn every 15 minutes following the injection for 4.5 to 9.5 hours depending on the plasma calcium response. All samples were then prepared with three drops of heparin (1000 U/ml) from a 20 gauge needle to prevent coagulation. The samples were centrifuged and the plasma immediately removed and frozen at -70°C. All food and water was withheld from the calves during the experiment. Samples were analyzed for plasma calcium concentration and iPTH.

"In Vitro" Perfusion

Bovine parathyroid glands were collected at a local abattoir. Immediately upon decapitation (5 to 15 minutes after killing the animal) the superior parathyroid glands were removed. A description of the gland location and identification in the slaughtered bovine has been reported previously (54). The glands were removed with their arterial connection to the carotid artery attached and immediately placed in cold (0-4°C) Hanks Balanced Salt Solution (HBSS) buffered to pH 7.4 for transportation to the laboratory.

The parathyroid artery was cannulated with "intramedic" polyethylene tubing P.E. 50 (I.D. 0.023", O.D. 0.038") and all accessory or branch arteries tied off. Any extraneous tissue was removed. The glands were then perfused with HBSS oxygenated with 95 percent O_2 and 5 percent CO_2 in a membrane oxygenator (Rocky Mountain Scientific Glassblowing Co., Denver, Colorado) and buffered with sodium bicarbonate to pH 7.4. Two perfusion rates, 0.5 and 1.0 ml/minute, were tested using a peristaltic, low-pulsatile pump (Sage model 375A). The perfusion pressure was monitored by a U-shaped mercury manometer. Two syringe pumps were connected into the perfusion system to enable the addition of test compounds. The glands were perfused in a reaction vessel surrounded by approximately 0.3 ml of HBSS. An overflow sidearm allowed collection of the effluent (Figure 1). The entire system was maintained in an environment chamber at 37°C. Parathyroid effluent and perfusate samples were collected in one dram glass vials and immediately frozen at -70°C.

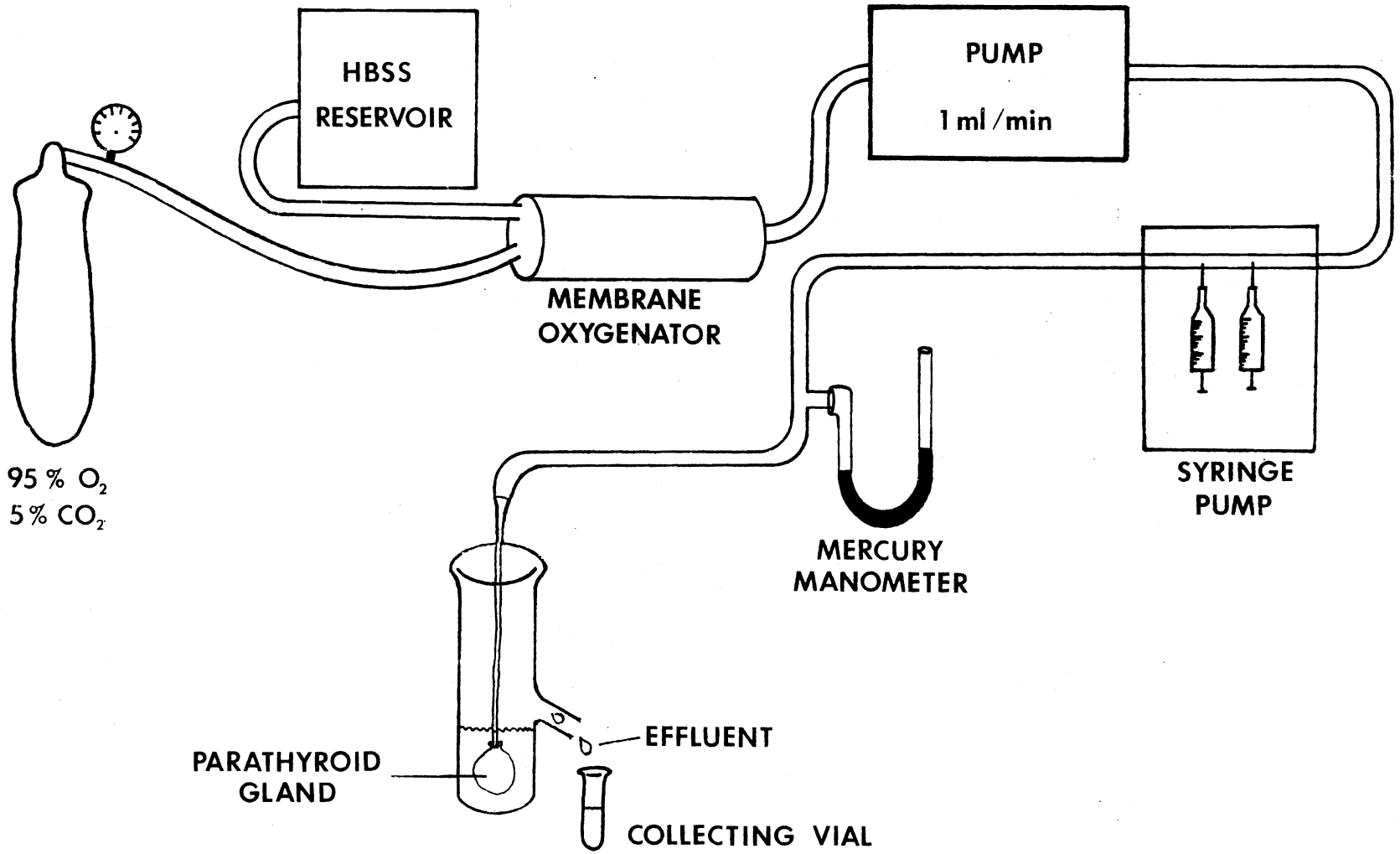


Figure 1. Perfusion System

In experiments which required a change in calcium concentration, either $CaCl_2$ or Na_2EDTA was added with a syringe pump (Harvard model 975). Calcium concentration was decreased from the 4.5 mg/100 ml HBSS by adding Na_2EDTA in increasing steps every 10 minutes. An increase was affected by adding $CaCl_2$ in increasing amounts to the HBSS perfusate at 10 minute intervals. Samples were collected at the end of every 10 minute period. The glands were perfused, therefore, at each calcium concentration for 10 minutes prior to sample collection.

Seven pituitary preparations dissolved in 0.9 percent saline and a control solution of 0.9 percent saline were individually added to the HBSS to test for secretagogue activity. Table I shows the major known hormonal activity and the concentration at which each preparation was perfused through the parathyroid glands. (Appendix B through H describes each preparation in detail.) In addition, Preparation A was repeatedly frozen and thawed (12 cycles) and tested on the "in vitro" perfused parathyroid glands. Each preparation was tested for twenty minutes.

Radioimmunoassay

A radioimmunoassay (RIA) of parathyroid hormone was first described in 1963 (17). The specific procedures used in the work in this dissertation have been fully detailed elsewhere (48) (84) (89).

Of great importance for the current studies, was the antibody specificity used. Two antibody populations with different antigenic specificity were used: GP-5 and R-4. The PTH molecule is an 84 amino acid polypeptide, the first 34 of which comprise the biologically active portion (75). Antibody R-4 was cultivated by use of the I-34

TABLE I

LIST OF PREPARATIONS TESTED FOR SECRETAGOGUE ACTIVITY

Preparation	Major Known Activity	Concentration Perfused
A	TSH	0.007, 0.011, 0.015, 0.023, and 0.030 mg/ml
B	TSH	0.017 IU/ml
C	GH	0.004 IU/ml
D	LH	12.2 ng/ml
E	PROLACTIN	0.25 IU/ml
F	ADH	0.0018 IU/ml
G	OXYTOCIN	0.0025 IU/ml

animo acid sequence terminus of the PTH molecule in a rabbit. It is specific for this biologically active fragment and its antigen detection in this work will be referred to as "n-iPTH". The GP-5 antibody was cultivated against the entire 1-84 amino acid sequence in guinea pigs. Fortuitously, the GP-5 antibody population appears to be specific for the carboxy (animo acid sequence 35-84) terminus of the antigen PTH molecule. The antigen detected by the GP-5 antibody which reacts only to the non-biologically active portion will be referred to as "c-iPTH."

Calcium Determinations

Calcium concentrations were determined by a flourometric titration method (19). This technique utilizes a dye, calcein, which flouresces in the presence of calcium ions. The mixture is then titrated with EGTA until the flourescence disappears. Measurements were automated with a "Fiske Calcium Titrator, model 700."

Enzyme Determinations

Enzyme analysis was performed with "Dow Diagnostic Kits" for serum glutamic oxalacetic transaminase (GOT), lactate dehydrogenase (LDH), and acid phosphatase. The GOT (3) (4) and LDH (100) tests were based on ultraviolet spectral detection of NADH changes. The acid phosphatase determination was based on production of thymolphthalein and subsequent combination with a blue chromogen (79).

Histological Preparations

Tissue for histological examination was fixed in 10 percent buffered formalin for 48 to 60 hours. Paraffin embedded sections were stained with hematoxylin-eosin. Examination was made of freshly removed parathyroid glands, glands perfused for eight hours at 1.0 and 0.5 ml/minute, and non-perfused glands which were left in a 37°C HBSS bath for eight hours.

CHAPTER IV

RESULTS

"In Vitro" Perfusion Technique

Figure 2 represents the c-iPTH secretory response over an eight hour perfusion period. These four glands were perfused with 4.5 mg calcium/100 ml HBSS at a rate of 1.0 ml/minute. The secretory rate fluctuated less than ± 10 percent from the average over the entire perfusion period. The perfusion pressure under these conditions also remained relatively constant until the 7 to 8 hour interval when it increased from an average of 45 to 85 mm Hg.

Perfusion under the same conditions except at a slower rate (0.5 ml/minute) resulted in a different response. Figure 3 depicts this pattern which was primarily a decreasing output of c-iPTH with time. The average secretory rate of these five glands increased, then was reduced a total of 75 percent in eight hours. Concurrently, the average perfusion pressure increased progressively for a total change of 20 mm Hg. Enzyme analysis of perfusate from these glands are shown in Figure 4. No LDH was detected. GOT and acid phosphatase concentrations did not change significantly during the eight hour perfusion period.

The response of four glands exposed to anoxia for approximately one hour and perfused at 1.0 ml/minute is illustrated in Figure 5. These glands responded to the anoxia by decreasing their c-iPTH

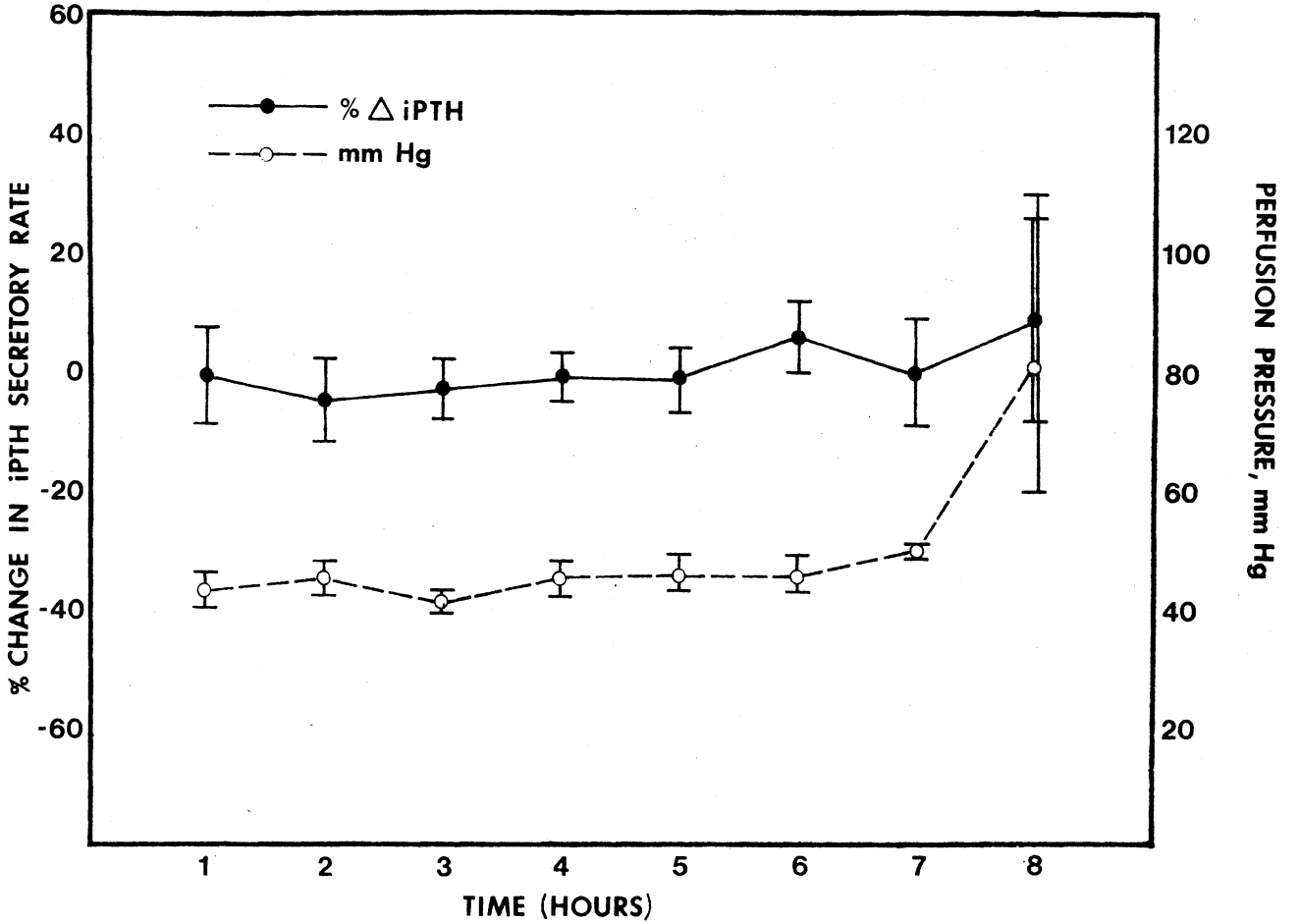


Figure 2. Parathyroid glands perfused "in vitro" with oxygenated HBSS. (Calcium concentration = 4.5 mg/100 ml, temperature = 37°C, pH buffered to 7.4 with sodium bicarbonate, and a perfusion rate of 1.0 ml/minute, average secretory rate = 5 ng/100 mg tissue/minute.)

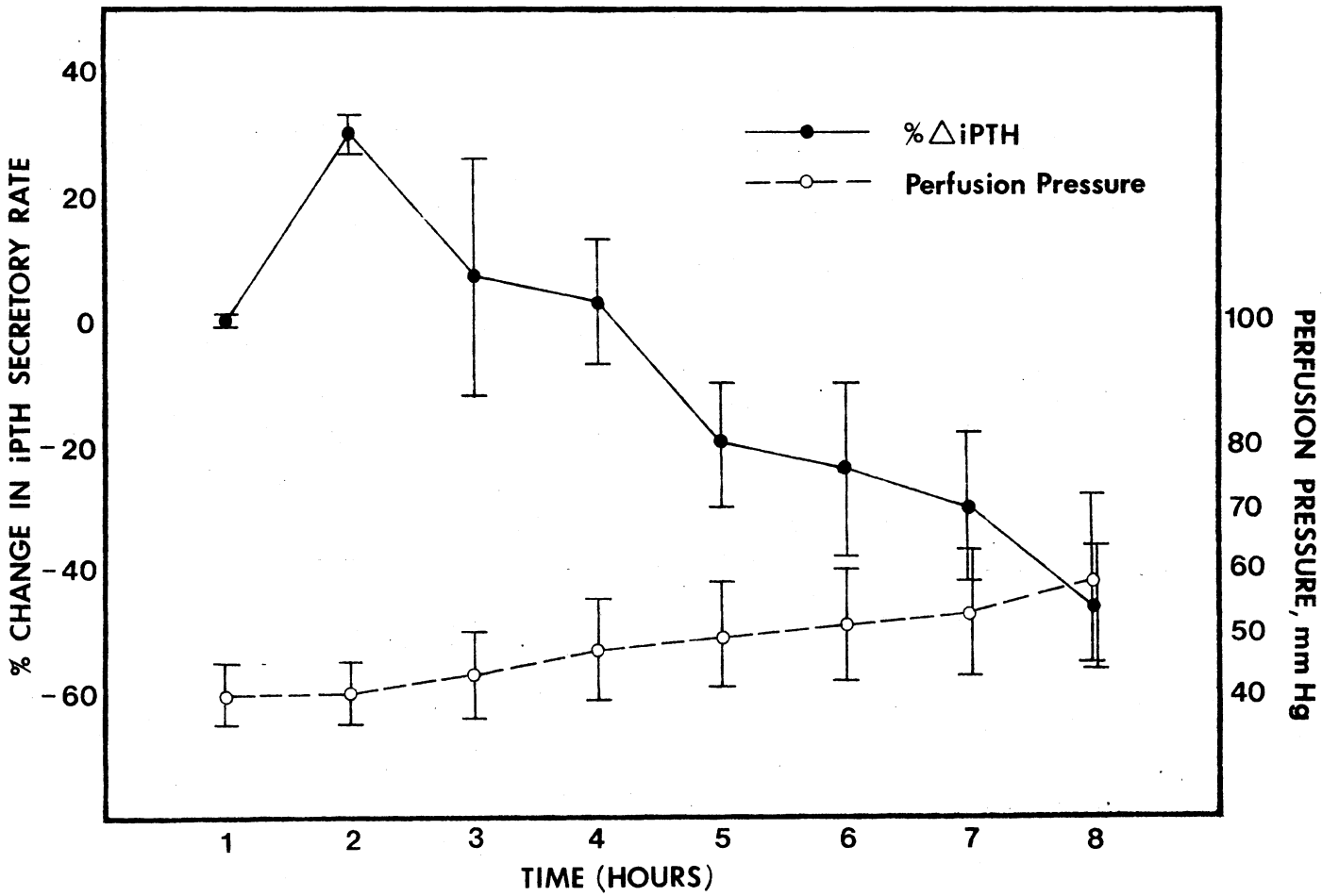


Figure 3. Parathyroid glands perfused "in vitro" with oxygenated HBSS at a rate of 0.5 ml/minute. Average initial secretory rate was 5 ng/100 mg tissue/minute.

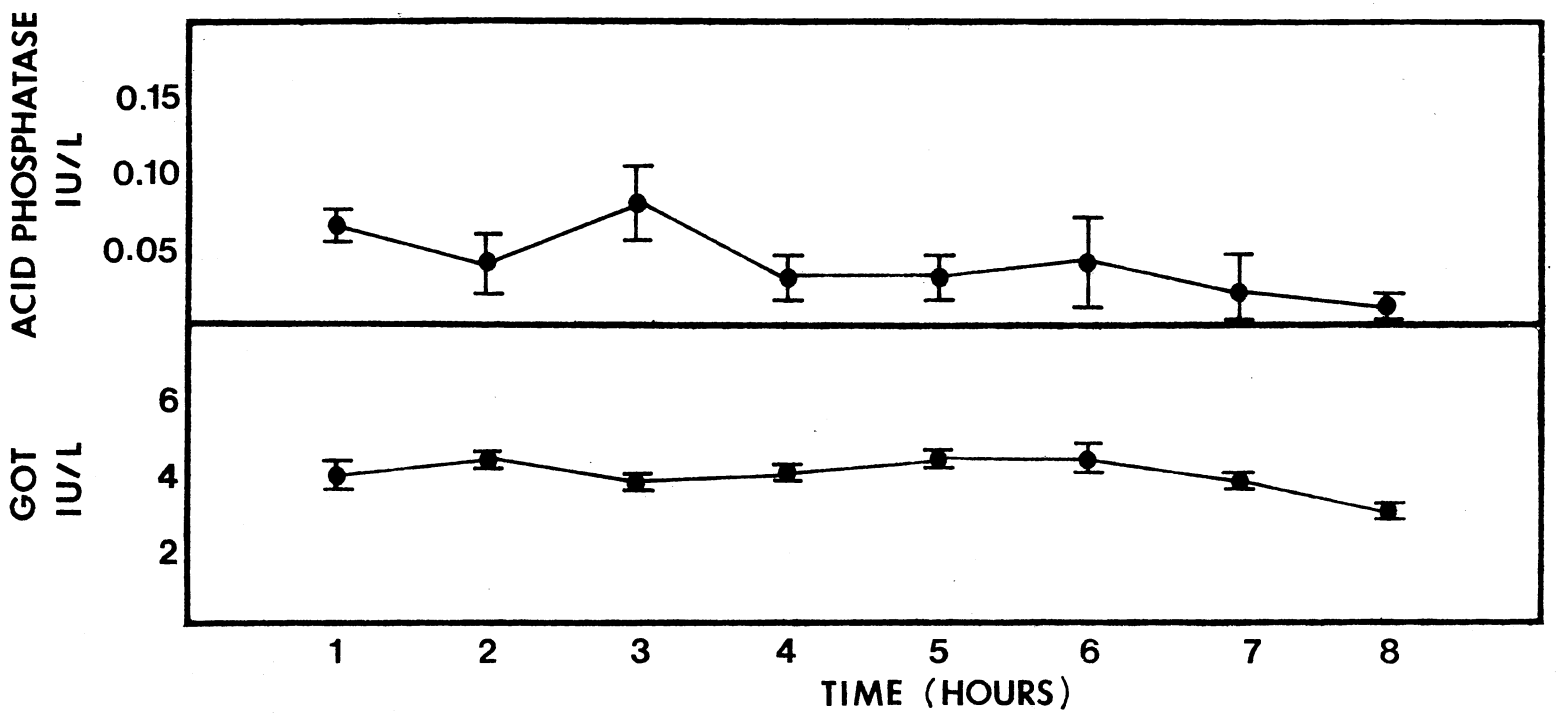


Figure 4. Enzyme output of parathyroid glands perfused at 0.5 ml/minute. (No LDH was detected.)

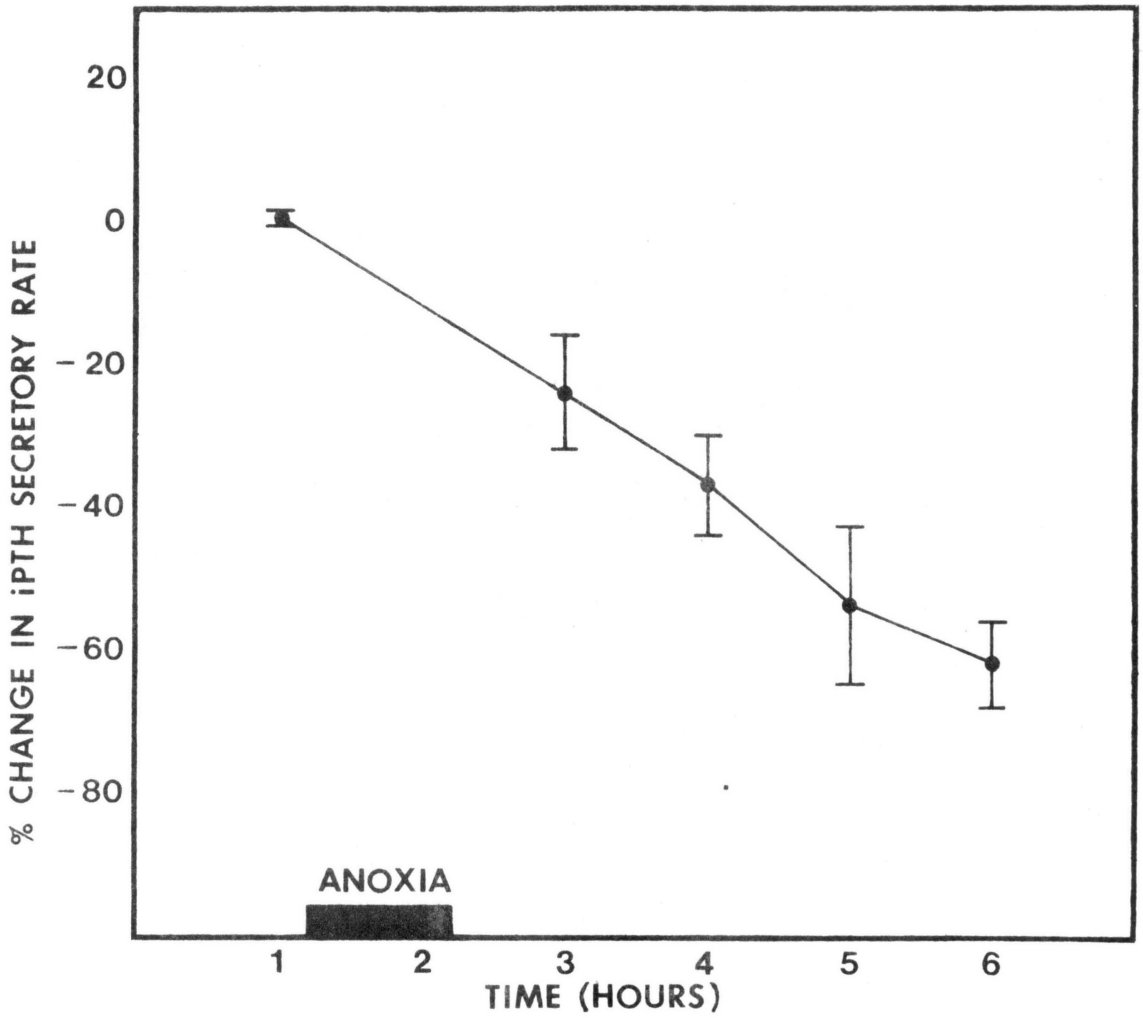


Figure 5. Effect of Anoxia on parathyroid gland secretion when perfused at 1.0 ml/minute. Average initial secretory rate was 5 ng/100 mg tissue/minute.

secretion even after the oxygen was restored. In the six hour period the hormone output was reduced by 60 percent.

Changes in c-iPTH secretion in response to changes in perfusate calcium concentrations for nine glands are represented in Figure 6. A decrease in calcium concentration stimulated c-iPTH secretion while an increase inhibited secretion. The basal secretory rate upon which the percent stimulation or inhibition was based was the secretory rate exhibited at the simulated normocalcemic concentration of 5.0 mg/100 ml. An increase in total calcium above 6.5 mg/100 ml failed to elicit further suppression of c-iPTH secretion. These glands were perfused at 1.0 ml/minute.

Table II compares the histological appearance of the parathyroid tissue under various conditions. Although the appearance of all the experimental tissue samples deviated from the freshly fixed tissue, only that perfused at 1.0 ml/minute had foci of acini and cords of normal appearing chief cells. This tissue did, however, reflect the greatest degree of separation of the connective tissue stroma.

Samples (3.5 ml) of effluent were collected in empty glass vials, vials coated with bovine serum albumin (BSA), and vials containing 0.1 ml of human plasma and frozen immediately to -70°C. Assayed for c-iPTH 48 to 60 hours later, the samples revealed no significant differences. Other samples collected in glass vials were repeatedly assayed for c-iPTH over a six-month period. No significant differences in the hormone concentrations were found with time.

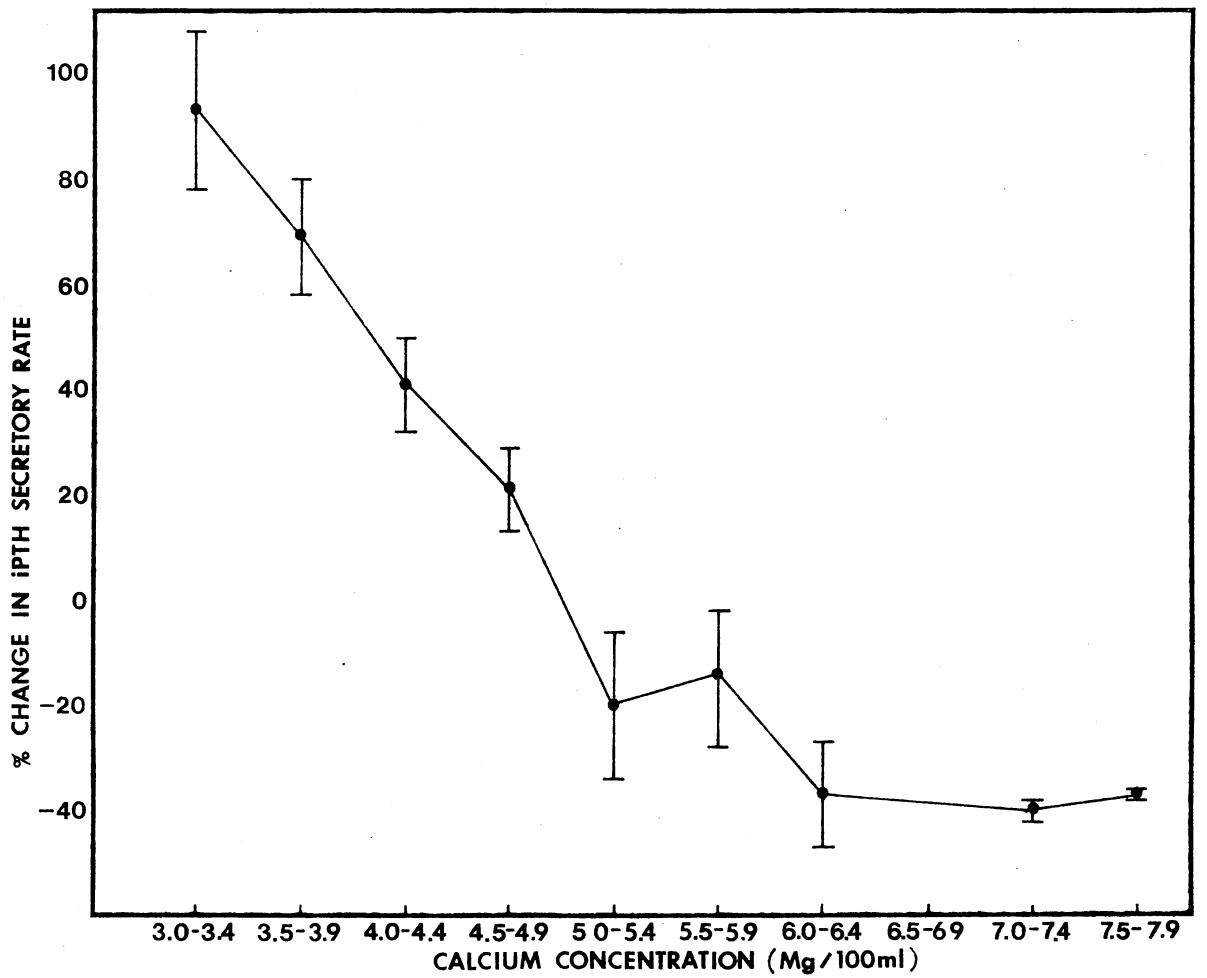


Figure 6. Dose response of parathyroid gland secretory rate to calcium concentration. Perfusion rate was 1.0 ml/minute.

TABLE II

HISTOLOGY OF PARATHYROID GLANDS

	Control	8 hour Incubation	Perfused 1 ml/min.	Perfused 0.5 ml/min.
Nucleus:				
Distinct Basophilia	x		x	
Pale Basophilia		x	x	x
Distinct Nucleolus	x	x	x	x
Pyknotic			x	x
Chromatin Clumping on nuclear membrane		x		
Cytoplasm:				
Distinct Eosinophilia	x		x	
Pale Eosinophilia		x	x	x
Vacuolation			x	x
Connective Tissue Stroma:				
Compact	x	x		
Moderately Compact				x
Loose			x	
Vasculature:				
Arteriolar Endothelium Intact	x	x	x	x
Capillary Endothelium Intact	x	x		
Capillary Endothelum Disrupted			x	x

Hypophysectomy

As reviewed in Chapter II, ablation of the pituitary has been reported to yield contradictory results. Figure 7 represents the results of this current study on hypophysectomy of rats. The calcium concentration decreased within 24 hours following extirpation and by 36 hours was 1.0 mg/100 ml plasma below the concentration of the control animals. The concentration appears to either stop decreasing or at least slow its rate of decrease by 36 hours and remains significantly below (10 mg/100 ml) the intact rats (11 mg/100 ml). The difference in plasma calcium concentration is statistically significant at $P < 0.01$.

Injection of Pituitary Preparation A

Injection of Preparation A (1.0 mg/67 kg body weight) into calves resulted in an increase in the plasma calcium concentration (Figure 8). The calcium increased an average of 1.1 mg/100 ml plasma. The time required to detect an increase varied from 0.75 to 2.5 hours post injection. Calcium concentration returned to the original value 3.5 to 9 hours after the injection. The duration of the induced hypercalcemia ranged from 2.5 to 6.5 hours.

The peripheral plasma c-iPTH concentration was also measured following the injection. No significant change was detected between the time of injection and the beginning of the calcium concentration increase.

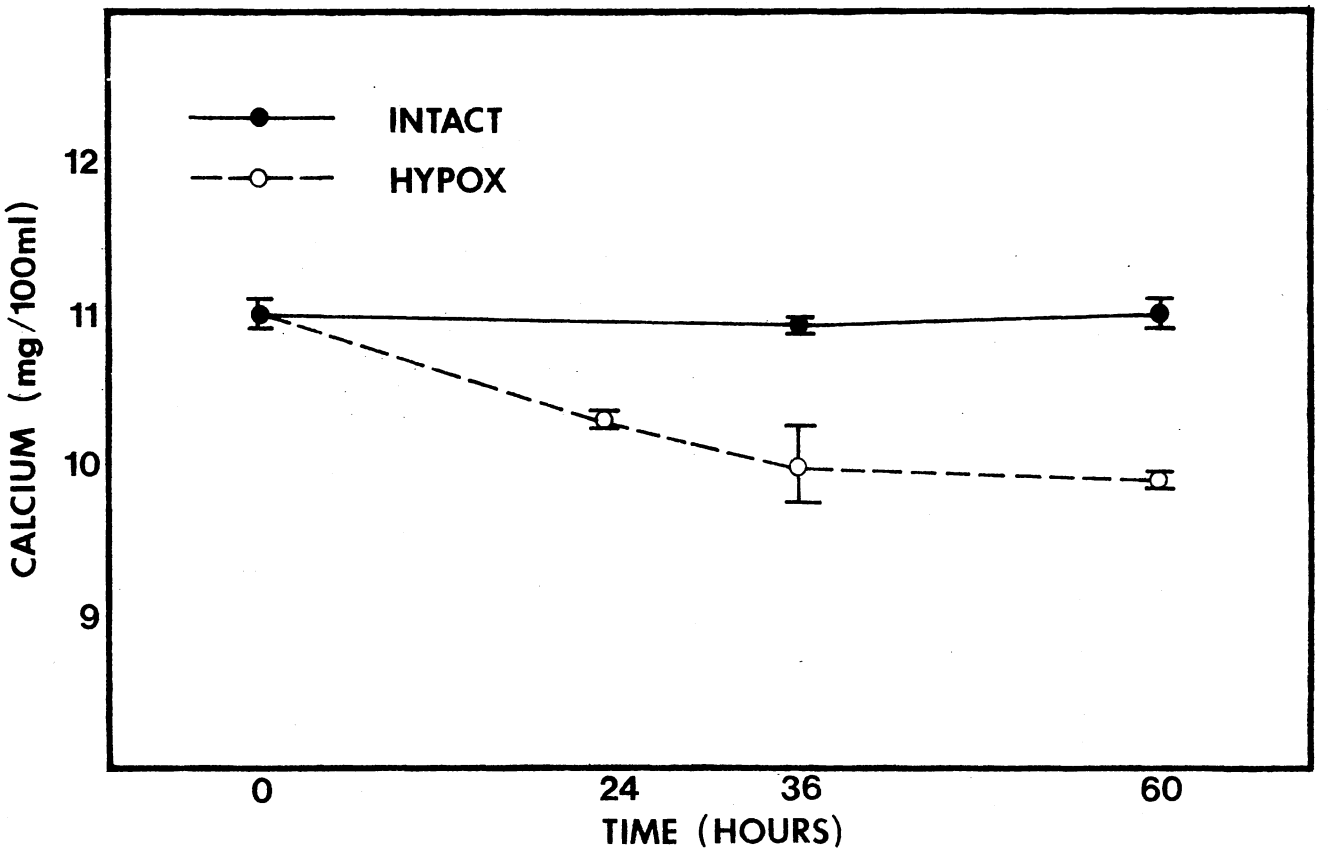


Figure 7. Effect of hypophysectomy on plasma calcium with time. ($P < 0.01$)

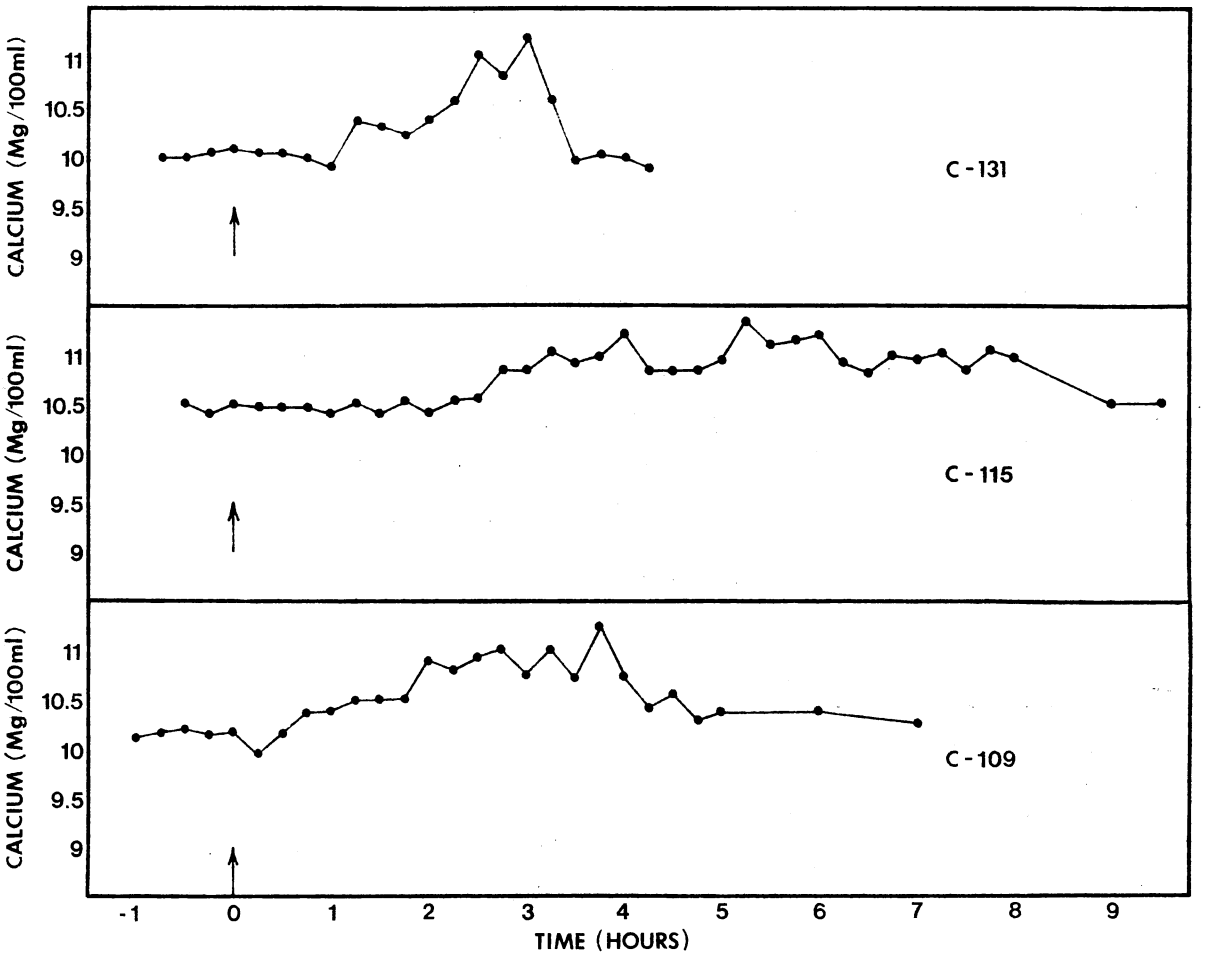


Figure 8. Effect of injection of pituitary Preparation A on plasma calcium concentration in three calves. Arrow indicates time of injection of Preparation A.

Response of "In Vitro" Perfused Parathyroid Gland to Suspected Secretagogues

Parathyroid gland secretory rate response to Preparation A was examined at simulated normocalcemia (4.5 mg/100 ml) and simulated hypercalcemia (6.5 mg/100 ml). The glands were perfused at 1.0 ml/minute. Stimulation of seven glands for 20 minutes with 0.015 mg/ml at 4.5 mg calcium/100 ml resulted in an average increase of 30 percent in the c-iPTH secretion (Figure 9). The same samples measured for n-iPTH showed a 56 percent average stimulation (Figure 10). The c-iPTH required 50 minutes after cessation of stimulation to return to the prestimulation secretory rate. In contrast, the n-iPTH required only 20 minutes. Stimulation of six glands for 20 minutes with 0.015 mg/ml of Preparation A at 6.5 mg calcium/100 ml HBSS resulted in an average increase of 60 percent in c-iPTH secretion (Figure 11). The same samples measured for n-iPTH showed an 88 percent average stimulation (Figure 12). As with the stimulation at normocalcemia, the hypercalcemic samples displayed a quicker return to prestimulatory rate of secretion (10 minutes) for n-iPTH than for the c-iPTH, which had not completely returned by 50 minutes.

A partial dose response curve under normocalcemic conditions is depicted in Figure 13. The curve was constructed based on 19 responses distributed over the five dosages tested.

The response of five glands to the carrier media (0.9 percent saline) were tested (Figure 14). No stimulation or inhibition of c-iPTH was found. Six glands tested for secretagogue activity of Preparation A (0.015 mg/ml) which had undergone 12 freeze-thaw cycles

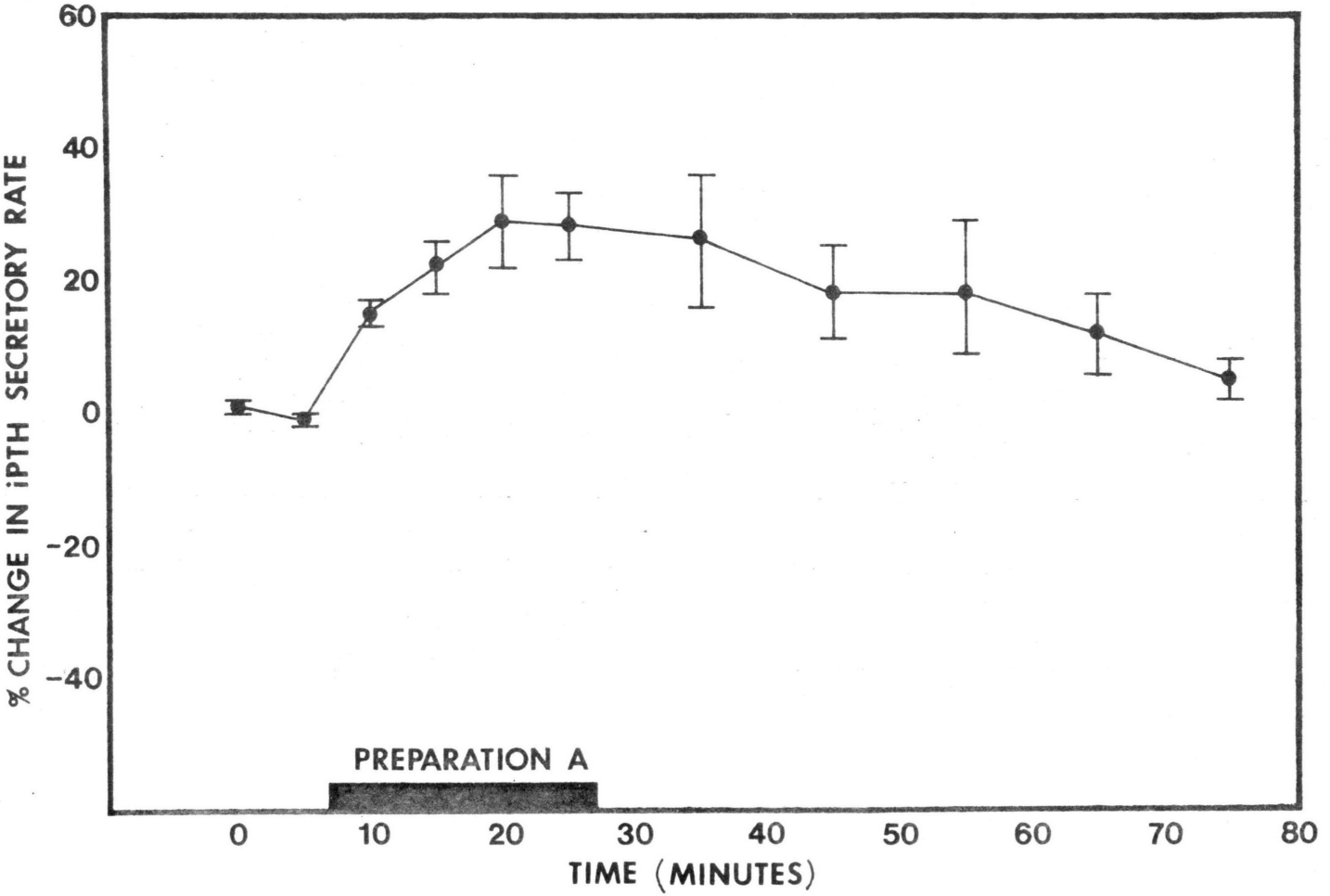


Figure 9. Direct stimulation of c-iPTH secretion in "in vitro" perfused parathyroid glands by pituitary Preparation A. (Perfusion rate = 1.0 ml/minute, calcium concentration = 4.5 mg/100 ml.) Pre-stimulatory rate was 5 ng/100 mg tissue/minute.

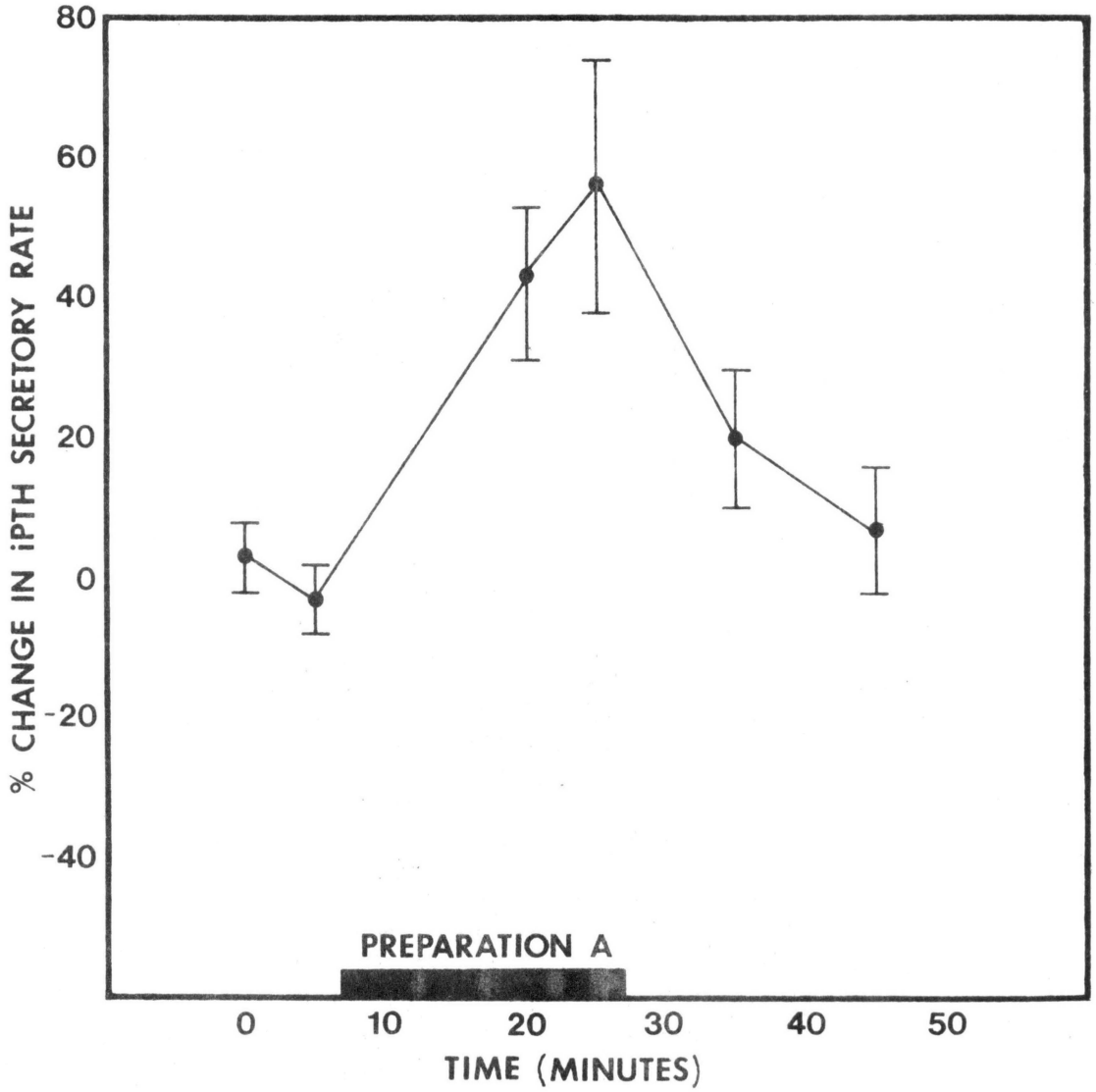


Figure 10. Direct stimulation of n-iPTH in "in vitro" perfused parathyroid glands by pituitary Preparation A. (Perfusion rate = 1.0 ml/minute, calcium concentration = 4.5 mg/100 ml.)

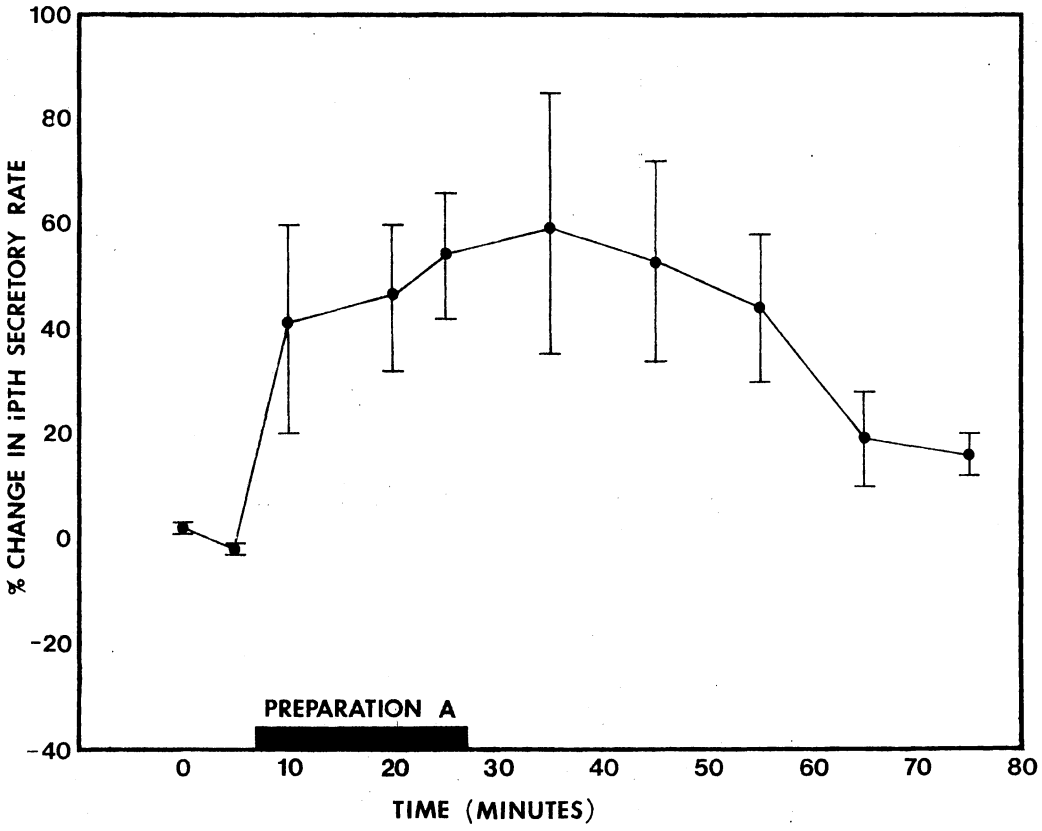


Figure 11. Direct stimulation of c-iPTH under hypercalcemic conditions (6.5 mg/100 ml) in "in vitro" perfused parathyroid glands by pituitary Preparation A. (Perfusion rate = 1.0 ml/minute.) Pre-stimulation secretory rate was 5 ng/100 mg tissue/minute.

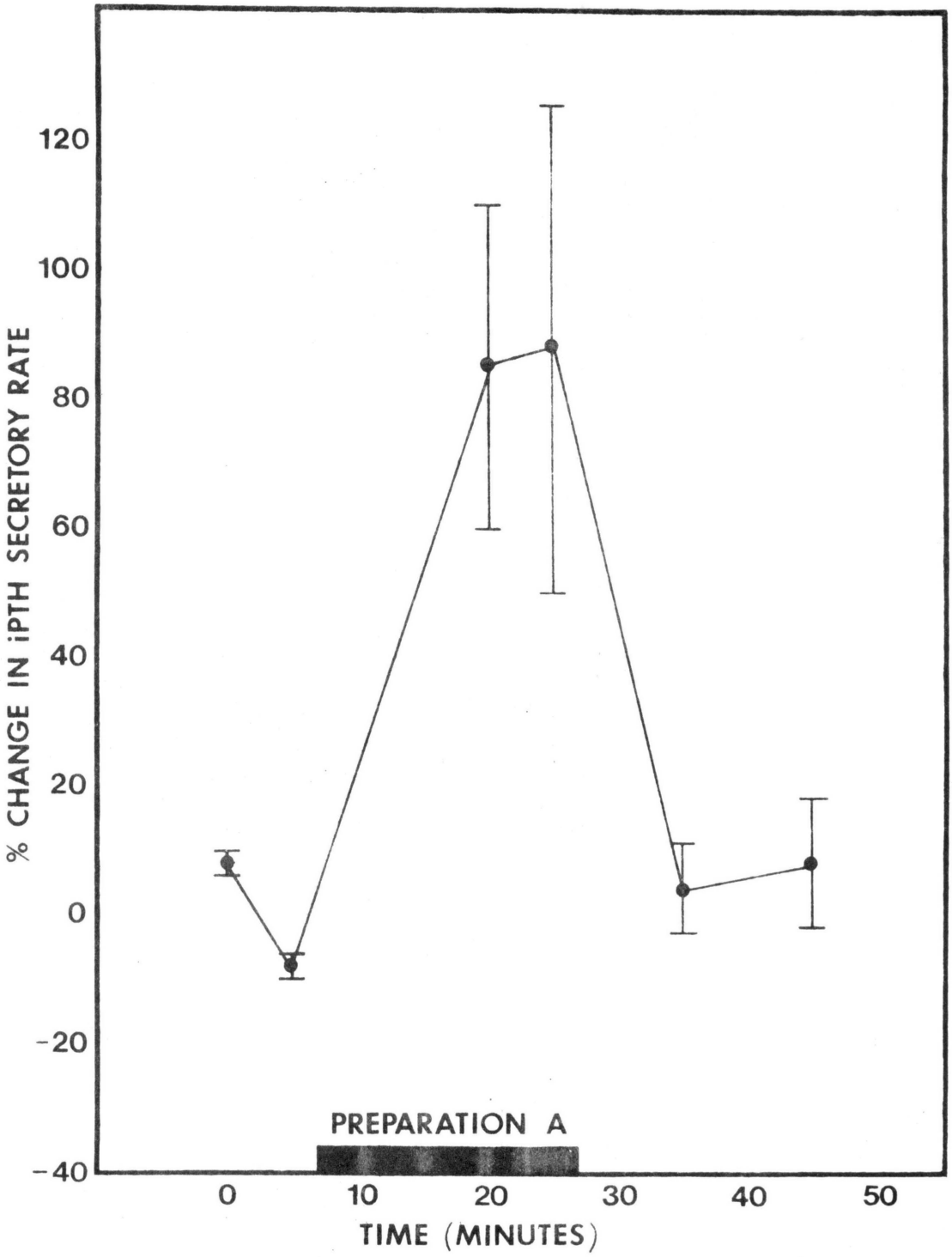


Figure 12. Direct stimulation of n-iPTH under hypercalcemic condition in "in vitro" perfused parathyroid glands with pituitary Preparation A. (Perfusion rate = 1.0 ml/minute.)

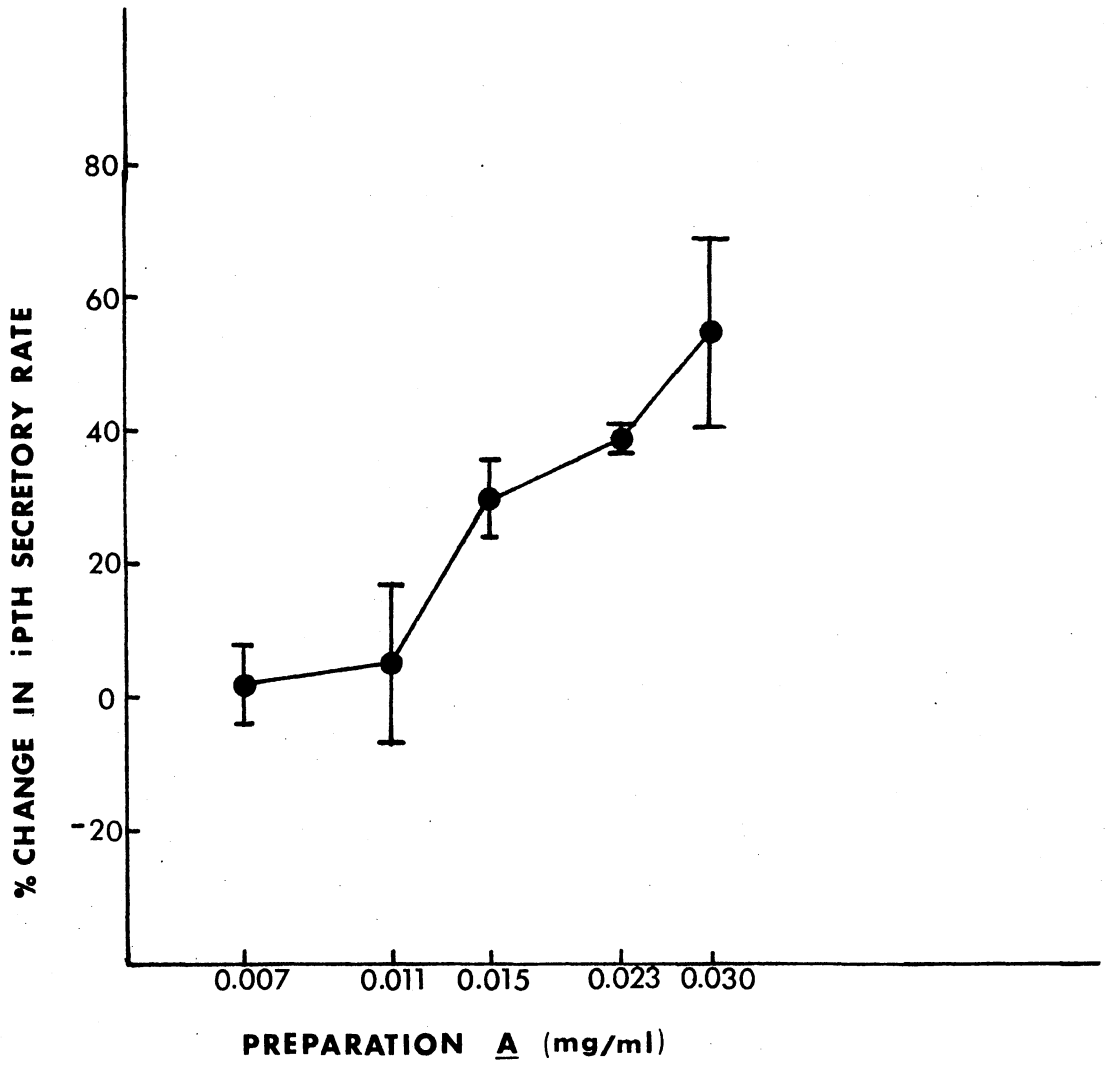


Figure 13. Dose response curve for the effect of pituitary Preparation A on c-iPTH secretion in "in vitro" perfused parathyroid glands. (Perfusion rate = 1.0 ml/minute, calcium concentration = 4.5 mg/100 ml.) Average secretory rate at 0.007 mg/ml of Preparation A was 5 ng/100 mg tissue/minute.

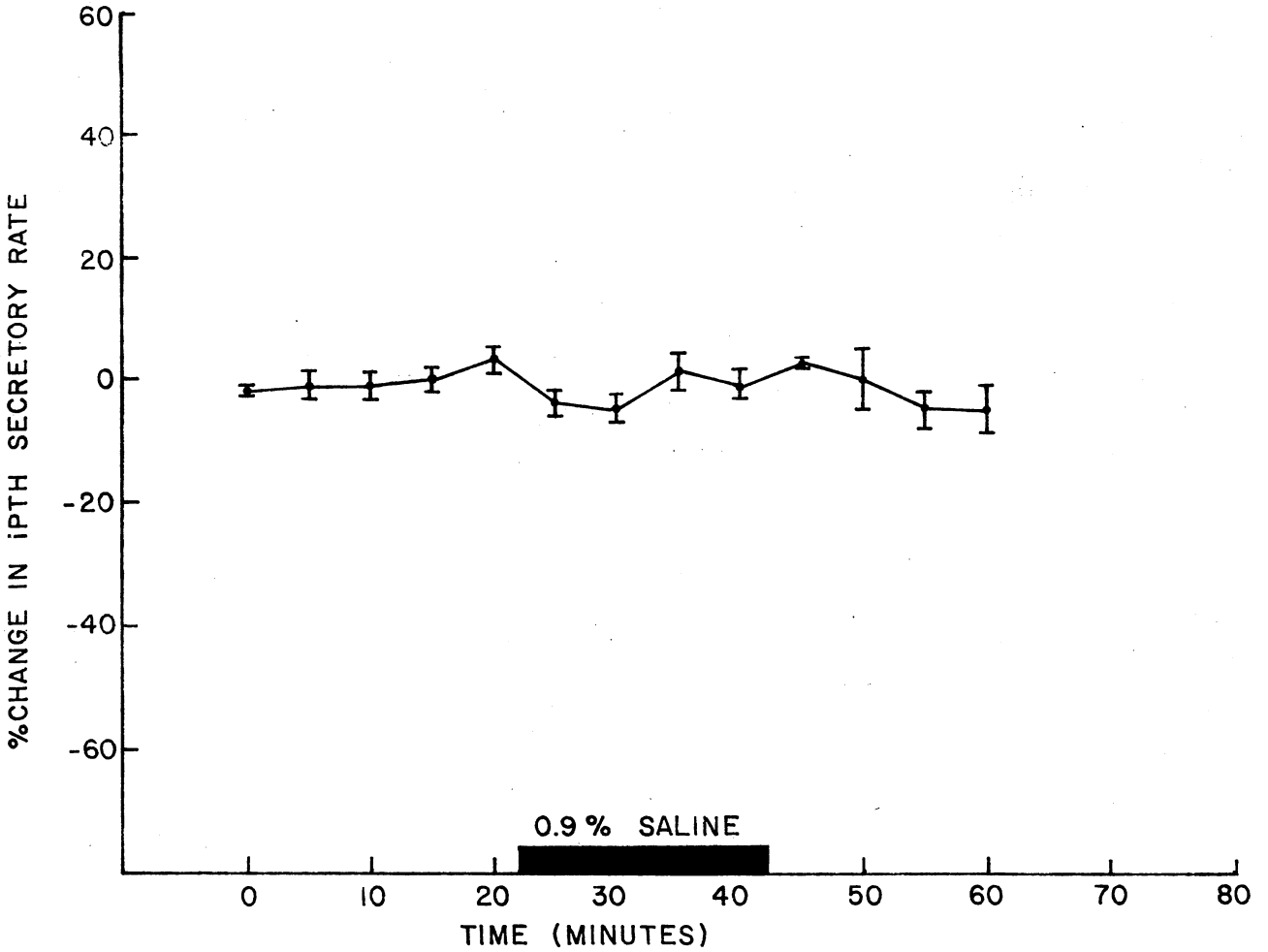


Figure 14. C-iPTH secretion observed with exposure of "in vitro" parathyroid glands to 0.9 percent saline. (Perfusion rate = 1.0 ml/minute, calcium concentration = 4.5 mg/100 ml.)

gave essentially the same negative response (Figure 15). That is, there was no stimulation or inhibition of c-iPTH or n-iPTH activity observed.

Each of the Preparations B through G were tested individually on five glands at the concentrations indicated in Table I. No apparent stimulation or inhibition of either c-iPTH or n-iPTH was observed for any of these pituitary preparations (Figures 16 through 21).

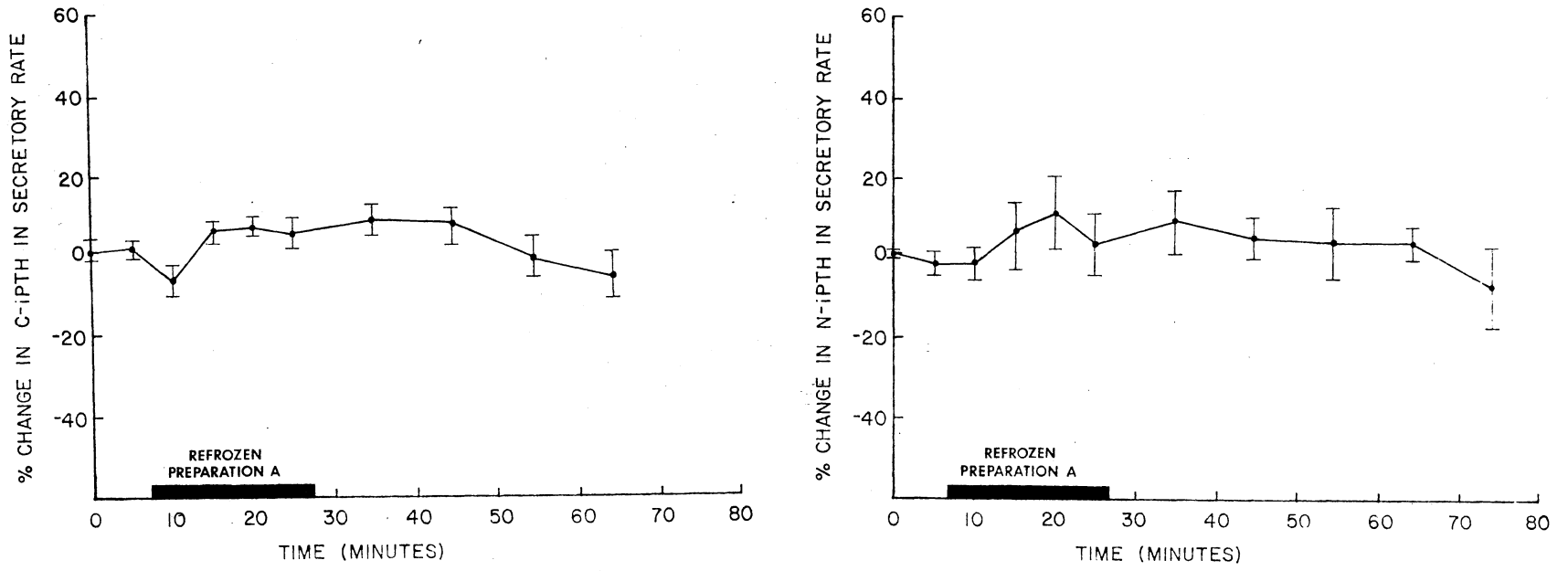
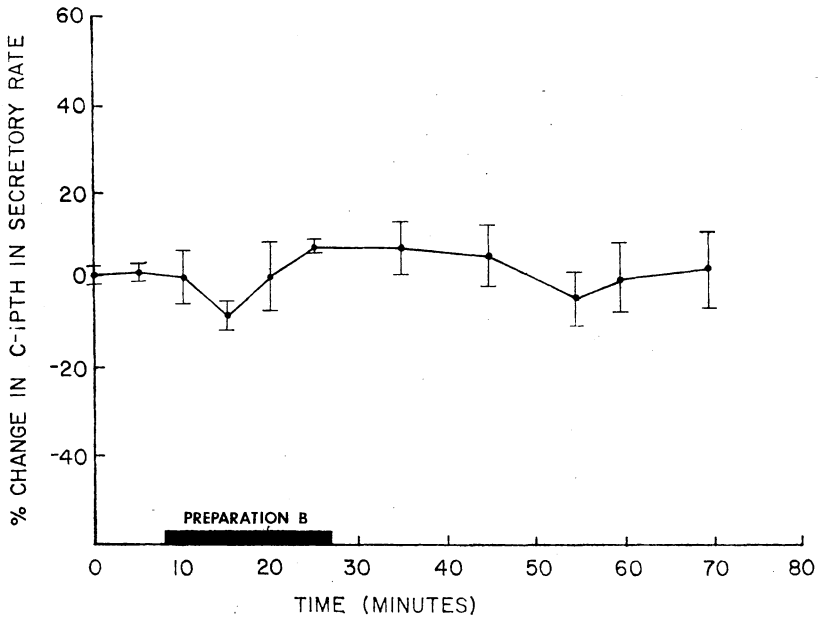


Figure 15. "In Vitro" secretion of c-iPTH and n-iPTH in response to refrozen (12X) Preparation A. (Normocalcemia)



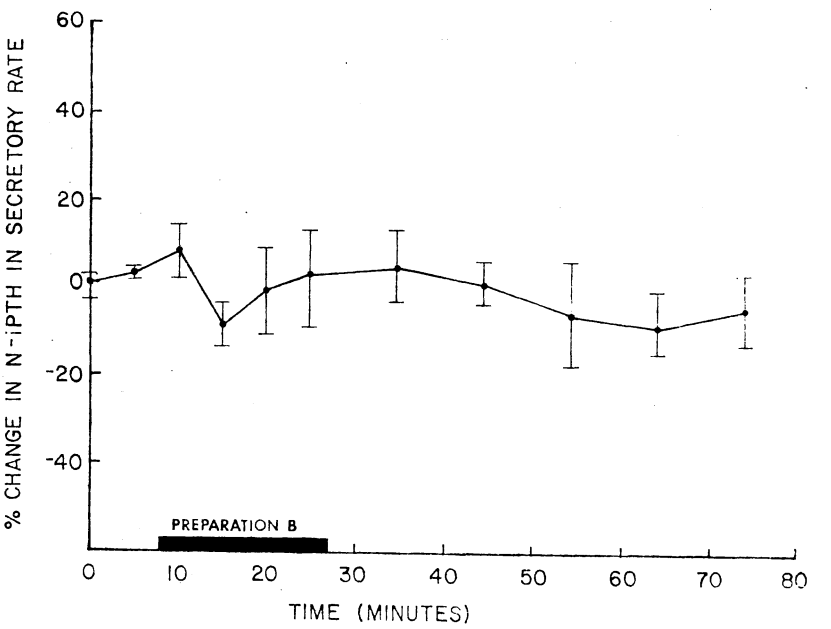


Figure 16. "In Vitro" secretion of c-iPTH and n-iPTH in response to Preparation B. (Normocalcemia)

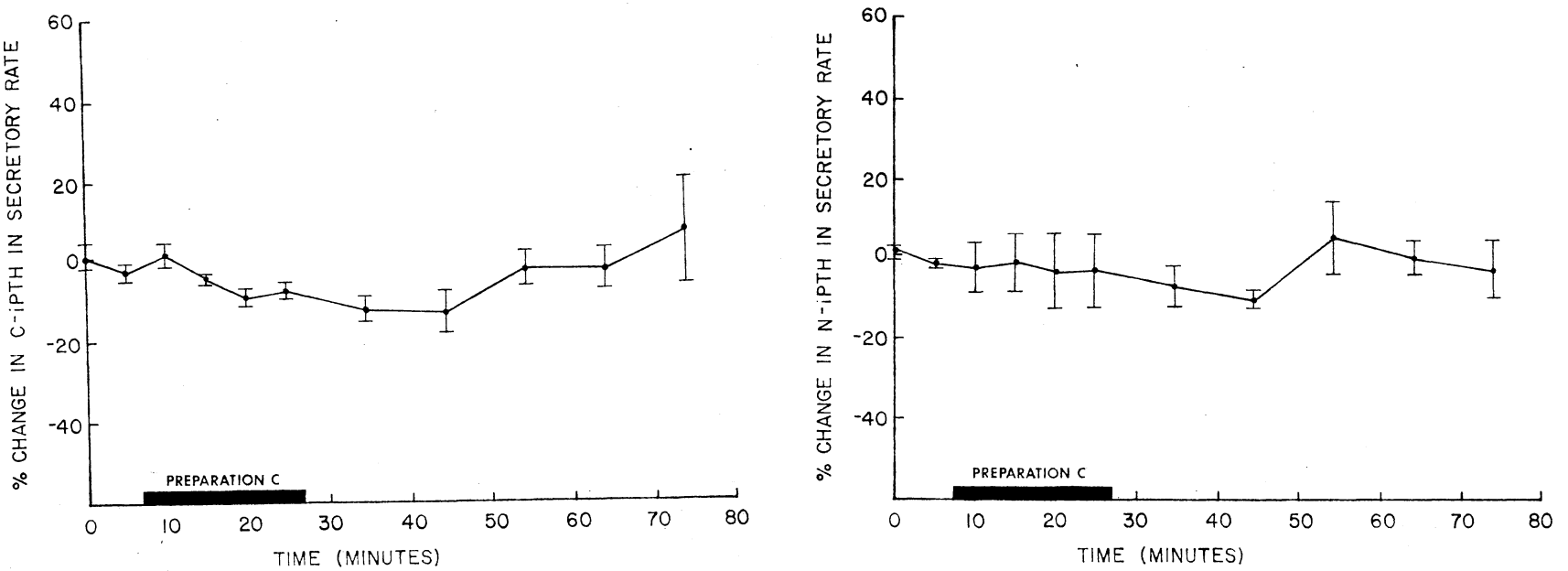


Figure 17. "In Vitro" secretion of c-iPTH and n-iPTH in response to Preparation C. (Normocalcemia)

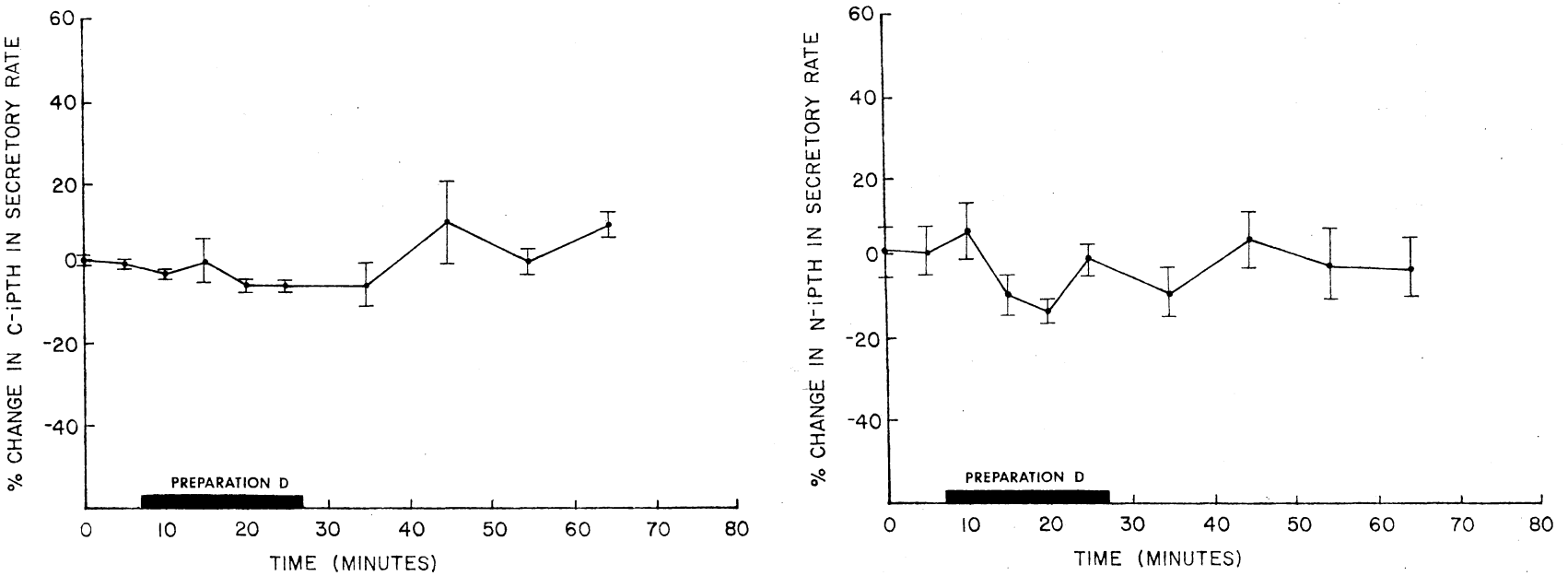


Figure 18. "In Vitro" secretion of c-iPTH and n-iPTH in response to Preparation D. (Normocalcemia)

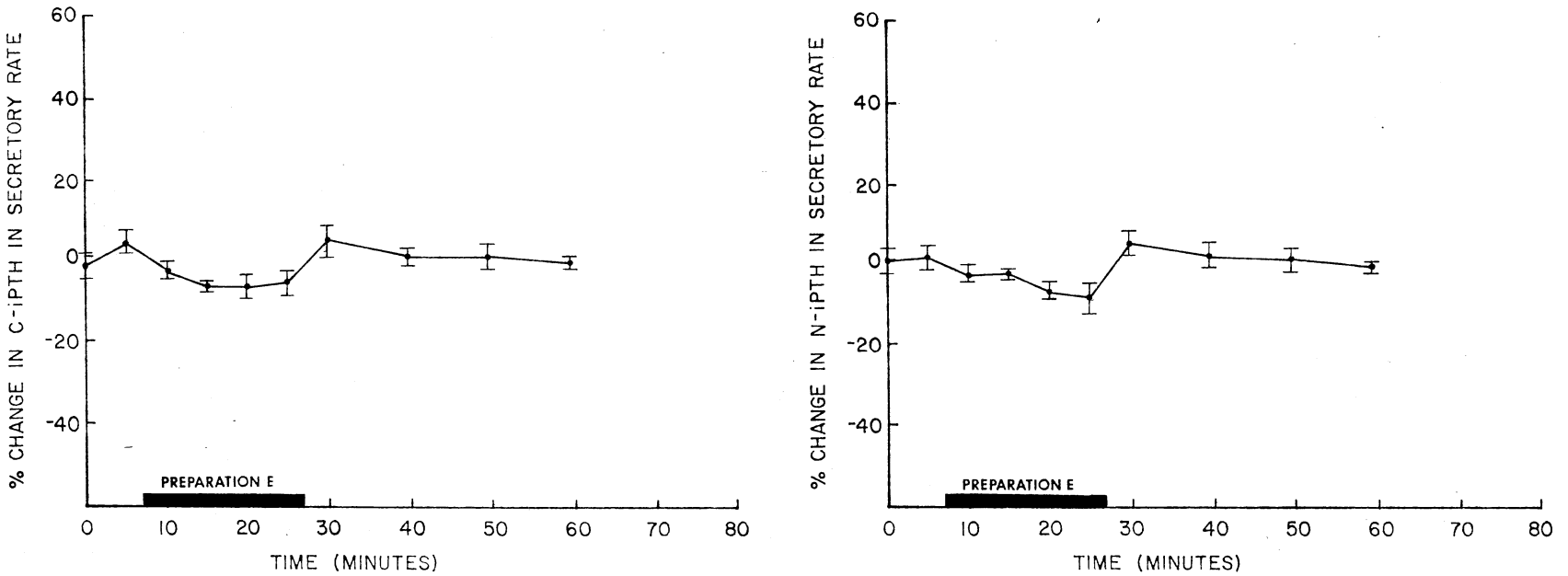


Figure 19. "In Vitro" secretion of c-iPTH and n-iPTH in response to Preparation E. (Normocalcemia)

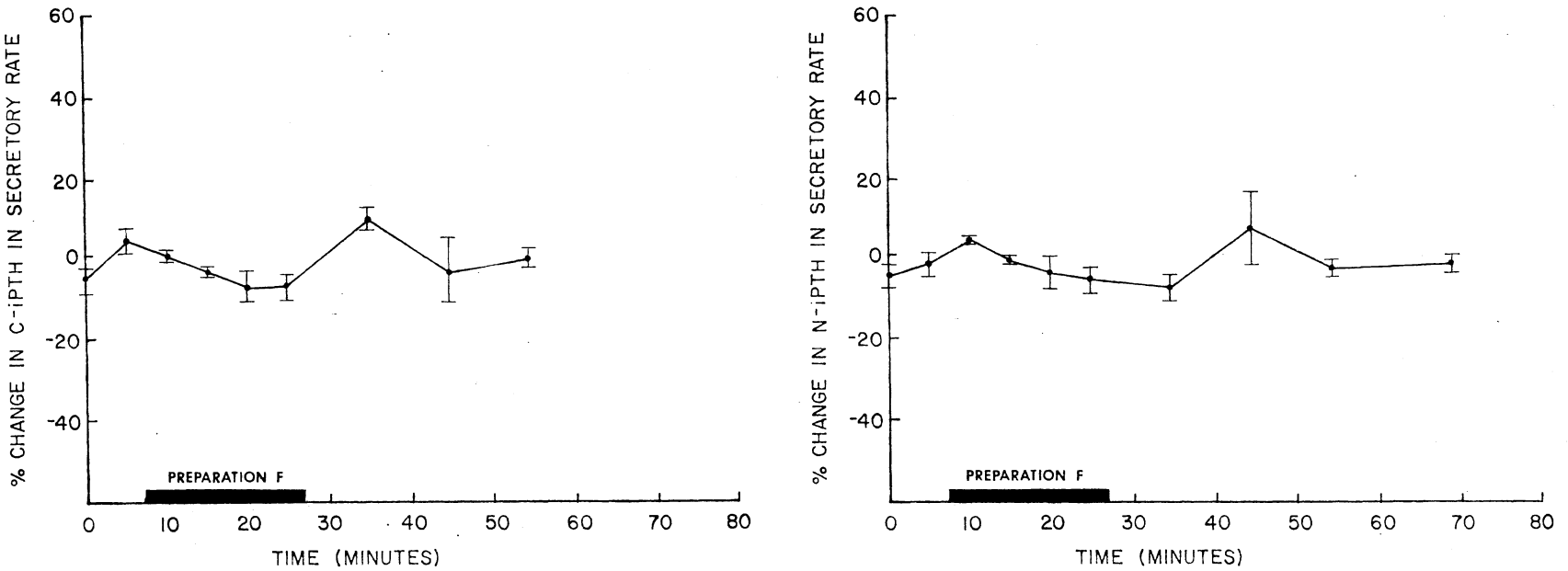


Figure 20. "In Vitro" secretion of c-iPTH and n-iPTH in response to Preparation E. (Normocalcemia)

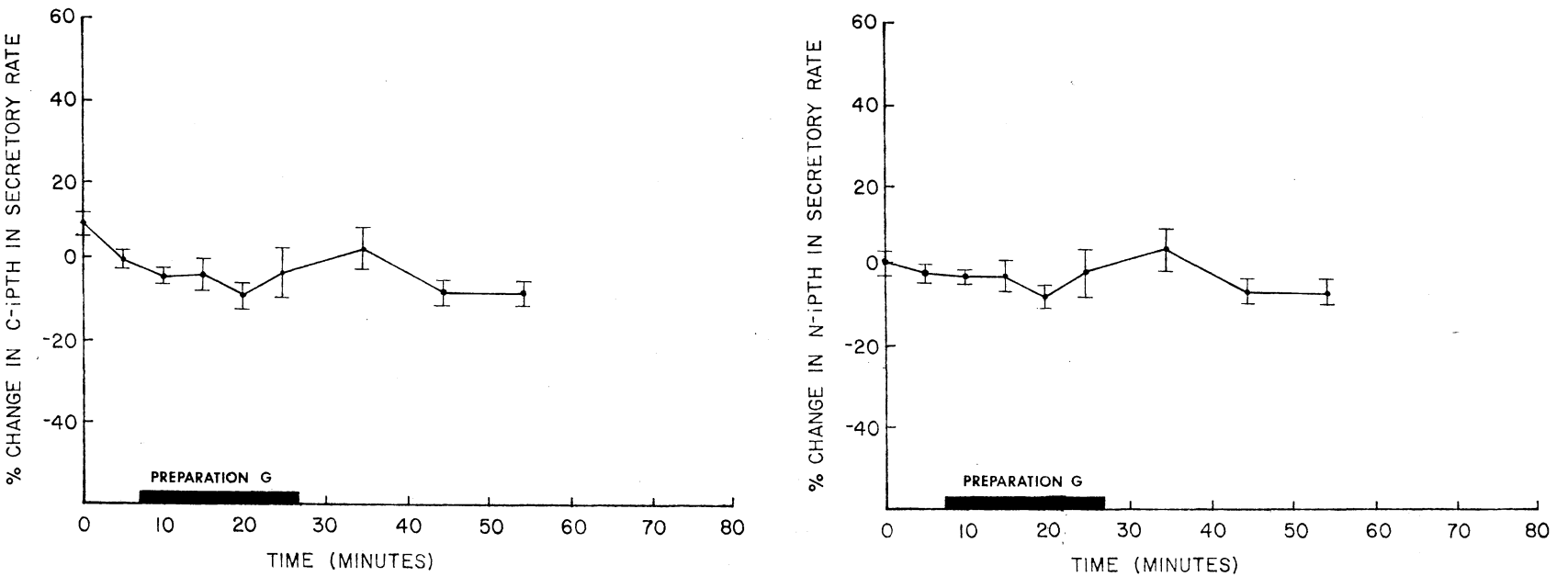


Figure 21. "In Vitro" secretion of c-iPTH and n-iPTH in response to Preparation G. (Normocalcemia)

CHAPTER V

DISCUSSION

"In Vitro" Perfusion of Parathyroid Glands

At least four methods for the isolated or "in vitro" study of parathyroid function have been developed. An "in situ" perfusion technique was developed in 1942 (74) and later modified for improved control (31). Parathyroid gland tissue slice incubations were used in 1934 with limited success (94). Modifications of this approach have increased its utility (12) (40) (49). Disperse cell systems or cultures have also proved of limited effectiveness (33) (66) (99). Most recently a parathyroid explant superfusion culture system has provided some advantages over the previous methods (71). These techniques, either singularly or in combination, exhibited one or more of the following disadvantages: 1) parathyroid tissue was not completely isolated from other glandular tissue which may interfere with interpretations of results, 2) minute to minute changes in secretory activity could not be measured, 3) PTH was not always secreted, and/or 4) they required sterile conditions.

The purpose of developing a new technique for examining parathyroid hormone (PTH) secretory activity was to eliminate these disadvantages. Additionally, the desire was to reduce the need for experimental animals; to use a simple, but well defined perfusion media; to be able

to conduct experiments for six or more hours on a functionally stable and responsive gland; and to have a simple and economical technique.

Criteria used to evaluate the viability of this new "in vitro" perfusion system were in general agreement with those suggested by Bernstein (16): 1) Organ function as indicated by secretion of iPTH, 2) Haemodynamic factors as indicated by perfusion pressure changes, 3) Evidence of cell destruction as indicated by enzyme release, 4) Anatomic studies as indicated by histologic appearance, and 5) Metabolic considerations loosely indicated by changes in color of the pH indicator phenol red.

Both "in vivo" (68) and "in vitro" (41) work appears to demonstrate that if the concentration of calcium and other affectors of PTH secretory rate are held constant, the secretory rate is constant. The parathyroid glands perfused at 1.0 ml/minute with oxygenated HBSS reflected this pattern. Those glands deprived of oxygen for one hour failed to maintain their initial secretory rates. The parathyroid gland has also been shown to respond "in vivo" to changes in calcium ion concentration in an inverse sigmoid manner (67). Since Figure 6 demonstrated that glands perfused at 1.0 ml/minute responded to changes in the calcium concentration, the criteria concerning organ function appears to be satisfied for this perfusion rate.

A progressive increase in resistance to perfusate flow is often a problem in experimental perfusion systems (32). The glands perfused at the 1.0 ml/minute rate maintained a relatively constant pressure until the seven to eight hour perfusion period. Glands perfused at 0.5 ml/minute showed a steady increase in pressure and therefore are not as satisfactory (73).

An increase in the release of cellular enzymes has commonly been used as an indicator of the loss of tissue viability (2). The enzymes chosen for monitoring in this study did not show a progressive increase, a pattern of release indicative of tissue failure in the glands perfused at 0.5 ml/minute. Monitoring these enzymes, therefore, cannot be used as a measure of gland viability in this system.

A perfusion rate of 0.5 ml/minute is approximately the "in vivo" rate for thyroid tissue on an equivalent per unit weight basis (56). This rate, however, was not sufficient to maintain parathyroid organ function in the "in vitro" perfusion system. Although the 1.0 ml/minute rate caused more connective tissue separation, it tended to maintain the chief cells in a more normal appearing state.

As indicated by color change of the phenol red, no pH alterations were noted in any gland effluents. This may be due to the high perfusion rate and relatively small mass of tissue. However, it should not go without note that if sufficient Na_2EDTA was added to drop the pH below 7.0, the secretory rate of iPTH was drastically reduced. This is contrary to the increase expected from the resulting calcium ion concentration decrease.

A secretory rate in one superior parathyroid gland of approximately 1.5 ng/Kg body weight/minute has been reported for calves "in vivo" at a calcium concentration of 9.0 mg/ml of plasma (67). This can be computed to equal 77 ng/100 mg gland weight/minute if the ratio of 1.93 mg of parathyroid tissue/Kg body weight (95) is assumed. An "in vitro" culture method has yielded a rate of approximately 400 ng/mg dry weight of gland/24 hours under similar conditions (41). This rate can be calculated to be 9.5 ng/100 mg/minute if the dry weight is assumed to

represent 33 percent of the wet weight. The average c-iPTH secretory rate recorded with the "in vitro" perfusion (1.0 ml/minute) system reported here at comparable calcium and magnesium ion concentrations was 5 ng/100 mg/minute. The range was quite wide, from 2 to 20 ng/100 mg/minute.

The problem of loss of PTH during storage by adsorption to glass has been well established. Loss has been reduced by use of bovine serum albumin (BSA) and almost eliminated by the addition of human serum (49). No loss of iPTH activity was observed under the conditions of the present study and neither BSA nor human plasma had an effect. The iPTH activity is very stable for at least six months when frozen at -70°C.

Pituitary Stimulation of Parathyroid Glands

The decrease in plasma calcium concentration in hypophysectomized rats verifies the results of positive reports reviewed in Chapter II. The transient increase in serum calcium concentration in calves following a pulse injection of pituitary Preparation A also confirms some of the reports reviewed in the literature survey. Both of these results are consistent with the concept of a parathyroid stimulating hormone located in the pituitary. The experiments do not, however, demonstrate a direct effect of the pituitary on the parathyroid secretory rate. In fact the lack of detection of an increase in c-iPTH in peripheral plasma of the calves prior to the calcium concentration increase raises some questions concerning this hypothesis. The peripheral plasma iPTH concentration, however, has been shown to be a relatively insensitive measure of changes in iPTH secretory rate (68).

There was, therefore, a need to directly examine the changes in the iPTH secretory rate in response to suspected secretagogues.

Preparation A directly stimulated the "in vitro" perfused parathyroid glands at simulated normocalcemia and hypercalcemia. Both c-iPTH and n-iPTH showed a significant increase in secretory rate corresponding to the period of stimulation. And in both instances the stimulation was greater during hypercalcemia. This implies that the pituitary factor is capable of overwhelming maximal calcium suppression of the secretory rate. Previous work has shown that dibutylryl cyclic AMP (which mimics the effect of cyclic AMP) can stimulate the parathyroid gland under hypercalcemic suppression (103). Cyclic AMP is extremely sensitive to inhibition by calcium ions (34). The physiological significance of this greater response may be in establishing an heirarchy of parathyroid secretion regulating mechanisms. That is, the generally accepted concept of calcium as the predominate control may now be questioned. In addition, a cellular mechanism through which this stimulant acts may be sought.

The importance of a different degree of stimulation of c-iPTH and n-iPTH observed in this experiment is evident in light of other work (10) (47) (91). Although it is known that fragments of the intact PTH molecule (amino acid sequence 1-84) can be degraded peripherally (47), secretion of fragments may also occur (91). The data presented in this dissertation indicates that not only can the pituitary stimulate secretion of c-iPTH (active and non-active molecules), but that n-iPTH (biologically active molecules) secretion was stimulated to a much greater degree. This preferential secretion occurred at both normo- and hypercalcemia. This is consistent with the concept that the

parathyroid gland can change its effective secretory rate by either changing the total secretory rate or the proportion of biologically active molecules it secretes (46) (84).

Failure of the repeatedly frozen and thawed sample of Preparation A to elicit a secretory response indicates that the physiological activity of the secretagogue can be denatured or degraded and it implies that the stimulating effect is a specific property, not merely a general property of all proteins. Since the carrier media (0.9 percent saline) did not stimulate the gland when given alone, it can be eliminated as the secretagogue.

None of the known hormones tested at concentrations approximately equal to that found in Preparation A elicited an increase in the iPTH secretory rate. Although not known to be present in Preparation A, Prolactin also gave negative results when tested at a concentration reported in lactating females (9). Adrenocorticotrophic hormone (ACTH), melanocyte stimulating hormone (-MSH), and -lipotropin have been reported (44) to decrease plasma calcium. Additionally, they do not appear to work via the thyroid, parathyroid, or adrenals. Therefore, they can also be eliminated as probable stimulants to parathyroid secretory activity.

The occurrence of calcium and parathyroid anomalies coincident with other hormonal anomalies, as reviewed in Chapter II, may represent a multiple endocrine syndrome or a parathyroid response to extremely high levels of other pituitary hormone. Since the occurrence of parathyroid lesions and plasma calcium abnormalities do not consistently occur in conjunction with hyper- or hyposecretion of the pituitary hormones, as reviewed in the literature survey, it is doubtful that

the known pituitary hormones are the cause. Although a massive dose of GH given to rats over a 4 to 6 week period did appear to increase plasma calcium and iPTH (61), this may be an indirect effect or the GH preparation may have been contaminated with sufficient amounts of a separate parathyrotropic substance to produce the effect. The action of GH on plasma calcium has been attributed to an indirect effect (35). According to this theory, GH causes a decrease in plasma phosphate which indirectly results in an increase in the plasma calcium concentration. As noted in Chapter IV, attempts to directly stimulate parathyroid secretion with large doses of TSH failed. Parathyroid involvement in multiple endocrine disorders are common. In one study of 85 cases, 88 percent involved the parathyroid and pituitary glands together (14). Of these same 85 cases, 22 involved only the parathyroid and pituitary with no other hormonal involvement known.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The "in vitro" parathyroid gland perfusion system described in this dissertation has the following characteristics:

1. maintains a functionally stable gland for 6 to 8 hours;
2. the glands become functionally stable within 1 to 1.5 hours after perfusion begins;
3. the glands are functionally responsive to stimuli;
4. a loss of functional stability after 6 hours or when induced, as with anoxia, can usually be detected by an increase in perfusion pressure;
5. dynamic secretory responses may be monitored on a minute by minute basis;
6. when stored at -70°C, iPTH samples remain stable for six months.

The pituitary gland may have a direct stimulatory influence on the parathyroid secretory rate under both physiological and pathological conditions. The following observations have been made about the pituitary-parathyroid relationship:

1. Hypophysectomy in rats may reduce serum calcium concentration, but there appears to be other mitigating variables.
2. Injection of a pituitary preparation can increase the serum calcium concentration in calves.

3. The parathyroid gland, "in vitro" can be stimulated directly by a pituitary extract.
4. The parathyroid stimulating factor in the pituitary preparation is not TSH, GH, LH, PROLACTIN, ADH, OXYTOCIN, ACTH, -MSH, or -lipotropin.

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APPENDIX A

"Some General Features of Endocrine Influence on Metabolism." W.B. Cannon. 1925. Trans. Cong. Am. Physicians and Surgeons. XIII:31-53.

"The first proposition is that in an open system, such as our bodies represent, complex and subject to numberless disturbances, the very existence of a poised or steady state is in itself evidence that agencies are at hand keeping the balance, or ready to act in such a way as to keep the balance. The agencies may act antagonistically, ...atonic action is also possible...."

"A second proposition is that, if the state remains steady, there is an automatic arrangement whereby any tendency towards change is effectively met by increased action of the factor or factors which resist the change."

"A third proposition is that any factor which operates to maintain a steady state by action in one direction does not act at the same point in the opposite direction."

"A fourth proposition, related to the third, is that factors which may be antagonistic in one region, where they effect a balance, may be co-operative in another region. It is necessary, therefore, to define closely the field of action of opposing factors in discussing a physiological equilibrium."

"A fifth proposition is that the system of checks which determines a balanced state may not be constituted of only two antagonistic factors; on either side there may be two or more, brought into action at the same time or successively."

"A sixth proposition is that when a physiological factor is known which can shift a steady state in one direction, it is reasonable to look for a physiological factor or factors having a contrary or counterbalancing effect."

APPENDIX B

PITUITARY PREPARATION A*

Known Hormones Present	Specific Activity
TSH	1.0-1.2 IU/mg
ACTH	$<$ 0.05 IU/mg
GH	$<$ 0.2 IU/mg
LH	$<$0.2 IU/mg
Oxytocin	$<$ 0.1 IU/mg
ADH	$<$ 0.1 IU/mg

Method of purification described in U.S. Patent No. 2,871,159 (January 17, 1959).

Distributed by ICN Pharmaceutical, Inc., (2621 Miles Road, Cleveland, Ohio) as "Thryotropic Hormone," Catalog No. 103057.

*This information supplied by ICN Pharmaceuticals.

APPENDIX C

PITUITARY PREPARATION B*

Known Hormones Present	Specific Activity
TSH	3.49 IU/mg
LH	<0.01 NIH-LH-S1 U/mg
FSH	<0.025 NIN-FSH-S1 U/mg

Method of preparation as described by Reichert, L.E. 1975. "Purification of Anterior Pituitary Hormones (Ovine, Bovine, Rat, Rabbit)," in Methods in Enzymology, 37:360.

Supplied courtesy of NIAMDD, as Bovine, NIH-TSH-B8.

*This information supplied by NIAMDD.

APPENDIX D

PITUITARY PREPARATION C*

Known Hormones Present	Specific Activity
GH	0.81 IU/mg
TSH	0.05 USPU/mg
LH	0.025 NIH-LH-S1 U/mg
Prolactin	0.50 IU/mg

Method of preparation as described in Reichert, L.E. 1970. J. Clin. Endo. and Metab. 31:331.

Supplied courtesy of NIAMDD, as Bovine, NIH-GH-B18.

*This information supplied by NIAMDD.

APPENDIX E

PITUITARY PREPARATION D*

Known Hormones Present	Content
LH	2.5 ug (normal plasma concentration 1-10ng/ml)

Prepared by Dr. L.E. Reichert, Emory University, Atlanta, Georgia.

Supplied courtesy of L.E. Reichert, Emory University, through Dr. R.J. Orts, Oklahoma State University as LH-LER (Batch No. 1056C-2).

*This information supplied courtesy of Dr. L.E. Reichert.

APPENDIX F

PITUITARY PREPARATION E*

Known Hormones Present	Specific Activity
Prolactin	29.2 IU/mg

Prepared by Sigma Chemical Co., St. Louis.

Distributed by Sigma Chemical Co. (P.O. Box 14508, St. Louis, Missouri) as "Luteotropic Hormone" catalog no. L4876.

*This information supplied by Sigma Chemical Company.

APPENDIX G

PITUITARY PREPARATION F*

Known Hormones Present	Specific Activity
ADH	370 IU/mg

Prepared synthetically by Bach Chemical.

Distributed by Bach Chemical (4077 Glencoe, Marina Del Rey, California) as "(Arg^8)-Vasopressin", Catalog no. 8747.

*This information supplied by Bach Chemical.

APPENDIX H

PITUITARY PREPARATION G*

Known Hormone Present	Specific Activity
Oxytocin	500 IU/mg

Prepared synthetically by Bach Chemical.

Distributed by Bach Chemical (4077 Glencoe, Marina Del Rey, California) as "Oxytocin", Catalog no. 5693.

*This information supplied by Bach Chemical.

VITA

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Doctor of Philosophy

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