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The effects of stress on the blood calcium level in the male white rat (Rattus norvegicus)

Howard Perry Cobb

Abstract

THE EFFECTS OF STRESS ON THE BLOOD CALCIUM LEVEL IN THE MALE WHITE RAT (Rattus norvegicus) by Howard Perry Cobb III was written as part of the requirements for a Master of Science degree in Biology at the University of Richmond (May, 1985). The present experiment was designed to determine whether parathyroid hormone (PTH) can be considered a "stress" hormone. Parathyroidectomized (PX) male rats (160-200 g) were injected with 10, 20, or 30 USP units of PTH per 100 g body weight and subjected to confinement/UHF stress for a 1.5-h period. Serum calcium levels of these PX groups were compared to sham-operated rats stressed in the same manner. Serum calcium levels of the stressed uninjected PX rats and those injected with 10 USP PTH dropped by 7.7% and 14.7% respectively whereas serum calcium levels of the PX+20 USP PTH dropped only by 3.3%. Serum calcium levels of the PX+30 USP PTH showed an increase similar to the sham-operated rats (5.2% and 7.0% respectively). These findings clearly demonstrate a role for PTH in the stress response.

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by

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Preface

Today, it is well known that parathyroid hormone (PTH) plays a prominent role in controlling serum calcium levels through its actions on bone, intestine, and kidneys. The parathyroid glands were first discovered anatomically by Sandstrom in 1880, but little attention was paid to this discovery. They were rediscovered by Gley, in 1891, and by Rohn, in 1895. In 1909 MacCallum and Voegtlin observed that tetany after parathyroid destruction was due to hypocalcemia and that the infusion of calcium salts restored thyroparathyroidectomized dogs to normal. Greenwald in 1911 reported a decrease in the excretion of inorganic phosphate in urine due to thyroparathyroidectomy. Although extracts of the parathyroid gland were isolated by Hanson in 1923 and Collip in 1925 the chemical identification of PTH was much later by Aurbach in 1959. Albright and Ellsworth, in 1929, proposed that PTH acts directly on bone on the basis of their observation of the presence of absorption cavities in bone from a patient with idiopathic hypoparathyroidism. Patt and Luckhardt were able to show in 1942, by perfusion of the parathyroid glands with serum depleted of calcium, that these glands increased secretion when stimulated by a lowered concentration of calcium in the serum. It is from these preliminary experiments that parathyroid research gained a solid footing.

The number of parathyroid glands varies in mammals,

with most having two (e.g., rats), but some having four (e.g., humans). They are usually embedded in the thyroid gland and are surrounded by a connective tissue capsule from which septae extend inward dividing the gland into lobules. The glands contain oxyphil cells which have an unknown function, and chief cells which synthesize and secrete parathyroid hormone. The blood supply is mainly from the anastomosing branches of the superior thyroid arteries (Turner and Bagnara, 1976; Martin, 1976). The thyroid and parathyroid glands can be differentiated histologically by the fact that the thyroid cells are arranged in follicles whereas the parathyroid cells are closely packed (mainly chief cells) and are not arranged into follicles (DiFiore, 1980).

The cells of the parathyroid synthesize a pre-pro-parathyroid hormone (109 amino acids) which is enzymatically cleaved to produce the 90 amino acid pro-parathyroid hormone. The majority of the pro-PTH is converted into PTH (84 amino acids) which is the major form of the secreted hormone. Fragments of PTH (1-84) are produced by liver, kidney, and bone. These fragments of PTH comprise a substantial percentage of the circulating hormone. However, any fragment of PTH, in order to be biologically active on bone and kidney, must consist of a continuous peptide sequence beginning with residue 2 (valine) and extending to residue 26 (lysine) (Goltzman et al., 1984; Frieden, 1976).

As indicated above, PTH stimulates the mobilization and resorption of calcium from bone directly (Bentley, 1982). PTH also stimulates calcium reabsorption in the thick ascending limb of the distal tubule of the kidney (Williams, 1981). Indirectly, PTH also influences calcium absorption in the intestine through Vit. D_3 by promoting 1 α-Hydroxylation of 25-hydroxycholecalciferol into the active metabolite which then acts to stimulate calcium absorption in the intestine (Fraser, 1980).

It should be noted that cAMP plays a major role in PTH action though its mode of action is not known. The postulated sequence of events in PTH-driven, cAMP-mediated calcium (and phosphate) transport can be summarized as follows. PTH binds to its receptor site on the membrane activating adenylate cyclase which in turn converts ATP into cAMP. The cAMP then binds to the inhibitor protein of the calcium pump causing the inhibitor protein to dissociate from and thereby activate the calcium pump (Turner and Bagnara, 1976).

Serum calcium consists of the ionized fraction (50%), the fraction bound to protein [40% (70-90% to albumin and 10-30% to globulins)], and the fractions associated with citrate and phosphate (10%) (Cohen & Kayne, 1983). Although the ionized serum calcium is the biologically significant fraction of serum Ca, the determination of total serum calcium is generally adequate for calcium studies. Ionized

calcium is important in muscle and nerve actions. In muscle contraction, calcium ions bind with the regulatory protein, troponin, which is bound to actin fibers. This changes the conformation of troponin so that it shifts another regulatory protein, rod-like tropomyosin, away from the myosin-binding sites on actin molecules. This permits crossbridge formation and filament sliding due to myosin-actin binding and thereby contracting the muscle. In neurons, the tips of the axons have synaptic knobs which contain neurotransmitters. When the action potential reaches these synaptic knobs, calcium ions enter the cytoplasm through calcium gates. This shift causes the vesicles to rupture and empty their neurotransmitter into the synaptic cleft which passes the action potential to the next neuron. Therefore, calcium ions are probably even more essential in muscle and nerve action during physical exertion.

The hormonal stress response in most mammals starts within seconds with the liberation of adrenal catecholamines (epinephrine and norepinephrine). These hormones enable the body to meet conditions of stress such as shock, cold, pain, intense muscular excitation, and emotional excitement. Resistance to infection is also markedly diminished in their absence. The catecholamines achieve these actions by setting into motion a large number of physiological mechanisms required to sustain vigorous activity. They stimulate glycogenolysis and gluconeogenesis in the liver,

and the activation of lipases (Martin, 1976). However the main actions of the catecholamines are to stimulate the heart, increase cardiac output, and constrict blood flow to structures not needed in times of stress.

The next major defense the body has against stress is the glucocorticoids which also enhance resistance to physical "stress" within minutes. Glucocorticoids increase the amount of energy available during times of stress by increasing blood glucose levels, and by accelerating the metabolism of fat and protein. The primary stimulus that initiates glucocorticoid secretion is any kind of stress, especially any type of body damage. The stress probably causes glucocorticoid secretion by initiating nerve impulses that are transmitted from the periphery into the hypothalamus. The hypothalamus then secretes an ACTH- releasing hormone which stimulates the release of ACTH from the pars distalis of the hypophysis. The ACTH stimulates the release of glucocorticoids from the adrenal cortex.

It occurred to me that there might be another hormonal involvement in stress response. It is my theory that PTH is part of the defenses against stress. It is proposed that PTH hormonal actions would take place about one hour after the stressful situation. A slight increase of PTH secretion would produce a mild state of hypercalcemia which would enhance many of the major stress responses such as muscle contraction and neuron activity. Also, a slight

PTH secretion would affect other stress responses such as blood clotting, enzyme activity, insulin output, and other hormone-target reactions. The following experiment was designed to examine this hypothesis, i.e., whether PTH is part of the stress response and whether it should be considered a "stress" hormone.

The definitive exposition of this master's thesis follows the format required for publication in the "Rapid Communications" section of the journal Endocrinology, a section reserved exclusively for discoveries at the cutting edge of the discipline. This format has been followed with the intent of submitting the paper immediately upon successful defense for the master's degree.

Abstract

The present experiment was designed to determine whether parathyroid hormone (PTH) can be considered a "stress" hormone. Parathyroidectomized (PX) male rats (160-200g) were injected with 10, 20 or 30 USP units of PTH per 100g body weight and subjected to confinement/UHF stress for a 1.5-h period. Serum calcium levels of these PX groups were compared to sham-operated rats stressed in the same manner. Serum calcium levels of the stressed uninjected PX rats and those injected with 10 USP PTH dropped by 7.7% and 14.7% respectively whereas serum calcium levels of the PX+20 USP PTH dropped only by 3.3%. Serum calcium levels of the PX+30 USP PTH showed an increase similar to the sham-operated rats (5.2% and 7.0% respectively). These findings clearly demonstrate a role for PTH in the stress response.

Introduction

This set of experiments was done to test the intuitive proposal that the stress response includes an increased availability of serum calcium mediated by an increase in parathyroid hormone (PTH) release.

Methods and Materials

Sixty-five 160-200 gram male rats, Rattus norvegicus (Sprague-Dawley), purchased from Dominion Laboratories (Dublin, Va.), were used in these experiments. The rats were given Purina Lab Chow and tapwater ad libitum, and were housed two to a cage in a photoperiod of 12L:12D. Initially three rats were used to test whether serum calcium levels would change in response to a stress that consisted of confinement in a body-tight plexiglas container and exposure to loud radio static for 1.5 h. The rats were lightly anesthetized with ether and blood samples were taken from the tail by cut-down right after they were placed in the plexiglas containers before the noise stress. Serum was obtained by centrifugation. After the 1.5 hours of combined confinement and noise stress, the rats were again slightly etherized to permit unhindered blood flow and blood was obtained in this and subsequent trials as before. Serum calcium levels were determined colormetrically by Connerty and Briggs' o-cresolphthalein complexone procedure (Sigma, 585-A).

This pilot experiment was repeated but this time it was designed to compare serum calcium levels in eight rats that had been parathyroidectomized (PX) by cautery while under sodium pentabarbital anesthesia (30 mg/KgBW) with that of five rats that were sham-operated and had undergone surgery identical to parathyroidectomy except that the

connective tissue near the thyroid gland was cauterized rather than the parathyroid glands. Three additional PX rats were then used to estimate a dose of PTH that would restore serum calcium levels to normal. PTH (Sigma, PO892) was dissolved in distilled water to give a concentration of 20 USP per 0.1 ml. Fifteen to twenty hours after the parathyroidectomy, two rats were injected with 20 USP units of PTH per 100 gram body weight (BW) and one with 10 USP units/100 g BW. Six hours after the injection, the pilot stress experiment was repeated on all 16 of these rats.

The main experiment was designed as follows. The rats were divided into five groups: sham-operated, PX, PX+10 USP PTH per 100 g BW, PX+20 USP PTH per 100 g BW, and PX+30 USP PTH per 100 g BW. The source of the noise stress was a commercial device, ULTRASON (Rat-X, Chicago), emitting ULTRA High Frequency sound (112 db @ 3 ft. @ 21 Kc) and designed as a rat eradicator. It should be noted that sham controls were injected with the PTH vehicle (A.D.). In addition to before and after stress testing, there were also two groups (a sham and a PX+10 USP PTH per 100 g BW) used to test for any stress occurring during blood sampling. This control experiment consisted of taking blood samples, as previously described, and then placing the animals in a quiet location for the standard 1.5-h period. The blood was again taken for calcium analysis and these values were used to determine any statistical differences between the

samples. Individual runs, conducted over a period of several weeks, always involved representatives of both control and experimental groups. Results were evaluated for statistical significance using the Mann-Whitney test and an analysis of variance.

Results

Stress increased serum calcium by 8% (9.4 vs 8.7 mg%) in the three intact rats used in the pilot study. Data from the subsequent experiments were combined and are shown in Figure 1. The stressed sham-operated rats showed a 7% increase in serum calcium, comparable to that observed in unoperated rats in the pilot study. However, starting levels in the unoperated rats were higher than in the shams and therefore only the shams are included in Fig. 1 as controls for nonspecific stress. It should be noted that the sham control values, examining the possibility of blood-sampling induced stress (samples taken before and after the 1.5-h quiet period), were not statistically different from the before-stress values in sham rats subsequently subjected to 1.5-h confinement/UHF stress.

Parathyroidectomy produced the expected decrease in the rats' serum calcium, and after stress, the serum calcium levels dropped even further (Figure 1). Serum calcium levels of PX rats were brought back to normal with 10 USP PTH but the serum calcium levels dropped 14.7% when the rats were stressed with confinement and UHF wavelengths.

It should be noted that the PX+10 USP PTH controls for blood sampling stress (samples taken before and after the 1.5-h quiet period) were statistically the same as the before stress levels in PX+10 USP PTH rats subsequently subjected to confinement/UHF stress and results were combined for statistical purposes. In contrast to PX+10 USP PTH, the serum calcium levels of the PX+20 USP PTH rats only dropped 3.3% from their unstressed levels. However, the stressed PX+30 USP PTH rats showed an increase of 5.2% thereby approaching the values observed in the sham rats. Statistical analysis revealed a p value of 0.15 between the stressed shams and PX+30 USP PTH rats.

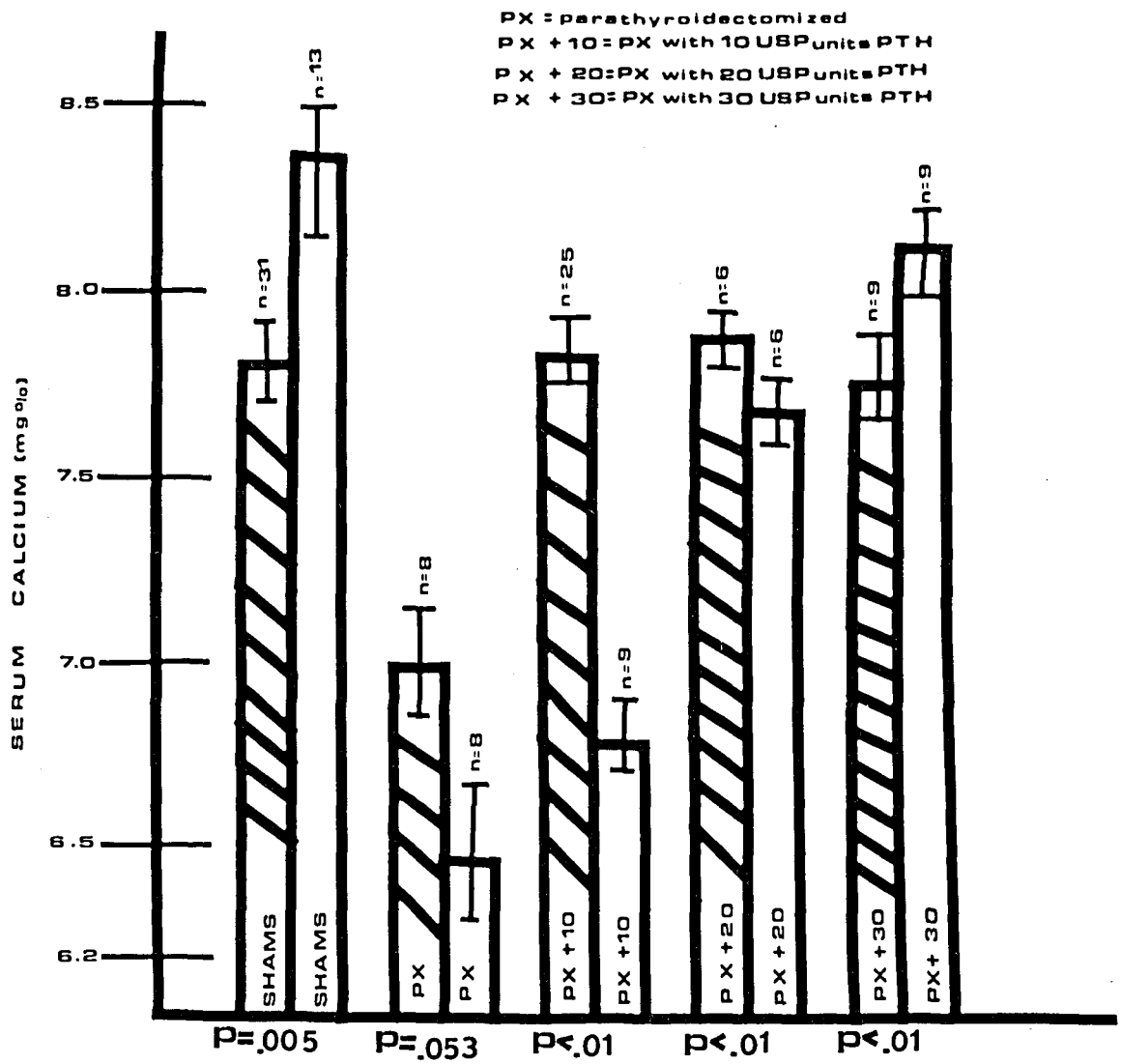

Figure 1. Changes in serum calcium levels in response to stress in sham-operated, parathyroidectomized (PX), and PX-treated with various amounts of PTH. Note the 14.7% drop in the PX+10 serum calcium compared to the 5.2% increase of the PX+30 and the 7.0% increase in the sham-operated rats' serum calcium. Striped boxes = unstressed and plain boxes = stressed. The brackets indicate the standard error of mean for the samples.

Discussion

The results of the work presented here fit precisely with the prediction that part of the stress response is a rise in serum calcium and that this rise is mediated by PTH. This was evident in stressed rats without parathyroid glands. These rats exhibited a marked decrease in serum calcium in contrast to the rise in sham-operated rats. This finding strongly suggested that calcium was being utilized peripherally but does not rule out the possibility of increased renal clearance. The PX+10 USP PTH rats had a before-stress calcium level similar to the sham-operated rats but, in contrast to the shams, stress caused a significant drop in serum calcium. From this, it would appear that the in situ parathyroid glands are able to respond to stress with an increase in hormone release and that the PX+10 USP PTH rats had enough PTH available to elevate their low serum calcium levels back to normal but not enough to respond to the stress thus eliminating renal clearance as a factor. This was further demonstrated by the PX+30 USP PTH rats which not only had enough PTH in their systems to elevate their serum calcium levels back to normal, but also to respond to stress by elevating their serum calcium levels.

A computerized literature search over the past 15 years revealed only four studies that have relevance to the present findings. The only in vivo study was reported

by Tigranian et al. (1980) who found a significant serum calcium increase in 15 male medical students following their medical board examinations but were unable to demonstrate PTH mediation of this response. The three *in vitro* studies appear to shed light on the mechanisms involved in the parathyroid's stress response. Adel et al. (1983) demonstrated enhanced PTH action on isolated perfused bone from glucocorticoid-treated dogs. Brown et al. (1977 and 1978) were able to show α-adrenergic stimulation of parathyroid hormone release from isolated bovine parathyroid cells. From these *in vitro* studies, it would appear that the two most well documented "stress" hormones, glucocorticoids and catecholamines, can mediate the role of PTH in two ways, first by direct stimulation of PTH release and second, by sensitizing bone to PTH action.

The present findings establish a new dimension of stress research in which studies on PTH will play a vital role.

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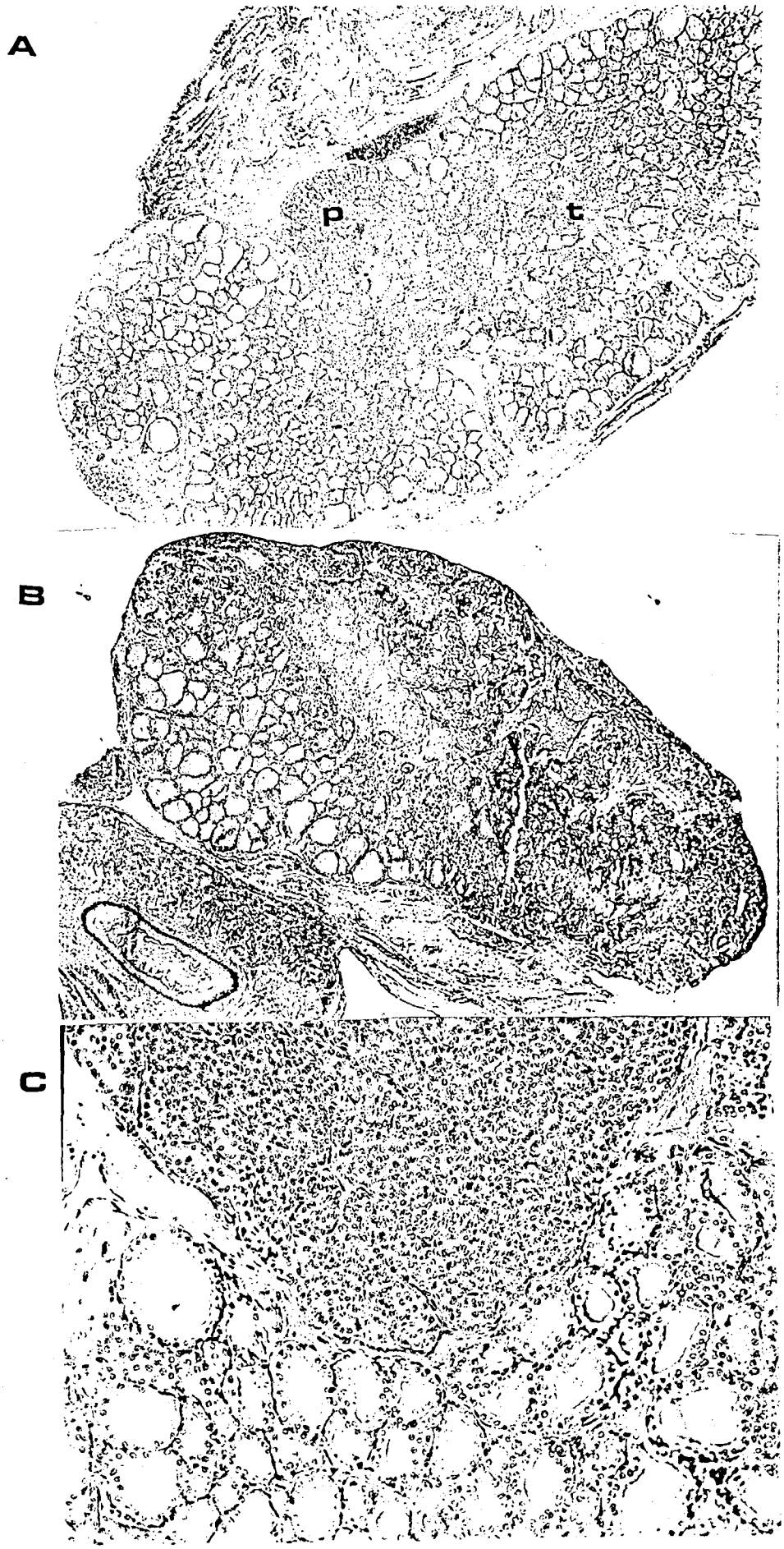
Appendix

It should be noted that in order to evaluate complete parathyroid removal other than by physiological means (serum calcium), the rats were killed by ether overdose and the thyroids removed and placed in Bouin's fixative. The tissue was later embedded in paraffin, sectioned in 10 m sections, stained with hematoxylin-eosin and examined microscopically (Figure 2).

Figure 2. A. photomicrograph of a sham rat's thyroid (T) and parathyroid (P), x 40; B. photomicrograph of a thyroid that was cauterized for parathyroid removal, x40; C. parathyroid/thyroid interface, x200; D. parathyroid/thyroid interface, x400; E and F. photomicrograph of cauterized area, x100.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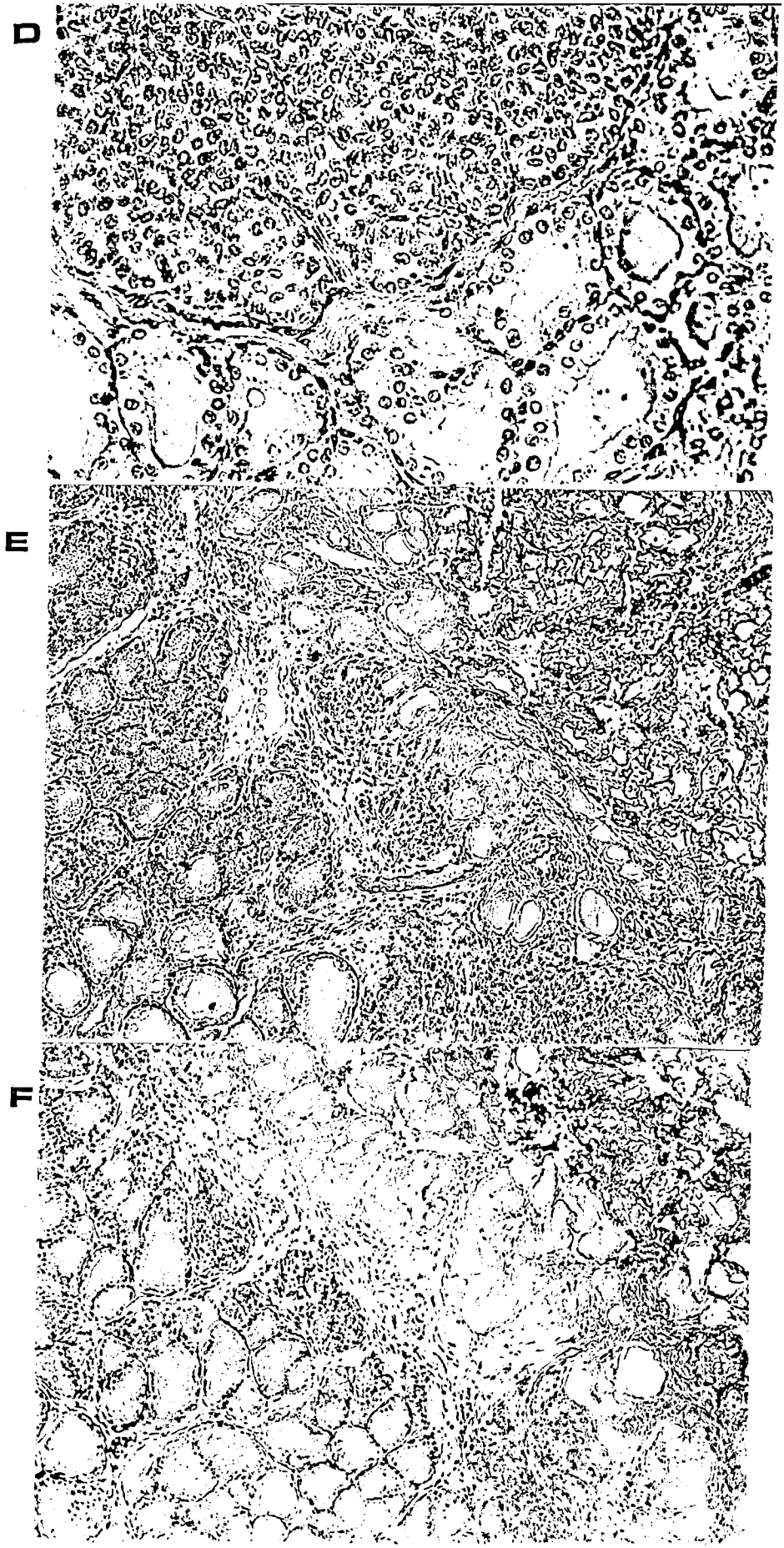
D
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Embedding procedure

Bouin's fixative	24 hours
Distilled H_2O	1 hour
50% ETOH	45 min.
70% ETOH	1 hour
95% ETOH	45 min.
100% ETOH	45 min.
xylene/100%	1-2 hours
xylene	30 minutes
xylene/paraffin	30 minutes
paraffin	30 minutes
paraffin	30 minutes

Staining

xylene	10 minutes
xylene	5 minutes
xylene	2 minutes
100% ETOH	2 minutes
100% ETOH	2 minutes
100% ETOH	2 minutes
95% ETOH	2 minutes
70% ETOH	30 minutes
50% ETOH	2 minutes
H_2O	2 minutes
Hematoxylin	2-5 minutes
H_2O	2 minutes
50% ETOH	2 minutes

70% ETOH	2 minutes
95% ETOH	2 minutes
Eosin	5 minutes
100% ETOH	2 minutes
100% ETOH	2 minutes
100% ETOH	2 minutes
xylene	2 minutes
xylene	2 minutes
xylene	2-5 minutes

Solutions

Bouin's Fluid

Sat. (aq) solution picric acid	75cc
Commercial formalin	20cc
Glacial acetic acid	5cc

Delafield's Hematoxylin-eosin

(A) Stock solution: eosin

Eosin Y	.5gms
95% ethanol	500ml

(B) Stock solution: Delafield's Hematoxylin

Hematoxylin, C.I. 75290	4g
95% ethanol	25cc
Ammonium alum	36gm
Distilled H_2O	400cc

Let stand exposed to light lightly covered.

Filter. Add:

Glycerine	100cc
Methanol, 100%	100cc

Let the solution stand 6 weeks to ripen. The stock will keep indefinitely.

VITA

Howard Perry Cobb III was born September 30, 1960 in Williamsburg, Virginia. He received his high school degree from Mahopac High School, Mahopac, New York in 1979. He then received his Bachelor of Science in Biology from Hampden-Sydney College, Hampden-Sydney, Virginia in 1983. Next, he attended graduate school at the University of Richmond, Richmond, Virginia and graduated with his Master of Science in 1985.