ULTRASTRUCTURAL LOCALIZATION OF CALCITONIN IN THE PARAFOLLICULAR CELLS OF PIG THYROID GLAND WITH CYTOCHROME c-LABELED ANTIBODY FRAGMENTS

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ABSTRACT

Parafollicular cells in mammalian thyroid glands are thought to be responsible for the secretion of calcitonin. In this study, calcitonin was localized in pig thyroid gland by an indirect immunocytochemical technique using rabbit antiserum directed against synthetic porcine calcitonin for the first step, and sheep Fab fragments prepared against rabbit Fab and coupled to cytochrome c for the second step. The antigenic determinants of calcitonin were present only in the parafollicular cells, whose secretory granules were heavily labeled. Labeling of the cytoplasmic matrix is thought to indicate a possible leakage of the polypeptide from the granules. A striking observation was the complete absence of labeling in the cisternae of the rough-surfaced endoplasmic reticulum and of the Golgi apparatus. It is concluded that the secretory granules of parafollicular cells contain calcitonin; the mechanism of synthesis of this peptide is not clearly understood.

INTRODUCTION

The first evidence for the existence of a second type of epithelial cell in the mammalian thyroid gland was provided by Nonidez (26) who rediscovered the "parenchymatous cells" of Baber (5) and interpreted their position as being parafollicular. More recently, numerous ultrastructural studies of the thyroid gland in various species (3, 4, 12, 27, 30, 32) have confirmed the existence of the parafollicular cells which are characterized by their position in the epithelium of the thyroid follicle; invested by the follicular basement membrane, they never come in contact with the colloid but are always separated from it by a cytoplasmic extension of adjacent follicular cells. Electron microscopy has also shown that the parafollicular cells contain cytoplasmic granules of the type found in endocrine cells of the APUD series as defined by Pearse (29).

Since the discovery of calcitonin (CT)[1] by Copp et al. (9), evidence has accumulated supporting the idea that CT is secreted by the parafollicular cells.

[1] Abbreviations used in the text: CT, calcitonin; DAB, 3,3'-diaminobenzidine; Fab, antigen binding fragment; IgG, immunoglobulin G; PAF, picric acid–formaldehyde; RER, rough-surfaced endoplasmic reticulum; THB, 0.2 M, pH 7.4 Tris-HCl buffer.

CT-containing cells (C-cells) have been identified by immunofluorescence (7, 20) in the pig thyroid gland. Since degranulation of the parafollicular cells has been observed after hypercalcemia (13, 23, 24), these cells were regarded as responsible for the production of CT and their cytoplasmic granules were assumed to represent a storage form of the hormone. Nevertheless, positive identification of CT in the granules has not yet been made. On the other hand, it has been recently established by electron microscope radioautography of rat thyroid gland that the cytoplasmic granules of parafollicular cells contain 5-hydroxytryptamine (14, 15, 18).

The purpose of this work was to localize the CT-antigen in pig thyroid gland by the use of a recently developed immunoenzymatic procedure (21) allowing sufficient resolution to confirm the identity of parafollicular cells and C-cells and to examine which organelles are involved in the production of this blood calcium–lowering principle.

MATERIALS AND METHODS

Animals

The study was performed on nine pigs (*Sus crofa domesticus* L.): three were adults whose tissues were obtained from a slaughter house, six were newborn piglets weighing between 600 and 2000 g each, of which three were less than 12 hr old and had been separated from the sow before any ingestion of colostrum; of the remaining three, two were 3 days and one was 6 days old, and all three had been suckled freely.

Tissue Preparation

The adult thyroid glands were removed 5–10 min after death. Some tissues were immediately frozen in liquid nitrogen; frozen sections were cut at 6–8 μ on a cryostat and prepared for immunofluorescence. Other specimens were fixed 3–6 hr by immersion in 2.3% glutaraldehyde in 0.1 M, pH 7.4 cacodylate-HCl buffer or 2.7% formaldehyde with 0.2% picric acid (PAF) and 5 mEq/liter CaCl₂ in 0.075 M, pH 7.4 cacodylate-HCl buffer and adjusted with NaCl to a final osmolarity of 1050 mosmols/liter. All fixations were performed in an ice bath. The piglet thyroid glands were fixed with the same solutions chilled in an ice bath and perfused into the abdominal aorta according to the method previously described (21); venous drainage was effected through the sectioned jugular veins. After the perfusion, the thyroid glands were dissected and cut into small pieces which were further fixed overnight by immer-

sion in the same fixative at 4°C. Some tissues fixed in PAF were postfixed immediately by overnight immersion in 3% glutaraldehyde buffered with 0.1 M, pH 7.4 cacodylate-HCl. After fixation, specimens were washed in several changes of 0.2 M, pH 7.4 Tris-HCl buffer (THB) or of 0.2 M, pH 7.4 cacodylate-HCl buffer and stored at 4°C. Tissues were processed for light and electron microscopy as indicated below. For immunoenzyme histochemistry, nonfrozen sections of tissues fixed only in PAF were cut at 30–100 μ on a Sorvall TC-2 sectioner (Ivan Sorvall Inc., Norwalk, Conn.).

Immunofluorescence

Cryostat sections were picked up onto slides and allowed to dry. Slides were then placed in a moist box, and sera were applied directly on the sections. An indirect technique (25) was employed using rabbit antisynthetic porcine CT serum (kindly provided by Dr. F. M. Dietrich from the CIBA Research Department, Basel, Switzerland) for the first step and rat IgG directed against rabbit gamma globulin and labeled with fluorescein isothiocyanate (Calbiochem, Los Angeles, Calif.) for the second step. Sections were incubated for 30 min and then rinsed in 0.01 M, pH 7.4 phosphate buffer in 0.15 N NaCl. The following controls were done: fluorescent rat IgG alone; nonspecific rabbit serum followed by fluorescent rat IgG; rabbit anti-CT serum followed by unlabeled rat IgG antirabbit gamma globulin followed in turn by fluorescent rat IgG. All controls were negative. Photographs were taken on Ilford HP⁴ films (Ilford Ltd., Ilford, Essex, England) through a Universal Zeiss microscope fitted with an HBO mercury lamp using blue light excitation and a barrier filter with a cut-off at 550 mμ.

Immunoenzyme Histochemistry

Nonfrozen sections of PAF-fixed tissues were treated by an indirect technique with cytochrome c–labeled antibody fragments as the tracer. For the first step, the sections were incubated 5 hr at room temperature with the rabbit anti-CT serum and then rinsed overnight at 4°C in THB with continuous stirring. For the second step, the sections were incubated in the same conditions with sheep Fab antirabbit Fab coupled with cytochrome c (Grade VI, Sigma Chemical Co., St. Louis, Mo.) by glutaraldehyde (2). This immunoenzymatic tracer was prepared as described in detail in another paper (21). The sections were then rinsed overnight at 4°C in THB with continuous stirring. After these immunological treatments some sections were postfixed 3 hr at 4°C in 3% glutaraldehyde buffered with 0.1 M, pH 7.4 cacodylate-HCl and were rinsed again repeatedly in cold THB. For the histochemical

demonstration of cytochrome c activity, the sections were preincubated for 45 min at room temperature in a 50 mg% 3,3'-diaminobenzidine (DAB) tetrahydrochloride solution (Fluka AG., Buchs, Switzerland) buffered with THB and then incubated for 30 min at 37°C with a solution of 50 mg% DAB in 0.05 M, pH 3.9 sodium citrate buffer containing 0.05% H_2O_2 (19) and adjusted to a final osmolarity of 400 mosmols/liter with NaCl. Finally, the sections were washed two times for 10 min each in THB and rinsed overnight in 0.2 M, pH 7.4 sodium cacodylate buffer.

In some cases, 0.2 M, pH 7.4 cacodylate-HCl buffer was used instead of THB for the rinses after fixation in PAF, the preparation of nonfrozen sections, and the incubations with immunological reagents.

Endogenous cytochrome c-like activity was investigated by incubation of sections in the DAB-citrate H_2O_2 solution without any previous treatment.

The followinng controls were performed: sheep Fab antirabbit Fab coupled with cytochrome c; nonspecific rabbit serum followed by sheep Fab antirabbit Fab coupled with cytochrome c (Fig. 4); rabbit anti-CT serum followed by unlabeled sheep Fab antirabbit Fab followed in turn by sheep Fab antirabbit Fab coupled with cytochrome c. All controls were negative.

Preparative Procedures for Light and Electron Microscopy

After rinsing in 0.2 M, pH 7.4 sodium cacodylate buffer, post-fixation was performed for 60 min in 2% OsO_4 buffered with 0.1 M, pH 7.4 sodium cacodylate. Tissues were then dehydrated in alcohol and embedded in Epon 812. Sections 1 μ thick for light microscopy and thin sections of chosen areas were cut on a Sorvall MT2 ultra-microtome. The 1-μ thick sections were stained with a solution of 1% azure II and 1% methylene blue in 1% borax and were examined on a Zeiss photomicroscope. Thin sections were prepared with a diamond knife and picked up onto grids covered with a Parlodion film (Mallinckrodt Chemical Works, St. Louis, Mo.) reinforced with carbon. Some sections were examined unstained; others were doubly stained using uranyl acetate followed by lead citrate. Micrographs were taken on a Philips EM 300 electron microscope at 80 kv with a 40 μ thin metal aperture in the objective.

RESULTS

Light Microscopy

After *immunofluorescence labeling*, strongly positive spots of approximately the size of a cell were seen in the walls of the follicles and the follicular pattern was weakly outlined by a slight fluorescence (Fig. 1). Even by combining phase-contrast microscopy, it was not possible to ascertain the precise location of the fluorescent spots in relation to the follicular structure.

With *enzyme-labeled tracers*, when tissues had been incubated in THB, heavy deposits of dark greenish-brown reaction product were present in elongated cells sectioned in different planes (Fig. 2). The parafollicular situation of the labeled cells was evident in most cases. Reaction product was also seen in the few remaining erythrocytes and leukocyte granules in sections incubated with tracers as well as in sections prepared as controls of endogenous cytochrome c-like activity. No enzyme activity was seen in other cells or structures. Control sections incubated with nonspecific sera showed no reaction product in parafollicular cells (Fig. 4). When cacodylate-HCl buffer was used for the rinses and incubations, penetration of tracers was very poor and nonspecific adsorption was marked at the edge of the nonfrozen tissues (Fig. 3).

Electron Microscopy

ULTRASTRUCTURE: A number of features were observed in both PAF- and glutaraldehyde-fixed tissues. Parafollicular cells with elongated extensions contained numerous granules 0.1–0.4 μ in diameter. The membrane limiting the granules was very thin and discontinuities were frequently observed (Figs. 7 and 10). Clusters of free ribosomes were scattered throughout the cytoplasm between the granules; some free ribosomes were located between cisternae of the rough-surfaced endoplasmic reticulum (RER). RER was distributed as isolated saccules or occasionally as stacks of parallel or concentric cisternae connected by numerous wide channels. The presence of free ribosomes between the innermost cisternae of concentric arrays could not be ascertained. Some smooth-surfaced vesicles or small vacuoles were seen to be in communication with elements of the RER, especially around the Golgi areas.

After glutaraldehyde fixation, the electron density of the granules varied and the very dense ones tended to be relatively small (0.1–0.2 μ). After PAF fixation (Figs. 5, 7 and 10) the contents of most granules were slightly more electron opaque than the cytoplasmic matrix, and very few
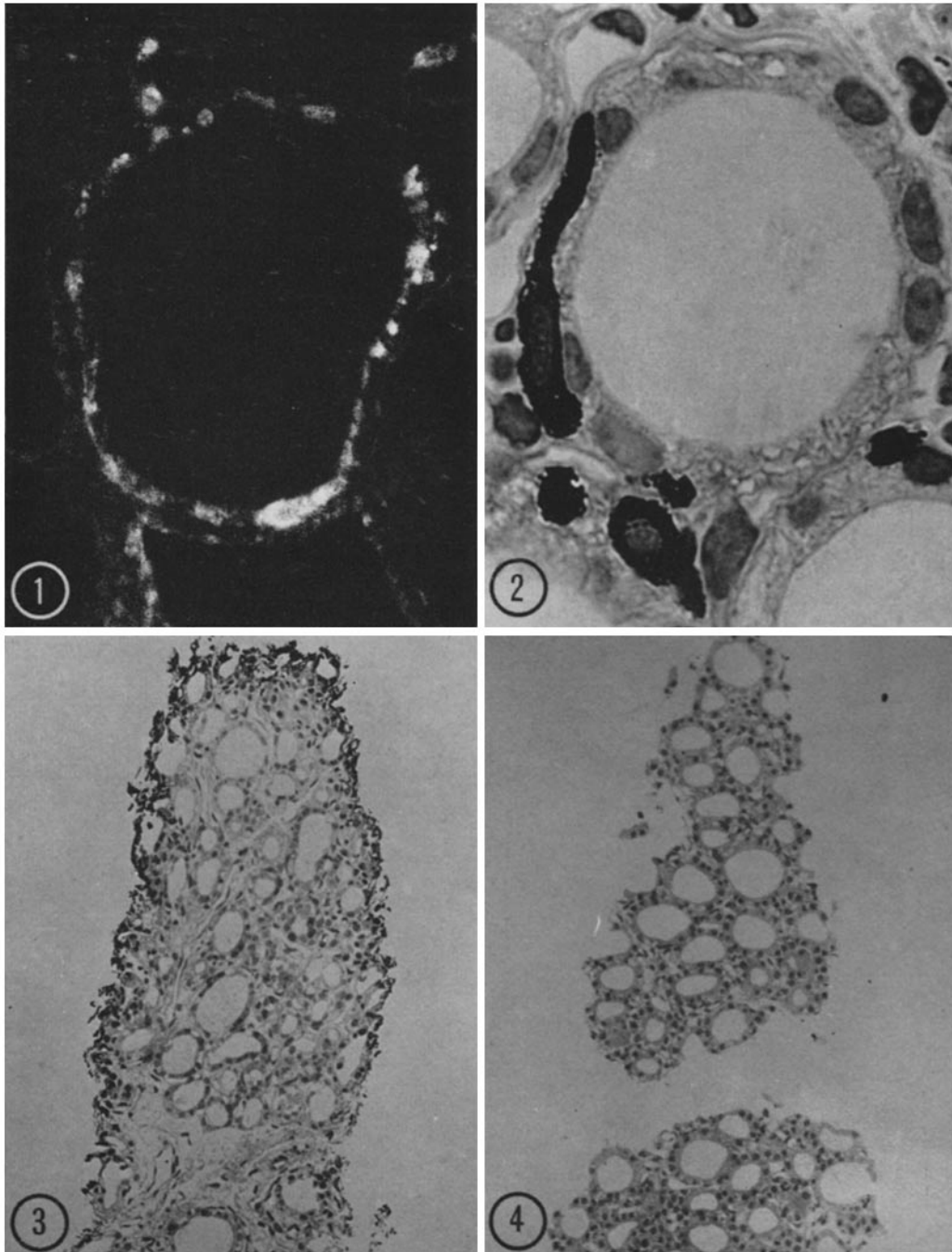
Key to *symbols used in the micrographs:*   *BM*, basement membrane; *Cap*, capillary lumen; *Col*, colloid; *F*, follicular cell; *G*, Golgi area; *gr*, secretion granule; *M*, mitochondrion; *P*, parafollicular cell; *R*, free ribosomes; *RER*, rough-surfaced endoplasmic reticulum.

FIGURE 1   Adult pig; light micrograph of immunofluorescent cryostat section. The position of the positive cells cannot be ascertained. × 600.

FIGURES 2–4   Light micrographs of 1-μ Epon sections stained with azure blue and methylene blue. PAF fixation.

Figs. 2 and 3, newborn pig; immunoenzyme labeling. Fig. 2, Section incubated in THB. Dark reaction product of cytochrome *c* labels elongated parafollicular cells, some of which are sectioned transversally. × 1400. Fig. 3, Section incubated in cacodylate-HCl buffer; nonspecific adsorption of tracer at the edge of the nonfrozen section. No labeling of the center of the section occurred. × 150.

Fig. 4, 3-day old piglet; control section of PAF-fixed tissue incubated in THB with nonspecific rabbit serum followed by sheep Fab antirabbit Fab labeled with cytochrome *c*. No labeling can be seen. × 150.
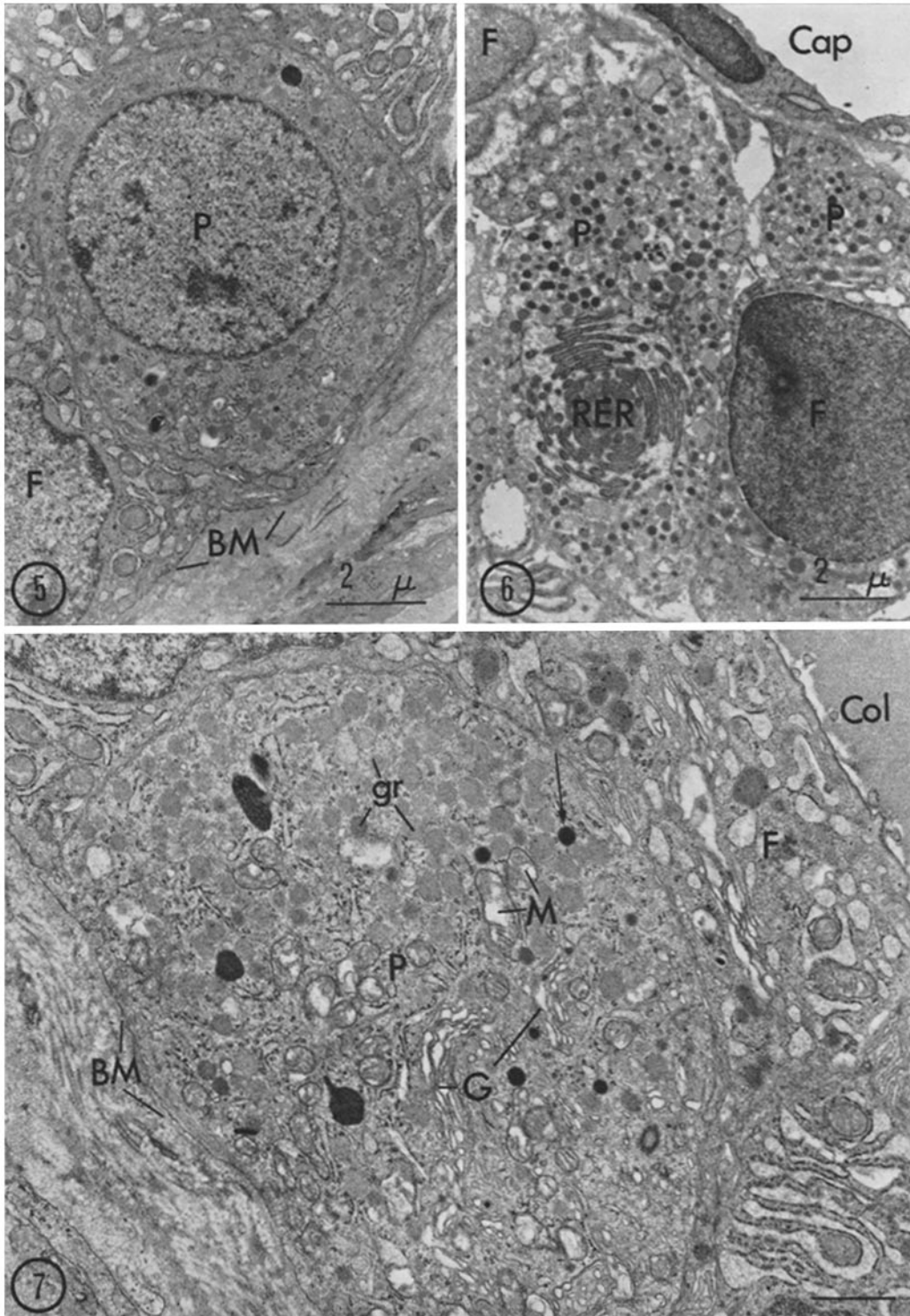
Figure 5 Adult pig; PAF fixation, no immunohistochemical treatment. Most granules of the parafollicular cell have a low electron opacity. Section stained with uranyl acetate and lead citrate. × 7500.

Figure 6 1-day old piglet; PAF followed by glutaraldehyde fixation, no immunohistochemical treatment. Most small granules are electron opaque. A concentric array of RER shows cisternae connected by wide channels. Uranyl acetate and lead citrate. × 6900.

Figure 7 Adult pig; PAF fixation, no immunohistochemical treatment. A parafollicular cell containing numerous granules with low density contents and very few dense granules (arrow). The membranes surrounding the granules are distinctly thinner than those of other organelles. Uranyl acetate and lead citrate. × 12,000.

granules contained highly dense material. Mitochondria showed occasional swellings. When PAF fixation was followed immediately by glutaraldehyde (Fig. 6), the ultrastructure was similar to that observed after glutaraldehyde alone.

IMMUNOCYTOCHEMICAL OBSERVATIONS ON UNSTAINED SECTIONS: All observations reported below were made on tissues incubated with immunological reagents in THB. On survey micrographs (Fig. 8–10) abundant reaction product was located in cells characterized by their parafollicular position. These cells often had elongated cytoplasmic extensions (Fig. 9). All observed parafollicular cells were labeled. The cytoplasmic matrix was diffusely labeled, excepting rare areas at the centers of concentric arrays of RER (Figs. 8

and 12). The intensity of labeling of cytoplasmic matrix varied, but tended to be high. The contents of numerous 0.1–0.4 μ granules were labeled by enzyme reaction product (Fig. 11). In areas where cytoplasmic matrix and granule contents were both strongly labeled, the granule membrane was clearly visible as a thin, circular, negative layer 100–200 A thick (Fig. 13). The contents of stacked RER cisternae were not labeled, nor were the perinuclear spaces or the contents of Golgi elements nor the mitochondrial matrix (Figs. 8, 9, 12 and 13). Some rounded elements were negative (Fig. 13); their precise identity could not be ascertained. No difference was noted in the pattern of labeling of cytoplasmic elements, regardless of the age of the animal. Nuclei were not labeled in ani-
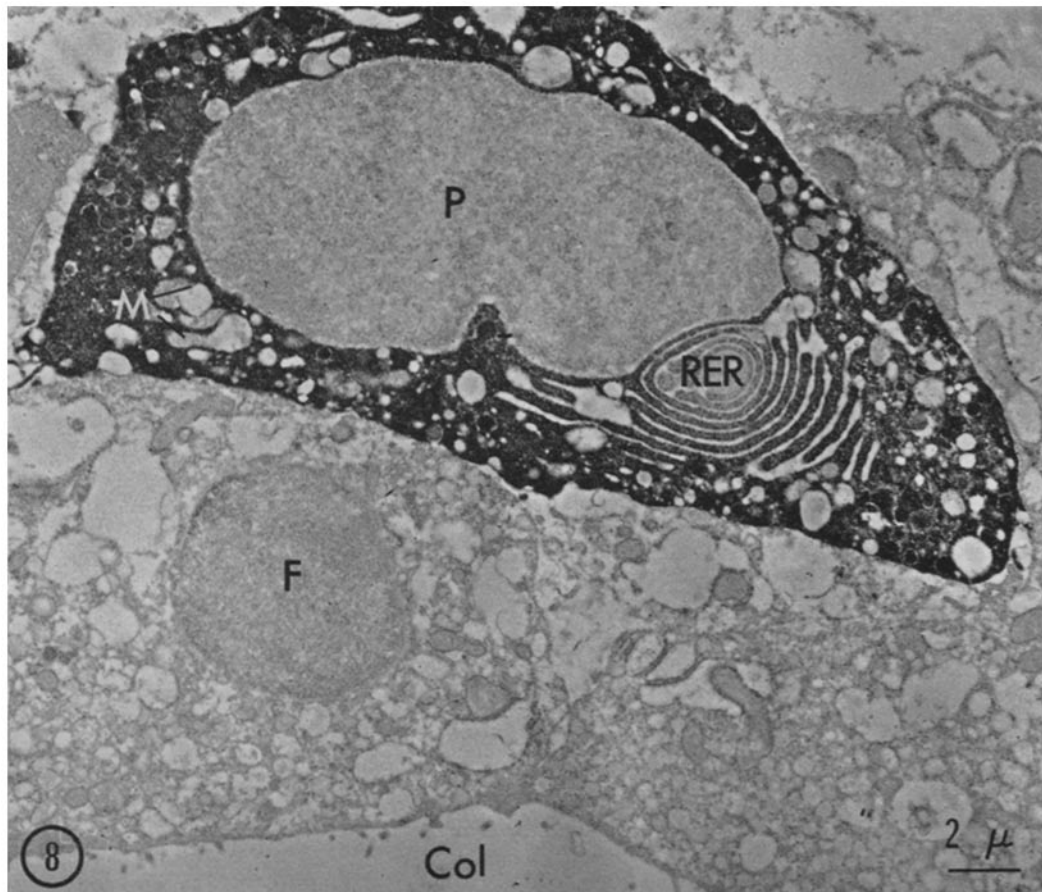

FIGURE 8   3-day old piglet; PAF fixation, immunoenzyme treatment. A parafollicular cell shows marked labeling of granules and cytoplasm. The mitochondrial matrix, the cisternae of the RER, some vacuoles, and the nucleoplasm are unlabeled. Cytoplasmic labeling is weak at the center of a concentric array of RER. Unstained section. × 9900.
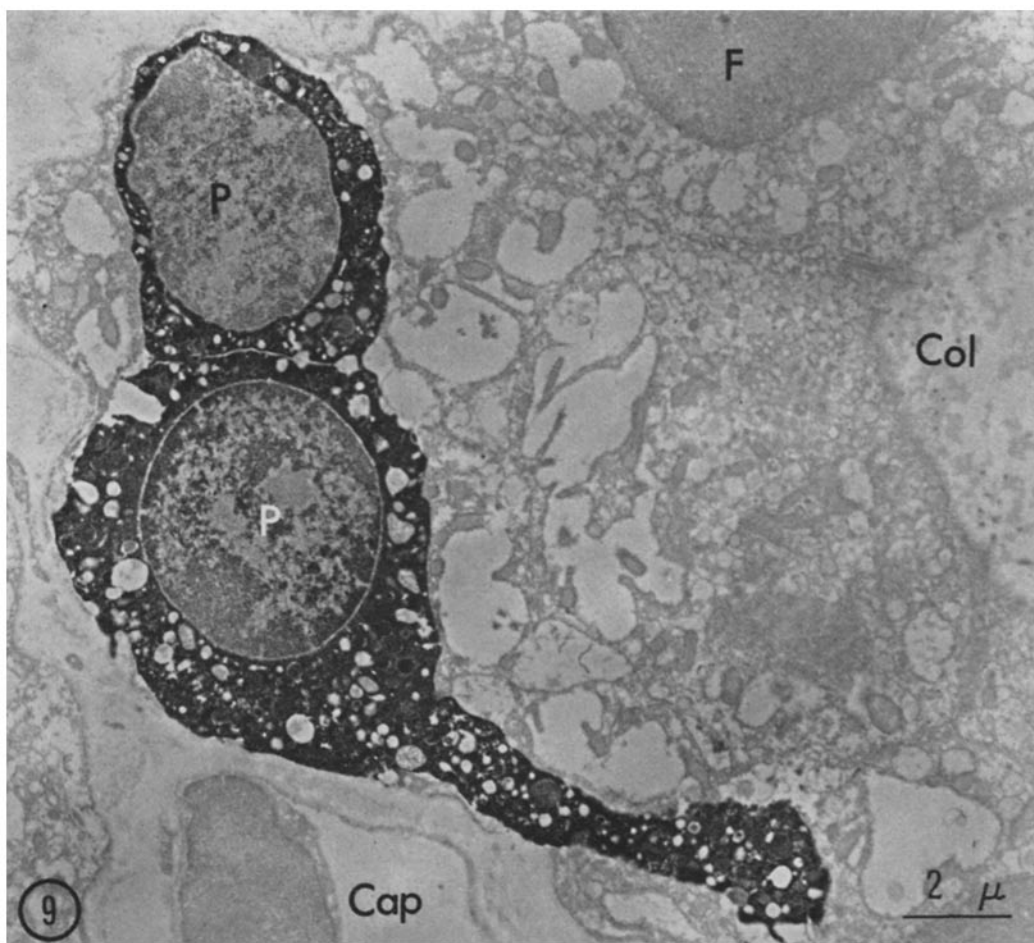
FIGURE 9 Newborn piglet; PAF fixation, immunoenzyme treatment. Two parafollicular cells with strong labeling of granules and cytoplasm and moderate labeling of nuclei. The perinuclear space is negative. Unstained section. × 7500.

mals aged 3 and 6 days (Fig. 8). In unfed newborn piglets aged up to 12 hr, some labeling was observed in the nuclei of all the parafollicular cells; clumps of reaction product tended to be more frequently located towards the periphery of the nucleus (Fig. 9). Endogenous cytochrome c–like activity in parafollicular cells was limited to the cristae of mitochondria (Fig. 8).

DISCUSSION

Picric acid–formaldehyde (PAF) was used as the fixative instead of glutaraldehyde, to improve the penetration of immunological reagents into the fixed tissues. However, if the incubations were carried out in cacodylate-HCl buffer, tracers pene-

trated poorly and were absorbed nonspecifically at the edge of the TC-2 sections. These difficulties disappeared if PAF-fixed tissues were rinsed and incubated in THB, possibly because remaining aldehyde groups were blocked by Tris, but tissue preservation was poorer than with cacodylate-HCl. Even in tissues osmicated and embedded immediately after PAF fixation, the membranes limiting the granules are frequently discontinuous. The contents of most granules are of low electron opacity after PAF fixation. After glutaraldehyde as the initial fixative or following PAF prefixation, some of the smaller granules are electron opaque. Granules with dense contents after glutaraldehyde fixation are thought to contain amines, particu-
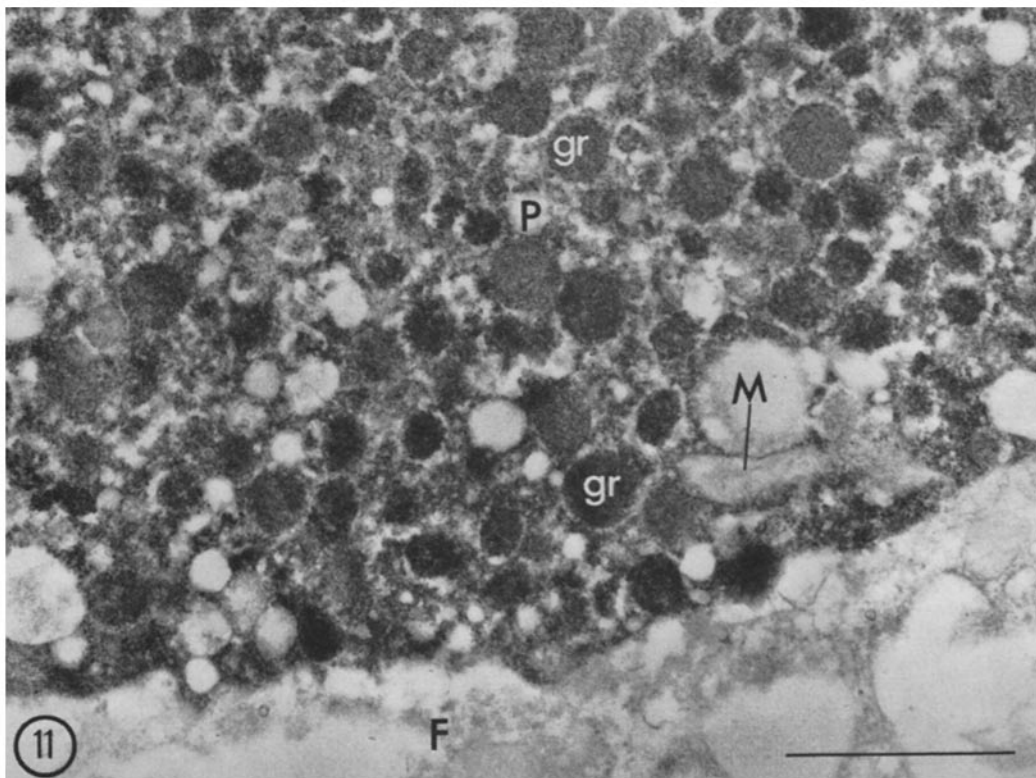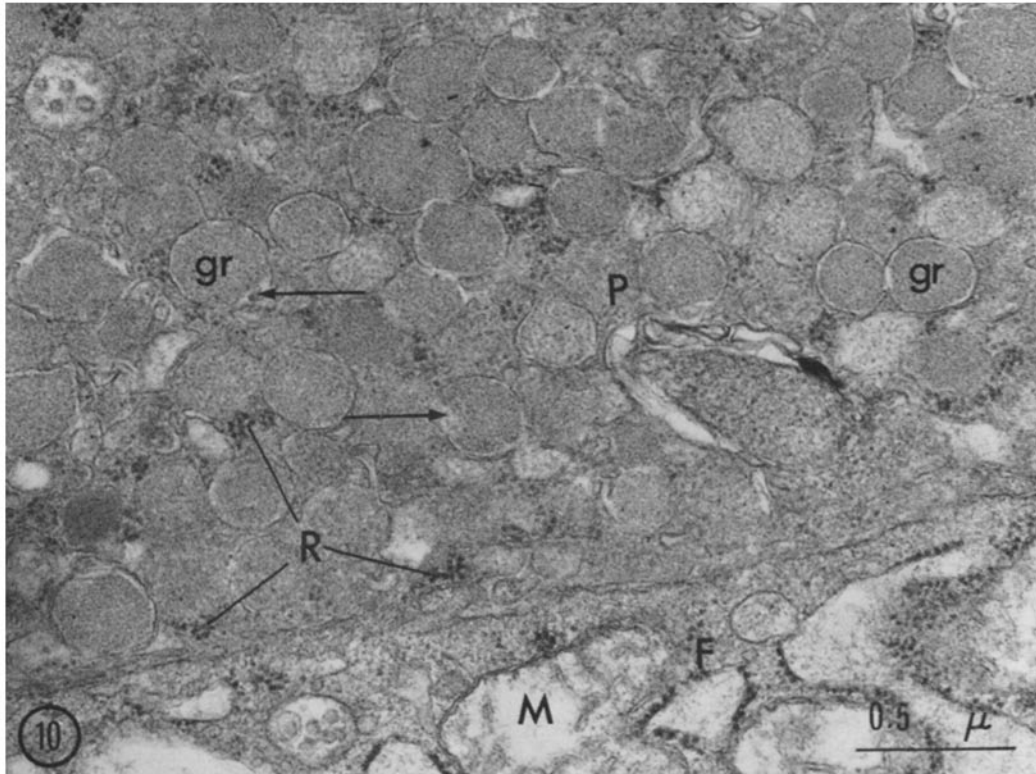
FIGURE 10 Adult pig; PAF fixation, no immunoenzyme treatment. Granules have contents of low electron opacity; their membranes are thin and show discontinuities (arrows). Small clusters of free ribosomes are seen between the granules. Uranyl acetate and lead citrate. × 42,000.

FIGURE 11 Newborn piglet; PAF fixation; immunoenzyme labeling of granules is marked. The cytoplasmic matrix is moderately labeled. The matrix of mitochondria is negative. Unstained section. × 30,000.
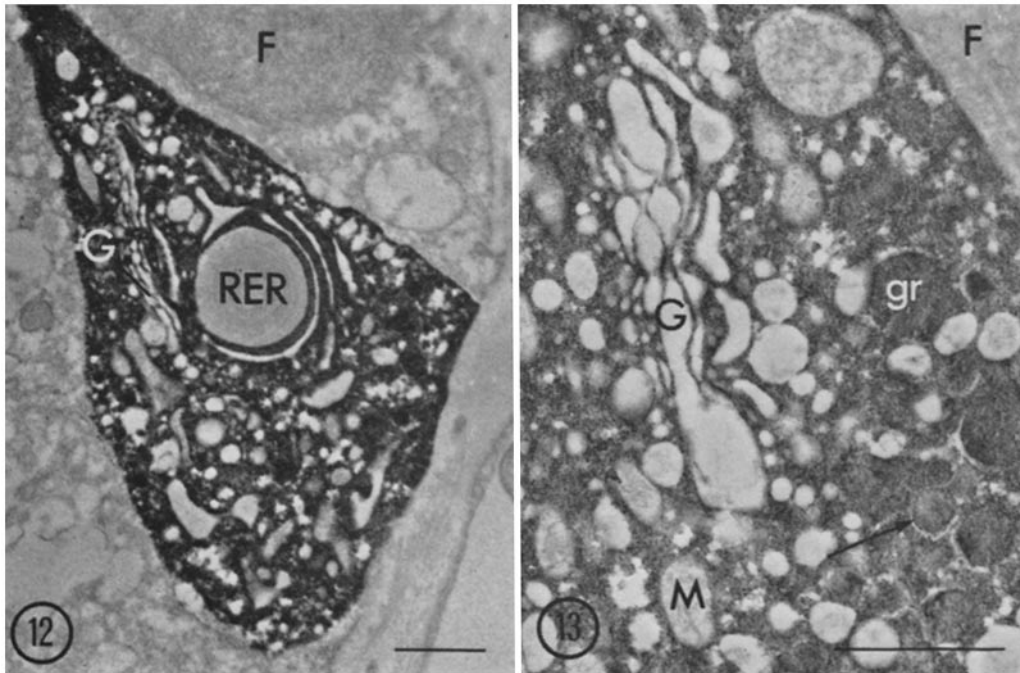
Figures 12 and 13   Newborn piglet; PAF fixation; immunoenzyme labeling of the cytoplasm and granules of parafollicular cells. Golgi cisternae and some vacuoles are negative. Fig. 12   RER cisternae are negative. The cytoplasmic matrix is unlabeled at the center of a concentric array of RER. Unstained section. × 12,000. Fig. 13   The membrane of some granules appears as a thin unlabeled line (arrow). Unstained section. × 23,000.

larly 5-hydroxytryptamine (10, 31, 33). It was shown in another study (21) that enzyme-labeled Fab fragments can allow high degrees of specific labeling at the ultrastructural level and that non-specific deposits can be reduced to a minimum if cytochrome *c* is used as the labeling enzyme. Furthermore, when enzyme-labeled Fab was used as the second-step tracer in the indirect method, the intensity of labeling appeared to be influenced by the composition of the first step; high labeling was observed if either an antiserum at an appropriate titer, or Fab fragments, or a papain-digested serum was used as the first-step reagent.

In the present study, as limited amounts of anti-CT serum were available, the indirect technique was chosen and the antiserum was used undigested and undiluted, as the results obtained by im-munofluorescence suggested that the titer was adequate. The antiserum was highly specific, as it had been obtained against synthetic porcine CT (11). As the endogenous cytochrome *c*-like activity in thyroid gland is very low, it was felt that deposits

of enzyme reaction product indicated the presence of antigenic determinants of CT. The only cells to be labeled were parafollicular cells containing granules. Most granules were labeled and were thus conclusively shown to contain CT. The variety of sizes of labeled granules and their mor-phology after glutaraldehyde fixation make it likely that some granules contain amines as well as CT.

Almost the entire cytoplasmic matrix was labeled. The most probable explanation is that the CT detected in the cytoplasm had been previously contained in granules. This interpretation is supported by two observations. The first is that the membranes limiting the granules are frequently discontinuous and appear to be far more fragile than the membranes of the RER, the Golgi apparatus, or the mitochondria. As stressed earlier, the cisternae of the RER and of the Golgi apparatus, and the mitochondrial matrix were consistently unlabeled. The second observation is the weaker labeling of cytoplasmic matrix in the cen-

tral areas of concentric arrays of RER; these areas do not contain granules. It cannot be determined whether this passage of CT from the granules to the cytoplasm is due to an artefact produced sometime between the beginning of fixation and the immunohistochemical procedures, or whether it could contribute to the physiological release of the hormone. Another hypothesis, which appears less likely, is that the CT found in the cytoplasmic matrix could have been synthesized by the free ribosomes which are present in parafollicular cells; the number of free ribosomes has been claimed to vary with the functional state of these cells (6, 22).

A striking finding was the complete absence of labeling in the cisternae of the RER and of the Golgi apparatus. According to the generally accepted pathway (8, 16, 17, 28), secretory proteins are synthesized in the RER, transported into the Golgi complexes, and packaged into secretion granules. From our findings, it could be thought that the RER of parafollicular cells is not involved in the synthesis of CT; this hypothesis appears improbable. Under the experimental conditions of this study, it could be that the C-cells were in a functional state in which synthesis of the hormone was not taking place. On the other hand, if the RER were synthesizing CT, the absence of labeling might be attributed to insufficient penetration of the immunological reagents, as an undigested antiserum was used; this explanation appears unlikely, since with the same technique intracisternal localizations of IgA have been observed in human salivary gland plasmocytes.[2] Another interpretation could be that the RER synthesized a polypeptide which is related to CT but in which the antigenic determinants of CT are not available. These determinants would become apparent after a transformation of the polypeptide, possibly taking place in peripheral golgi elements.

Labeling of nuclei of parafollicular cells was observed only in piglets aged up to 12 hr. The pattern of distribution of reaction product corresponded to the arrangement of chromatin; nucleoli appeared to be unlabeled, from the limited observations at hand. It has been claimed that nuclei can be the site of protein synthesis (1). Another interpretation could be that CT penetrates from the cytoplasm into the nucleus. This passage could be due to an artefact; it remains to be explained, however, why

this phenomenon is encountered only in newborn animals and, why again, in all the nuclei of parafollicular cells.

It may be concluded from this study that parafollicular cells with secretion granules are C-cells indeed, and that these granules contain calcitonin. It is probable that some granules contain both calcitonin and amines. Under the experimental conditions and with the techniques used in this study, it was not possible to determine the mechanism of synthesis and intracellular transport of calcitonin.

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