The Anatomy of the Parotoid Gland in *Bufonidae* with Some Histochemical Findings

I. *BUFO MARINUS*[1]

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ABSTRACT The gross and microscopic anatomy of the venom producing parotoid glands of *Bufo marinus* has been studied by light and electron microscopy and reactions for the presence of glycoprotein and mucopolysaccharides, the catecholamines, 5-hydroxytryptamine or dopamine, glucose-6-phosphatase, adenosine triphosphatase and the steroid nucleus and cholesterol and its esters have been performed. The gland is composed of numerous individual lobules, each lobule surrounded by a double cell layer. The interior surface of the outer layer is thrown into small cytoplasmic projections which traverse an intercellular space and interdigitate with microvilli formed by the outer plasmalemma of the inner layer. The outer layer resembles smooth muscle-like cells, is rich in adenosine triphosphatase, contains many pinocytotic vesicles and various organelles and may function in some aspect of venom synthesis, active cellular transport and contraction in the discharge of the secretory product. The inner layer shows a positive chromaffin reaction, contains various organelles, appears devoid of a plasmalemma on its inner surface and is involved in venom formation and release via an apocrine type of secretion. The intercellular space is rich in PAS positive materials, while the secretory product, itself, demonstrates a positive chromaffin reaction. The significance of these findings is discussed.

Most amphibians including the toads (*Bufo*) produce integumentary secretions that if not poisonous are usually irritating and sometimes sternutary (Albuquerque et al., '71; Lutz, '71). When and where toad venoms first were used medicinally is unknown, but the ancient Egyptians often represented toads in the form of amulets and the Orientals have employed dried toad venoms as therapeutic agents since antiquity (Lutz, '71). Attempts have been made to utilize the analgesic, hemostatic, heart-stimulating and wound healing properties of these venoms (deKlobusitzky, '71); however, more fundamental data regarding these anurans and their poisons are needed.

The marine toad, *Bufo marinus* is fairly large (66–220 mm; Smith, '51; Wright and Wright, '67). Its parotoid glands, a collection of tumid, granular glands extending from the rear of the tympanum to the sides of the body, produce a white, creamy secretory product composed of several venoms (Abel and Macht, '12; Cei et al., '67; Wright and Wright, '67).

Much is known regarding the biochemical (Chen and Osuch, '69; Kamano et al., '68) and pharmacological (Chen and Kovarikova, '67; Deulofeu and Rúveda, '71; Meyer and Linde, '71) characteristics of these venoms. They are composed primarily of two groups of toxic substances, the bufogenins and bufotoxins representing steroid derivatives (Barbier et al., '59; Chen and Chen, '33; Meyer, '51, '66) and basic components derived from phenylethylamine (Märki et al., '62; Pratesi et al., '58) and tryptamine (Erspamer, '54; Udenfriend et al., '52). Nonetheless, little is known concerning the gross morphology (Lutz, '71; Noble, '54), histology and histochemistry of the parotoid gland.

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This investigation presents the detailed anatomy of the parotoid gland in *B. marinus* at both the light and electron microscopic levels; it includes histochemical findings related to the cellular components of this gland.

MATERIALS AND METHODS

Adult marine toads (*B. marinus*) were obtained by personal collection (from southern Florida) or from the Lemberger Company (Oshkosh, Wisconsin). The animals were killed by overdose of sodium nembutal and the parotoid glands excised (in toto), cut into cubes and fixed in 10% neutral formalin, Zenker-formol solution, Bouin's solution or 80% ethyl alcohol and embedded in carbowax (Sidman et al., '61) or doubled embedded in paraffin (Cowdry, '52). Sections cut at 5 μ were stained with hematoxylin and eosin azan trichrome (Gurr, '62) or exposed to the periodic acid-Schiff/Weigert hematoxylin/picro-indigo carmine reaction (Wismar, personal communication).

Glucose-6-phosphatase activity was determined from fresh 10 μ cryostat sections using the method of Wachstein and Meisel ('56), while adenosin triphosphatase activity was demonstrated following fixation of tissue cubes in formol-calcium at 4°C for 36 hours, cutting 10 μ frozen sections and treating the free-floating sections according to Wachstein and Meisel ('57). Parotoid tissue also was subjected to the chromaffin reaction in which frozen sections were examined for the presence of catecholamines (adrenalin and nonadrenalin), 5-hydroxytryptamine or dopamine (Barka and Anderson, '63). The steroid nucleus and cholesterol and its esters was determined from 20 μ frozen sections using a modified Schultz method (Weber et al., '56).

For electron microscopy, parotoid glands were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer containing 2% sucrose at pH 7.3 utilizing three techniques to insure adequate tissue fixation and preservation of morphology. The glands either were: (1) excised, minced to 1 mm cubes and placed in fixative; (2) excised and individual portions of the gland injected with fixative via syringe or (3) excised following fixation of the entire animal by cardiac perfusion. The glands then were cut into 1 mm cubes and fixed at room temperature for four to six hours, put into 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2 for one hour; dehydrated in graded ethanols, placed in three, 20 minute changes of propylene oxide and infiltrated and embedded in Epon 812 (Luft, '61). Thin sections were cut on the Porter-Blum MT-2B ultramicrotome, stained with uranyl acetate and lead citrate and examined using the Philips 300 electron microscope. One micron epon sections were stained with 1% methylene blue or 1% toluidine blue in 1% sodium borate (Philpott, '66) for light microscopy.

OBSERVATIONS

Histological

In *B. marinus* the two distinct, rhomboid-shaped parotoid glands extend from the caudal aspect of the tympanum along the sides of the body (figs. 1, 2). Each gland measures approximately 2 cm by 7 cm; lies deep to the skin and subcutaneous connective tissue and consists of 50–60, 4 × 7 mm, separate, dissectable lobules, each having its own duct which opens onto the surface of the skin through a pore (figs. 2, 3). The lobules are closely packed in a honey-comb formation and contain the viscous secretory product which exudes onto the skin through all pores simultaneously, upon agitation of the animal.

Blood supply to the gland is from the suprascapular artery via the now-named parotoid artery; the latter courses deep to the gland, is covered by a single layer of mesothelium and divides into lateral, medial and caudal branches. These arterial branches subdivide into smaller arteries and ultimately into capillaries which supply the individual lobules by perforating the interlobular connective tissue. The parotoid vein accompanys its artery and drains into the subclavian vein.

Each lobule is an individual unit surrounded by a double cell layer (figs. 4, 11). The exterior surface of the cells composing the outer layer rests on a thin, continuous basement membrane, while the interior surface of these cells is thrown into small cytoplasmic projections which traverse an intercellular space and interdigitate with microvilli formed by the outer plasmalemma of the inner cell layer (figs. 4, 5, 6). Occasionally a punctate desmosome is formed where a cytoplasmic projection con-

tacts the inner cell layer (figs. 4, 5). Cells forming the outer layer are elongated, united laterally by desmosomes (fig. 5) and form a continuum resembling smooth muscle-like cells (fig. 11). The cytoplasm contains numerous longitudinally oriented myofilaments (figs. 5, 6). The irregular nuclei extend over one-third the length of the cells; the chromatin pattern is primarily a peripheralization of dark, finely granular material, while the central portion of the nuclei exhibit extremely fine chromatin granulation (figs. 7, 11). Nucleoli are seen where they are in the plane of section (fig. 11). A few round to rod-shaped mitochondria (fig. 11) are seen and by electron microscopy five to ten may be counted per cell. Golgi complexes occasionally are present on the intercellular space side of these cells (fig. 7). A few ribosomes, polyribosomes and short segments of granular endoplasmic reticulum are found throughout the cytoplasm, with the primary concentration of these organelles in the outer margins of the cell (figs. 6, 7). In addition, pinocytotic vesicles are observed on all surfaces of the cells and in the cytoplasmic projections (figs. 6, 7).

The cells composing the inner layer are unique in that the outer plasmalemma forms microvilli which interdigitate with the cytoplasmic projections of the outer cell layer (figs. 5, 6), while the portion of the inner layer toward the center of the gland appears devoid of a plasmalemma. The cytoplasm of the inner cell layer is continuous with the secretory product of the gland (figs. 4, 11). The large nuclei are irregular and branching with no predictable shape. The chromatin is primarily marginated, but with some granular, moderately dark chromatin dispersion (figs. 4, 11). Nucleoli are commonly observed (fig. 11). Most organelles are in the outer margin of the cells, including numerous cigar-shaped mitochondria usually present in close association with the nucleus and outer plasmalemma and frequent, distinct, scattered polyribosomes (figs. 4, 5, 6, 11). Golgi complexes are rarely observed. Occasional microfilaments are seen parallel to the outer plasmalemma, while large vacuoles (fig. 11), some of which contain small granules, are present primarily in the inner glandular portion of the cytoplasm. The secretory product is nearly homogenous with localized areas of vacuoles and granular material (fig. 11).

Histochemical

Repeated testing for the presence of glucose-6-phosphatase proved negative with no significant difference between test and control (without substrate) frozen sections. Adenosin triphosphatase activity was strongly positive in the outer cell layer while the inner layer showed little or no reaction (fig. 9). The chromaffin reaction demonstrated a gold to brown positive color within the inner cell layer as well as in the secretory product (fig. 8), but showed no reaction in the outer layer and surrounding connective tissue. Melanophores between the epidermis and dermis showed a positive reaction. After repeated runs, the modified Schultz method for steroids and cholesterol and its esters proved inconsistent and inconclusive with little positive reaction product produced.

The periodic acid-Schiff reaction (PAS) yielded a positive reaction in the area of the basement membrane. The intercellular space between the outer and inner cell layers contained PAS positive material (fig. 11, insert). The secretory product was negative.

DISCUSSION

The inner cell layer is involved in synthesis as indicated by the presence of the secretory product, organelles, frequent distinct nucleoli and granules within large vacuoles in the glandular part of the cytoplasm. Moreover, this cell layer, devoid of a plasmalemma on its inner surface appears to undergo an apocrine type of secretion (Copenhavar et al., '71) with a portion of the cell released with the secretory material. Furthermore, since the cell boundaries are indistinct it is assumed that each cell has one nucleus or that the nuclei collectively contribute to an inner cell layer syncytium. In addition, a positive chromaffin reaction within the inner layer and in the secretory product indicates the presence of basic derivatives of phenylethylamine and tryptamine known to be constituents of the venoms (Erspamer, '54; Marki et al., '62; Pratesi et al., '58; Udenfriend et al., '52).

Inasmuch as no pinocytotic vesicles are seen in the microvilli formed by the inner

cell layer, it seems likely that materials or the venoms at some stage of synthesis are transported from the cytoplasmic projections, across the intercellular space to the microvilli by a method not employing pinocytosis. These microvilli are not unlike those found in the small intestine of mammals (Cardell et al., '67; Eicholtz and Crane, '65; Ito, '65; Mukharja and Williams, '67) and perhaps absorption of materials by the microvilli in the parotoid gland is similar to that observed in the small intestine.

The presence of pinocytotic vesicles throughout the outer cell layer including the cytoplasmic projections suggests a way that materials necessary for venom synthesis may enter these cells and be transported via micropinocytosis (Anderson and Ussing, '60; Holter, '60; Palade and Bruns, '68). This possibility of active transport is further substantiated by the presence of mitochondria and adenosine triphosphatase in the outer cell layer (Essner et al., '58; Hall and Palmer, '69). In salivary gland cells Junqueira ('51) demonstrated a direct correlation between the number of mitochondria and glandular activity. Since parotoid glands only secrete upon agitation and physical stress of the animal and our animals had not been stressed for several months and the secretory product was abundant in the glands, the rather sparse number of mitochondria in the outer cell layer probably reflected the current glandular inactivity. In addition to active cellular transport, the presence of occasional Golgi complexes, some polyribosomes and short segments of granular endoplasmic reticulum suggest cells with a potential for some aspect of venom synthesis. The outer cell layer, because of its orientation and the presence of numerous myofilaments and pinocytotic vesicles resembles myoepithelial cells (Ellis, '65) or smooth muscle-like cells and it appears probable that contraction of this layer assists in discharge of the secretory product toward the duct. Furthermore, the arrangement of the basement membrane and outer and inner cell layers seems ideal to isolate the venoms from other parts of the body of the toad.

The inconclusive result for the presence of steroids and cholesterol and its esters suggests these compounds to be bound in a non-reactive product or simply to be in insufficient concentration to be detected by the modified Schultz method, as cholesterol is known to be a precursor in the synthesis of the bufogenins and bufotoxins (Siperstein et al., '57).

The positive periodic acid-Schiff reaction (PAS) in the intercellular space (as compared with the outer and inner cell layers and the secretory product which are negative) would indicate the presence of substances which can supply free aldehyde groups; although no conclusive data is available on the biosynthesis of the catecholamines in Bufo (or other toads) it can be assumed their production, if not exactly the same, utilizes similar pathways found in other animals, albeit species differences are expected (Axelrod, '59; Daly and Witkop, '63; Weiner, '64) and in man and the rat the free aldehyde groups can be formed by the oxidation of noradrenalin (Rosen and Goodall, '62); therefore, the positive PAS reaction in the intercellular space may reflect a step in catecholamine metabolism.

Perhaps more likely is that the PAS positive material within the intercellular space is sloughed off glycoprotein or mucopolysaccharide by-product (glycocalyx) of the cell surface (Bennett, '63) which is involved in selective absorption across the plasmalemma (Brandt, '62; Brandt and Pappas, '60; Stein, '67; Stoeckenius and Engelman, '69). A negative PAS reaction for the secretory product from *B. marinus* substantiates previous biochemical analyses demonstrating the probable absence of mucopolysaccharides (Daly and Witkop, '71; Deulofeu and Rúveda, '71; Meyer and Linde, '71), although they are present in the venoms from *B. vulgaris* and *B. viridis* (Vialli et al., '69).

Our observations show the parotoid gland of *B. marinus* to be composed of individual lobules, each lobule circumscribed by a double layer of cells, i.e., an outer cell layer which appears involved in active cellular transport, contraction for ejection of the secretory product and which may participate in some aspect of venom synthesis. The inner cell layer is involved in venom formation and release via an apocrine type of secretion. Further investigation is needed to demonstrate clearly the detailed mechanism of biosynthesis, regulation and discharge of parotoid venom.

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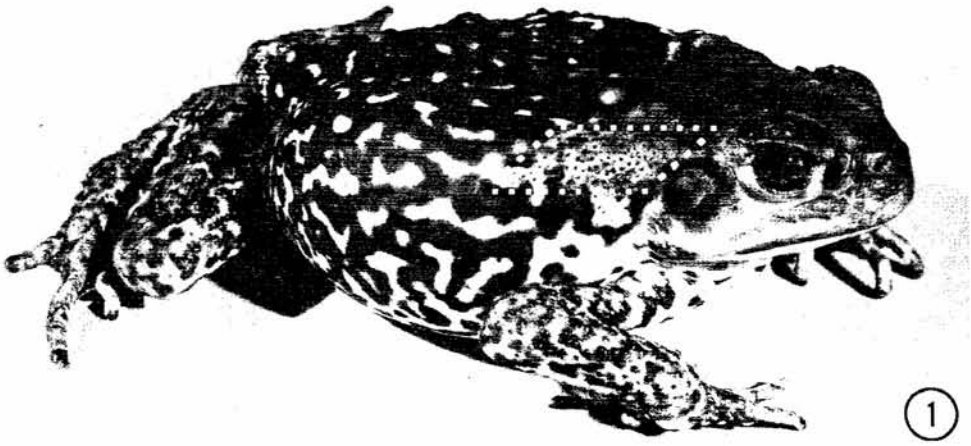
PLATE 1

EXPLANATION OF FIGURES

1 Adult *B. marinus* with parotoid gland outlined.

2 The parotoid gland is composed of individual lobules, each having its own duct which opens onto the surface via pores (arrows).

3 Each lobule (G1) of the parotoid gland has a duct which penetrates the subcutaneous connective tissue (Ct) to emerge through the skin (Sk) as a single pore (P). There are 50–60 individual lobules in each gland. Hematoxylin and eosin. × 40.



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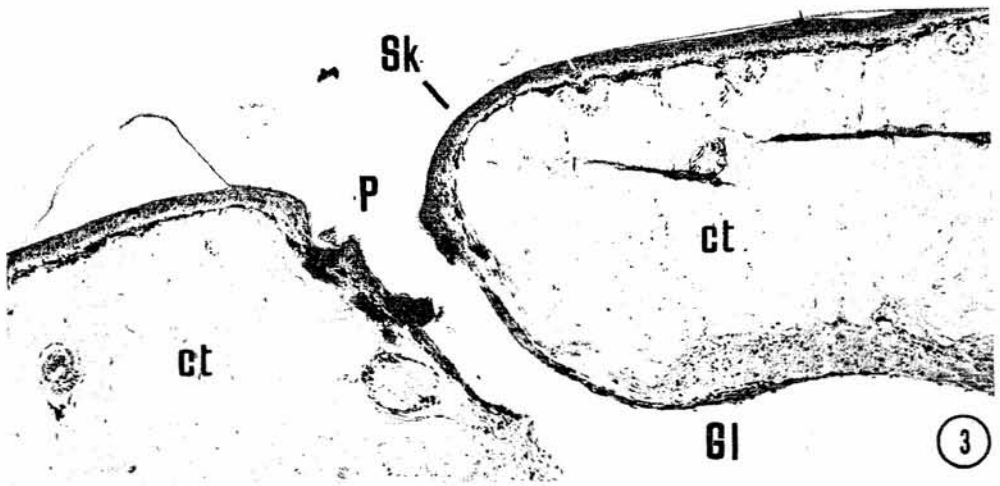

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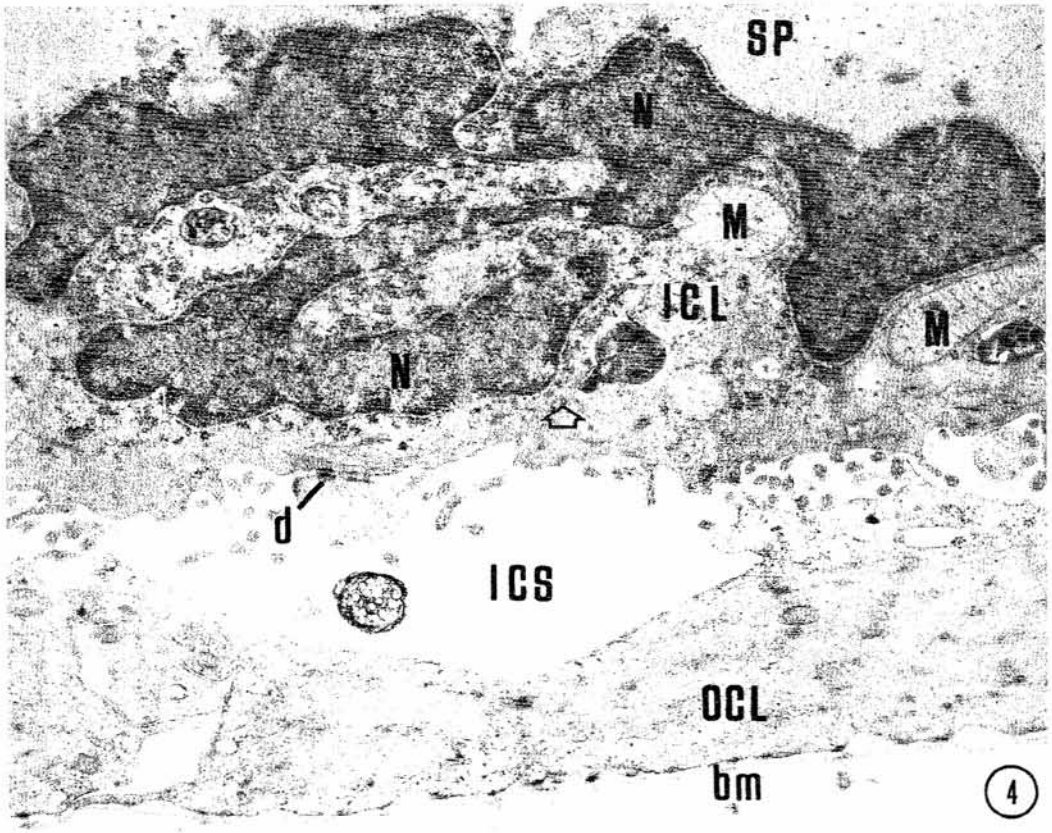
PLATE 2

EXPLANATION OF FIGURES

4 Each lobule is surrounded by a double cell layer. The outer cell layer (OCL) rests on a continuous basement membrane (bm) and its inner surface is directly apposed to an intercellular space (ICS). At varying points cytoplasmic projections from this cell layer contact the inner cell layer (ICL) forming punctate desmosomes (d). The inner layer produces the secretory product (SP). Note the irregular nucleus (N) and primarily marginated chromatin with some granular, dark dispersion; also position of mitochondria (M). × 11,400.

5 A cytoplasmic projection (CP and arrow) from the outer cell layer contacts the inner cell layer forming punctate desmosomes (d_1). Desmosomes (d_2) also are formed between the lateral margins of outer cells. Microvilli (mv) from the inner cells project into the intercellular space. Myofilaments (mf) in the outer cells and a mitochondrian (M) of the inner cells are seen. × 20,000.



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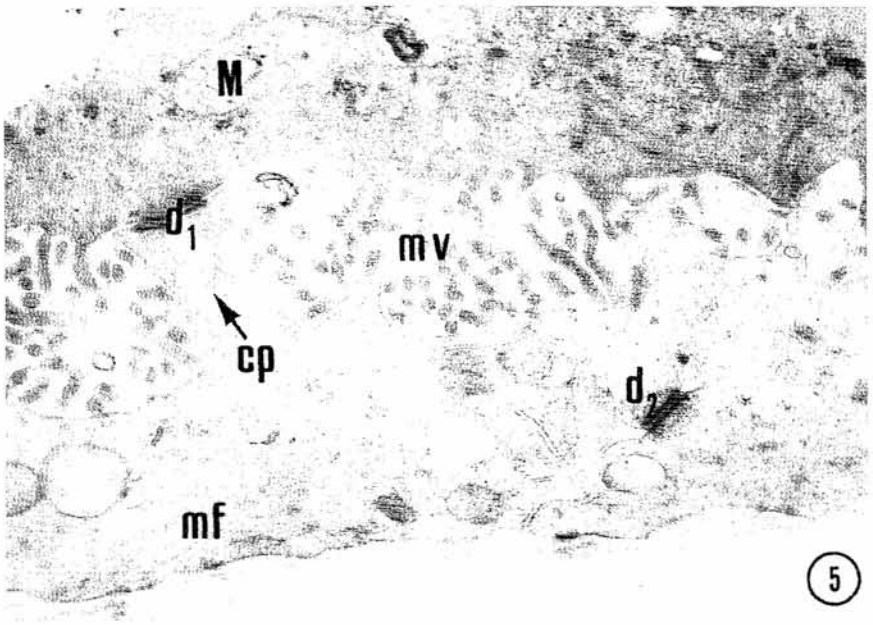

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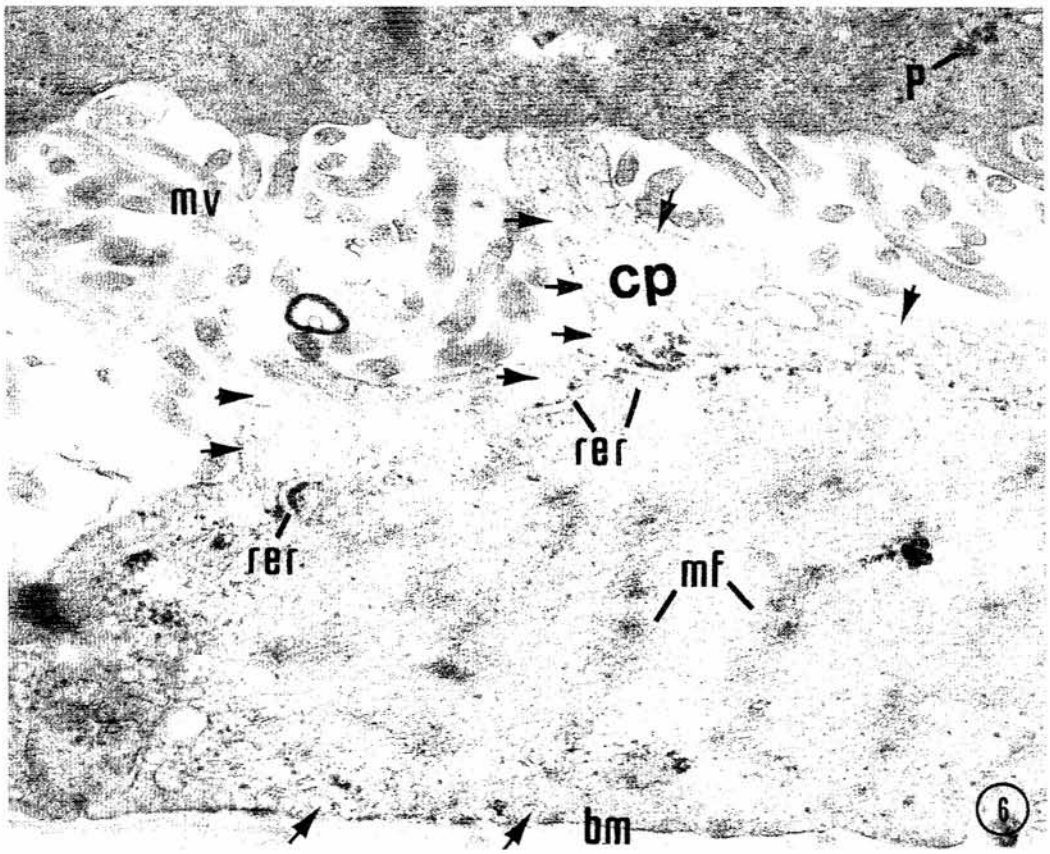
PLATE 3

EXPLANATION OF FIGURES

6 Scattered polyribosomes (p) are present in the inner cell layer, while microvilli (mv) project into the intercellular space. A cytoplasmic projection (CP), pinocytotic vesicles (arrows), myofilaments (mf) and short segments of granular endoplasmic reticulum (rer) are seen in the outer cell layer which rests on a basement membrane (bm). × 28,000.

7 A Golgi complex (G) occasionally is seen on the intercellular space side (ICS) of the outer cell layer. Note also polyribosomes (p), pinocytotic vesicles (arrows) on all surfaces and in a cytoplasmic projection. The nucleus (N) exhibits peripheral dark granulation and central fine chromatin. × 28,400.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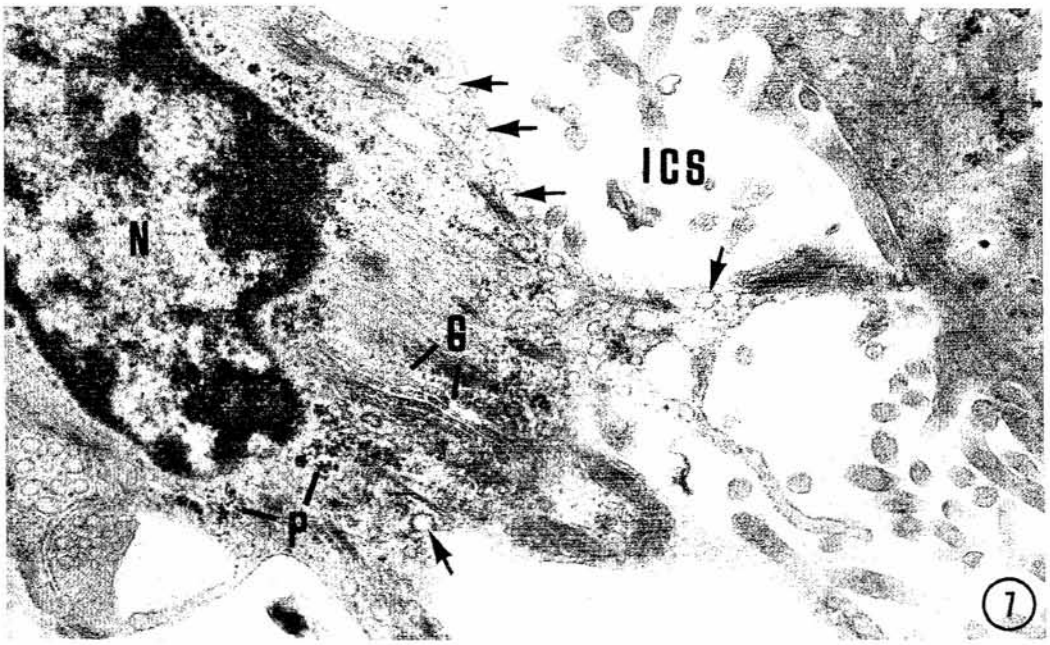
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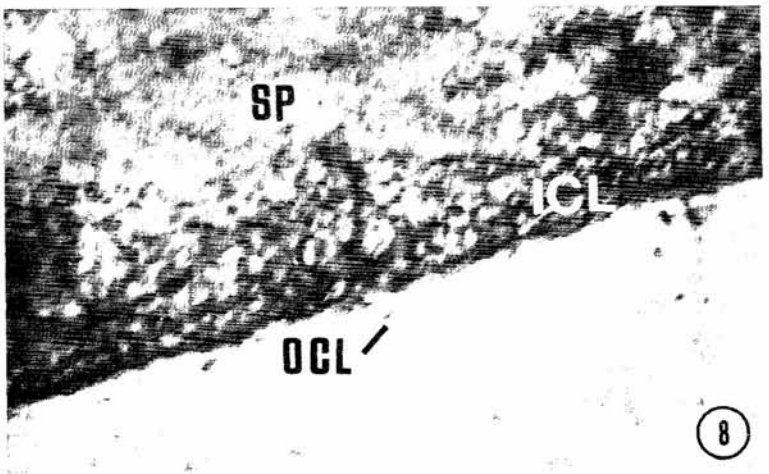
PLATE 4

EXPLANATION OF FIGURES

8 A frozen section from a gland lobule exposured to the chromaffin reaction. The inner cell layer (ICL) and secretory product (SP) are clearly positive as seen by the dark reaction product, while the outer cell layer (OCL) is non-reactive. × 800.

9 A frozen section from a lobule following the histochemical determination for adenosine triphosphatase. The outer cell layer (OCL) is strongly positive, while the inner cell layer (ICL) and secretory product (SP) are negative. × 420.

10 A frozen section from a lobule unexposed to any histochemical reaction. The cytoplasm of the inner cell layer (ICL) is continuous with the secretory product (SP). The outer cell layer (OCL) is a continuous thin band. × 420.


SP
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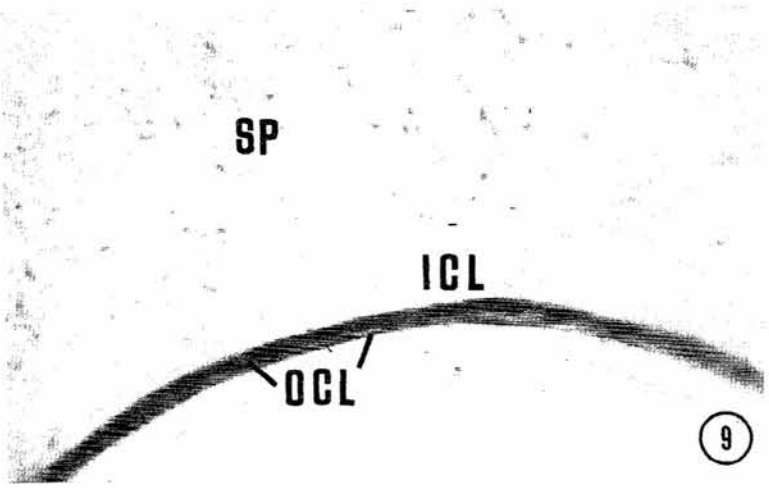
OCL
8


SP
ICL
OCL
9

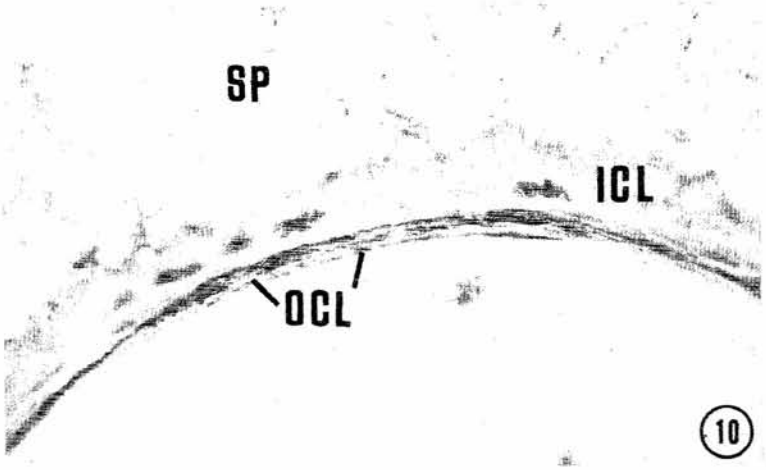
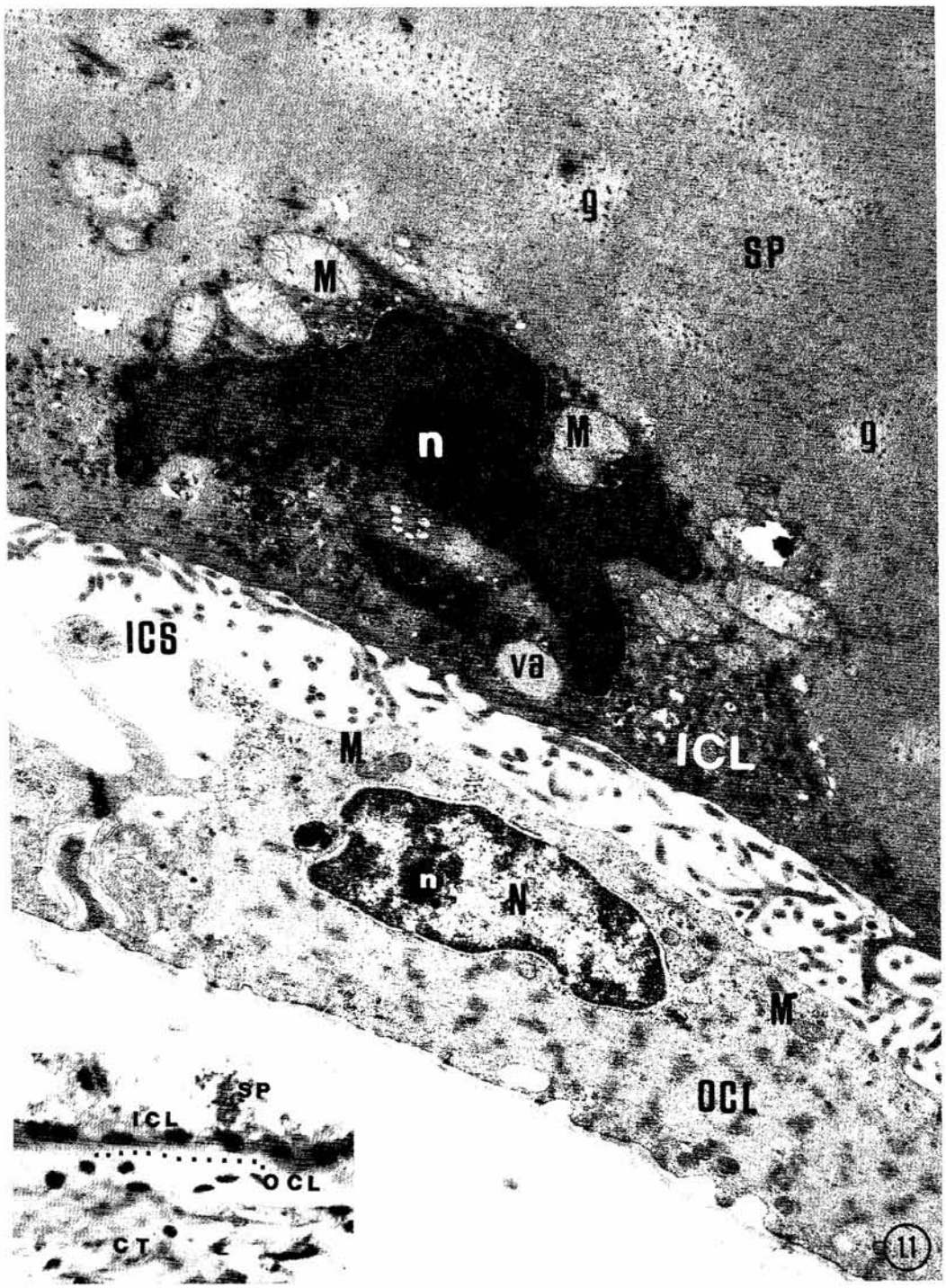

SP
ICL
OCL
10

PLATE 5

EXPLANATION OF FIGURE

11 The outer cell layer (OCL) forms a continuous band of smooth muscle-like cells. A nucleus (N), nucleolus (n) and two mitochondria (M) are indicated. Note the intercellular space (ICS). A vacuole (Va) is seen in the inner cell layer (ICL) as well as a nucleus, nucleolus (n) and mitochondria (M). Granules (g) are present within the secretory product (SP). × 11,600.

Insert: The outer (OCL) and inner cell layers (ICL), surrounding connective tissue (CT) and the secretory product (SP) are shown. The dotted line indicates PAS-positive material within the intercellular space. Periodic acid-Schiff/Weigert's hemotoxylin/picro-indigo carmine. × 620.


g
SP
M
n
M
g
ICS
va
M
ICL
n
N
M
OCL
SP
ICL
OCL
CT
11