Histochemistry of Granular (Poison) Secretion in the Skin of the Frog, *Rana pipiens*

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ABSTRACT There are two types of granular glands in the skin of leopard frogs (*Rana pipiens*). Cellular glands produce fine intracellular granules rich in carbohydrate, carboxylic acids, tyrosine and sulfur-containing amino acids. Luminal glands consist of peripherally located nuclei with scant cytoplasm. Fine granular material in these glands resembles cellular gland secretion, except that the carbohydrate is less susceptible to periodate oxidation and that a diphenol (DOPA?) is present instead of tyrosine. The prominent lumen contains large granules composed of phospholipoprotein, and are also rich in primary catecholamine and a sulfur-containing amino acid. There was no evidence of 5-hydroxytryptamine or steroids.

Adult amphibians generally possess two types of integumentary glands, mucous and granular (serous or poison) glands (Noble, '31; Quay, '72). It is commonly assumed that mucus aids in water balance and the granular secretion is protective because of its disagreeable or even lethal properties. Frog skin, particularly that from the leopard frog (*Rana pipiens*), has long been used to study water and electrolyte movements in living systems. Until recently, however, integumentary secretions were not considered, despite the apparently important roles they play (Watlington and Huf, '71).

Pharmacologic and biochemical properties are known for some anuran poisons (Michl and Kaiser, '63; Chen and Chen, '33; Albuquerque et al., '71), and biosynthetic pathways for certain biologically active components have been outlined (Erspamer, '71). Histochemical investigations have been limited to single compounds (Kramer, '70; Vialli and Quaroni, '56; Ferguson and McGadey, '70). Morphologically, granular glands undergo considerable change in size and appearance during their maturation (Muhse, '09; Noble and Noble, '44), but no correlation has been made between chemical composition of the secretion and developmental changes in the glands.

Because of the potential importance of integumentary glands in physiological research, there is need for a comprehensive study of their properties and functions. Mucous glands in *Rana pipiens* skin were described earlier (Dapson, '70). The present study provides a histochemical description of granular glands of this species.

MATERIALS AND METHODS

Several specimens of the leopard frog (*Rana pipiens*) were collected near Flint and Lapeer, Michigan; Aiken, South Carolina; and Port St. Joe, Florida. All were killed by decapitation, and areas of skin, including the dorsolateral folds, were excised from the back. A variety of fixatives was employed to meet the requirements of different tests: 10% formalin (F); 10% formalin neutralized and saturated with magnesium carbonate (NF); 10% formalin containing 8% cetylpyridinium chloride (F-CPC) (Conklin, '63); 10% formalin containing 2% calcium acetate (F-CA) (Lillie, '65); dichromate solution consisting of 100 ml 5% potassium dichromate and 10 ml 5% potassium chromate (D) (Barka and Anderson, '65); Moller's (Regaud's) fluid (M) (Lillie, '65); Carnoy A solution (C) (Lillie, '65). Tissues were fixed 24–48 hours in all cases except for the indole tests, when a 3–6 hour period was used. For chromaffinity tests,

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some material fixed 24 hours in formalin was then treated with dichromate solution for another 24 hours (NF + D). The reverse treatment was used on other material (D + NF).

Most tissues were embedded in paraffin (Paraplast) and sectioned at 6 μ. Some were infiltrated after fixation and washing with Cryoform embedding medium (4–6 hours at room temperature), then sectioned with a cryostat. This medium is presumably a glycol solution and acts like an antifreeze to reduce ice crystal artifacts (Knox, '70). The procedure also prevented the displacement of large luminal secretion granules, a serious problem with conventional frozen sections. Tests were performed on both pre-embedded and non-embedded frozen sections and no histochemical differences were found. With both paraffin and frozen material, sections were made in a nearly frontal plane to increase the number of glands per section. Permount and fructose syrup (Lillie, '65) were used as mounting media.

For convenience, data concerning type of section and fixative used with each of the histochemical procedures have been summarized in table 1. Further comments will be given only where warranted.

The tetrazotized benzidene method of Barka and Anderson ('65) produced intense staining of all tissue elements, even after 15 minutes exposure to acid alcohol. The final staining solution contained 0.44 mg/ml (nearly 2 m*M*) benzidene dihydrochloride, and was exposed to the tissue for 20 minutes. Greater specificity was achieved by tetrazotizing 0.5 m*M* (0.129 g) benzidene dihydrochloride (1.0 m*M* amine) in Lillie's ('65) diazo procedure, producing 129 mg/ml in the final staining solution. Tissues were stained 2–5 minutes, rinsed two minutes in 0.1% aqueous HCl, post-coupled two minutes with 4.0% H-acid (8-amino-1-naphthol-3,6-disulfonic acid) in 1 *M* sodium carbonate, then exposed to 1% HCl in 70% ethanol for 5 minutes.

An Osram HBO 200 W lamp was used in conjunction with a BG38 heat-absorbing filter, a UG 1 excitation filter, and a K430 or K460 barrier filter for fluorescence studies. A Leitz Orthoplan microscope with Orthomat camera was used with Kodak Panatomic X film.

RESULTS

Morphology

Granular glands were most numerous in the dorso-lateral folds, but could be found in the general surface skin. Two types were obvious and will be called cellular and luminal glands (figs. 1, 2). Cellular glands consisted of large cells full of fine, colorless granules which appeared the same with any of the fixatives used in this study (figs. 3, 4, 7, 8). Nuclei were usually obscured; however, figure 1 shows a prominent exception. A lumen was present, and usually was bounded by sharply-defined cell membranes. A finely granular precipitate was seen in many cellular gland lumens after all fixatives except M and D + NF (fig. 2). With C, it was especially abundant.

Luminal glands were usually bigger and were characterized by a large lumen surrounded by varying numbers of nuclei. A small quantity of perinuclear cytoplasm was usually visible (figs. 9, 11) but cell membranes were indistinct. Secretory material was demonstrable within the cytoplasm in some cases, and appeared finely granular (figs. 3, 11). The most characteristic feature of these glands was the luminal secretion: pale yellow, spherical granules several times larger than those in cellular glands (compare right and left sides of fig. 3). Large granules were dissolved by C. A fine colorless precipitate was present in the lumen around the large granules after most fixatives and was particularly abundant after D + NF and C (fig. 9).

Luminal glands varied in appearance from round and full of material to collapsed and devoid of secretion. Intermediate stages between these extremes were common (fig. 1).

Histochemistry

Results of all histochemical procedures are summarized in table 2, and are generally self-explanatory. Variations in staining intensity were due to different fixatives. With cellular secretion, the PAS test was strongest with CPC-F and weakest with C. Perinuclear material reacted only after F-CPC and M; fine precipitate in luminal

TABLE 1

Summary of histochemical procedures used in this study. Abbreviations of fixatives are defined in text. Types of sections include paraffin (P) and frozen (F).

Histochemical procedure	Fixative	Section	Reference
Azure A, pH 5, 4, 3, 2, & 1	F-CPC	P	Dapson, 70
Mild methylation with and without saponification followed by azure A, pH 4.0	F-CPC	P	Dapson, 70
PAS or Schiff without oxidation	F-CPC; C; M NF; NF+D; D+NF	P P; F	Dapson, 70
Strong acetylation with and without saponification, followed by PAS	F-CPC	P	Dapson, 70
Oil red O in triethyl phosphate with and without unmasking	NF	F	Barka and Anderson, '65
Sudan black B, with and without unmasking	NF	P; F	Chiffelle and Putt, '51
Luxol fast blue	NF F-CPC	P; F P	Pearse, '68
Schultz sterol tests (Mallory's, and Lewis and Lobban's variations)	F (48 hrs)	F	Lillie, '65
Mercuric bromphenol blue	F-CPC	P	Barka and Anderson, '65
Tetrazotized benzidene (1.0 m*M* amine)	NF	P	Barka and Anderson, '65 Lillie et al., '68
Diazo-safranin	NF; D+NF; M; C	P	Lillie et al., '68
Diazo-methylene violet	NF; D+NF; M; C	P	Rock et al., '68
Diazo-tyrosine	F-CPC	P	Barka and Anderson, '65
DMAB	F	P	Barka and Anderson, '65
Post-coupled DMAB	F-CA	P	Barka and Anderson, '65
Mercury orange alone or after sulfhydryl blockade (iodine or N-ethyl maleimide) and/or disulfide reduction (thioglycolic acid)	F-CPC	P	Barka and Anderson, '65 Lillie, '65
DDD alone or after sulfhydryl blockade (iodine or N-ethyl maleimide) and/or disulfide reduction (thioglycolic acid)	F-CPC	P	Barka and Anderson, '65 Lillie, '65
Chromaffin reaction	D NF+D M	F F; P P	Barka and Anderson, '65 Lillie, '65
Potassium iodate oxidation	fresh	F	Barka and Anderson, '65
Schmorl's ferric-ferricyanide	NF; NF+D; M; D+NF; C	P	Barka and Anderson, '65
Formalin-induced fluorescence	F NF; M; NF+D; D+NF C	P; F P; F P	Barka and Anderson, '65 Corrodi and Jonnson, '67

TABLE 2

Summary of results of histochemical procedures. Six categories were recognized; negative (−), questionable (?), and four grades of positive, from weak (±) to very strong (+++). Numbers in parentheses refer to photomicrographs; those marked with an asterisk are figures in Dapson ('70)

Histochemical procedure	Results			
	Cellular glands	Luminal glands		
		Perinuclear	Fine	Large
Azure A, pH 5	++	++	++	−
Azure A, pH 4 (2*)	+	+	+	−
Azure A, pH 3	±	±	±	−
Azure A, pH 2 and 1	−	−	−	−
Meth. + azure A pH 4 (5*)	−	−	−	−
Meth. + sap. + azure A pH 4 (6*)	+	+	+	−
Schiff	−	−	−	−
PAS (2, 3)	+ to +++	+ to +++	− to +++	−
Acet. + PAS (13*)	−	−	−	−
Acet. + sap. + PAS (12*)	+++	+++	+++	−
Oil red O	−	−	−	−
Oil red O after unmasking	−	−	−	−
Sudan black B, frozen sect.	−	−	−	−
Sudan black B after unmasking (4, 5)	−	−	−	++
Luxol fast blue (6)	−	−	−	++
Schultz sterol tests	−	−	−	−
Mercuric bromphenol blue (19*)	−	−	−	++
Tetrazotized benzidene	++	?	?	−
Diazosafranin (7)	+ to +++	++	+	−
Diazo methylene violet	+	+	+	−
Diazotyrosine (8)	+	?	?	−
DMAB	−	−	−	−
Post-coupled DMAB	−	−	−	−
Mercury orange, sulfhydryls	±	−	−	−
Mercury orange, disulfides (9)	+++	++	++	+++
DDD, sulfhydryls	±	−	−	−
DDD, disulfides	+++	++	++	+++
Chromaffin (10)	−	−	−	++
Iodate oxidation	−	−	−	−
Schmorl's test (11)	−	− to ++	− to ++	−
Formalin fluorescence (12)	−	−	−	+++

glands was reactive with PAS only after F-CPC.

Large luminal granules in paraffin sections stained deeply with Sudan black B (fig. 4) but were negative in frozen sections, suggesting masked lipids. Burnt Sudan and acetone Sudan procedures for masked lipids produced excessive precipitation of dye and were judged unreliable. Staining was obtained by passing frozen sections through ascending and descending series of ethanol, xylene, hot paraffin, xylene and ethanol. Exposure times were 3–5 minutes per solution. Large granules then stained blue-green with Sudan black B (fig. 5).

Both perinuclear and fine luminal material gave positive results with ferric-ferricyanide after all fixatives except D + NF (fig. 11).

With UV light, granules were intensely fluorescent after F (fig. 12): cream yellow with a K430 barrier filter, brighter gold yellow with a K460 filter. Most other formalin-containing fixatives produced similar results; however, D + NF caused more variable and less intense fluorescence. Regardless of treatment, all fluorescence was stable (non-fading) for at least 30 minutes. Unfixed cryostat sections were not autofluorescent.

DISCUSSION

Cellular gland secretions

Secretions within the cells and in the lumens of cellular glands were histochemically identical except that luminal material was not fixed with primary dichromate treatment. Tests with azure A at graded pH suggest the presence of carboxylic acids, and are corroborated by persistence of staining with this dye after methylation and saponification. Sulfate esters are absent, because they would have produced positive results at low pH and negative results after methylation and saponification (Fisher and Lillie, '54; Spicer, '60).

Positive results with PAS are ordinarily ascribed to *vic* glycols or amino-glycols on hexose sugars, and unsaturated fats. In view of the positive reaction for lipid in luminal gland granules, the second alternative deserves careful consideration. Acetylation is supposed to be a specific blocking procedure for carbohydrates, not lipids (Barka and Anderson, '65). Negative results in cellular glands after this blockade, and successful removal of the block with saponification argue strongly against lipids. Furthermore, no lipids of any kind were demonstrable with fat soluble dyes. Conklin's ('63) CPC-formalin was designed to preserve acid mucopolysaccharides; and it was after this fixative that the most intense PAS reaction was obtained. Thus, cellular gland secretions almost certainly contain carbohydrates, which may be associated with a carboxylic acid moiety.

Barka and Anderson ('65) list nine amino acids that can react with tetrazotized benzidene; however, Lillie ('65) attributes his more specific diazo reactions to tyrosine, histidine and tryptophan and some of their derivatives. If tryptophan had been present in cellular granules, the DMAB tests would have been positive; if 5-hydroxytryptamine (5-HT), the diazo methods would have produced blue or black results and cellular glands would have been chromaffin. Unfortunately, the role of histidine remains unknown because of lack of histochemical tests for this amino acid. However, the positive diazotyrosine test indicates that tyrosine was the cause of positive diazosafranin, diazo methylene violet and post-coupled tetrazotized benzidene procedures.

Disulfide groups were present in fixed cellular glands, indicating the presence of cystine. Reducing substances were not found, even with dichromate fixation. Fluorescent biogenic monoamines were absent.

Cellular gland secretions thus contain a carbohydrate, carboxylic acids, and protein rich in tyrosine and a sulfur-containing amino acid. Whether these components reside on a single molecule or represent a mixture of two or more substances is unknown.

Luminal gland secretions: large luminal granules

Large luminal granules contained no demonstrable carbohydrates nor carboxylic acids, but did consist of lipids, sulfur-containing amino acids, and a biogenic monoamine.

Results of various lipid tests suggest a phospholipoprotein. Oil red O does not stain phospholipids readily (Pearse, '68), and gave no reaction with luminal granules. On the other hand, Sudan black B is soluble in phospholipids (Pearse, '68) and, after unmasking procedures, stained luminal granules. Further evidence for a phospholipid component is given by the positive reaction with Luxol fast blue (Lycette et al., '70). Pearse ('68) states that sudanophilia of formalin-fixed paraffin sections indicates "lipoprotein whose component is usually phosphatide or cerebroside." The protein apparently masks the lipid as well as preventing dissolution of lipids during paraffin embedding. This phospholipoprotein was alcohol-soluble unless previously fixed in formalin. Steroids were not demonstrated.

Disulfides were present in large luminal granules, again suggesting the presence of cystine, but no other amino acid was specifically demonstrated. Positive results with mercuric bromphenol blue cannot be explained readily. If the reaction was due to cystine, cellular granules should have been positive also.

Formaldehyde-induced fluorescence is specific for certain biogenic monoamines (Corrodi and Jonsson, '67). With mild treatment, only primary catecholamines

fluoresce intensely; 5-HT and secondary catecholamines require more energetic reaction conditions. Catecholamines have an emission maximum at 480 nm and usually appear green or green-yellow with K430 and K460 barrier filters. However, they can appear bright yellow when the catecholamine concentration is high, because of the increased intensity of light (Corrodi and Jonsson, '67). The fluorescent derivative of 5-HT undergoes rapid photodecomposition, while catecholamine-based fluorescence is more stable.

The non-fading, intense yellow fluorescence of luminal granules suggests a primary catecholamine, and other tests support this conclusion. Diazosafranin and diazomethylene violet were negative; if 5-HT were present, these tests would have produced black coloration (Lillie et al., '68; Rock et al., '68). Furthermore, 5-HT gives a positive Schmorl test (Lillie, '65), and luminal granules were negative.

These results indicate noradrenalin. However, this primary catecholamine is able to reduce ferric ions after dichromate fixation, is oxidized by potassium iodate, and loses its chromaffinity after formalin fixation. None of these characteristics was seen in large luminal granules. Evidently formalin-induced cyclization did not hinder reaction with dichromate, but prior oxidation with dichromate did impede formalin condensation and produced irregular fluorescence.

Bjorklund et al. ('71) tested 15 phenylethylamines and found three that were intensely fluorescent after formaldehyde treatment: noradrenaline at 100 arbitrary units of fluorescent intensity, dopamine at 109, and 4-methoxy-3-hydroxyphenyethylamine at 227. The latter compound is of unknown biological significance. Dopamine and noradrenaline share many histochemical features, but dopamine does not give an iodate reaction (Barka and Anderson, '65). Thus, dopamine could be the monoamine in large luminal granules.

Luminal gland secretions: perinuclear and fine luminal material

These products apparently are intermediate in histochemical features between cellular gland secretion and large luminal granules. The carbohydrate and carboxylic acid moieties were present, although the former's susceptibility to periodate oxidation differed from cellular gland carbohydrate. These secretions further resembled cellular gland granules in lacking phospholipid and monoamine. However, the positive ferric-ferricyanide reaction indicates a diphenol. Since tyrosine was present in cellular glands and dopamine might be present in large luminal granules, DOPA might be represented in perinuclear and fine luminal secretions as an intermediate product. DOPA gives a diazosafranin reaction similar to tyrosine (Lillie, '65). Sulfur-containing amino acids were present. Perinuclear and fine granules apparently are identical.

Comparison with other amphibian skin secretions

A considerable amount of biochemical data is available for biologically active components of amphibian skin, but most of it comes from studies on toads (*Bufo*), and none covers *Rana pipiens*. Five general categories of active substances are known for anurans: steroids, including the unique derivative batrachotoxin; indolealkylamines (5-HT and related compounds); imidazolealkylamines (derivatives of histidine); phenylalkylamines (tyrosine and its derivatives, including catecholamines); and active polypeptides (Erspamer, '71; Michl and Kaiser, '63; Chen and Chen, '33; Albuquerque et al., '71). Of these five, steroids and indolealkylamines were not found in *R. pipiens*, imidazolealkylamines and active polypeptides were not tested, and phenylalkylamines were present.

Few histochemical studies have been made of mature granular secretions, and most indicated that 5-HT was present (Vanable, '64, *Xenopus;* Kramer, '70, *Xenopus* and *Bufo regularis;* Vialli and Quaroni, '56, *Rana esculenta, Discoglossus* and *Bombinator;* see also Pearse, '61 for photograph of *Bombinator* glands). Ferguson and McGadey ('70) found hydroxysteroid dehydrogenase activity in poison glands of an unidentified frog. Obviously, none of these studies show similarities to the results of the present report on *R.*

pipiens. Granular secretions in the cellular stage have not been studied previously.

Function of granular glands

There is little doubt that some species use granular secretions for defense. However, most species are not obviously poisonous and many are common food items for other vertebrates. Other functions are therefore indicated for these glands.

Exogenous catecholamine (norepinephrine) alters the electrical potential of the skin (House, '70); furthermore, it affects integumentary secretions by stimulating their rates of production and release and by increasing their ionic content (Watlington and Huf, '71; Benson and Hadley, '69). Under normal conditions, alterations induced by endogenous catecholamine could function in water balance while the animal is submerged and in evaporative cooling during basking (Lillywhite, '71). Granular gland secretion could be a major source of this catecholamine.

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PLATE 1

EXPLANATION OF FIGURES

1 Nearly frontal section stained non-specifically with Barka and Anderson's tetrazotized benzidene, showing three small, dark cellular glands (left), one light mucous gland (top, center), and several large luminal glands in various stages from full to nearly collapsed and empty. Blue filter. × 320.

2 F-CPC fixation, PAS. Intense reaction in cellular granules, weaker reaction in secretion in lumen of one cellular gland; large luminal gland granules negative. × 250.

3 F-CPC fixation, PAS. Cellular gland on left, with darkly stained, small granules packing the cells. Nuclei (arrows) and large granules are unstained in the luminal gland at right; moderate reaction in very fine, perinuclear granules. × 2,250.

4 F-CPC fixation, paraffin section, Sudan black B. Only large luminal granules are positive (blue-black). Nuclei and perinuclear cytoplasm are conspicuous, but unevenly distributed about the periphery of the luminal gland. Dark red filter (Wratten 25). × 640.

5 NF fixation, frozen section, unmasking procedure, Sudan black B. Luminal granules blue green; cellular granules (not shown) negative. Blue filter. × 640.

6 F-CPC fixation, Luxol fast blue. Positive reaction (bright blue) in luminal granules only. Perinuclear cytoplasm unstained but abundant in certain areas. The luminal gland is partially collapsed and nearly empty. Portions of two cellular glands can be seen at left and top. Orange filter (Wratten 22). × 640.

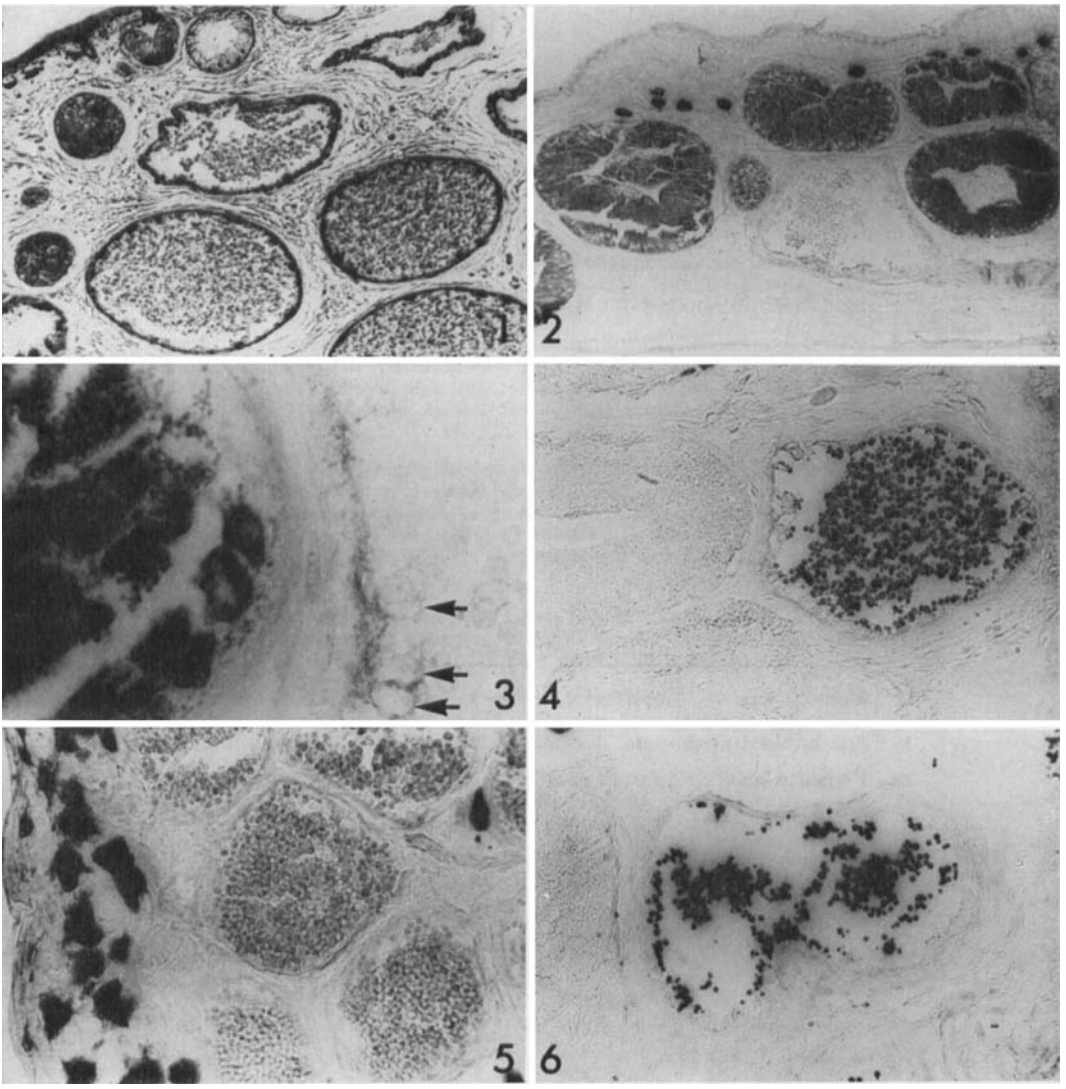
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PLATE 2

EXPLANATION OF FIGURES

7 F-CPC fixation, diazotyrosine. Pale violet coloration in cellular granules. Mucous glands (far left and upper right) are negative. Dark blue filter (Wratten 47B). × 640.

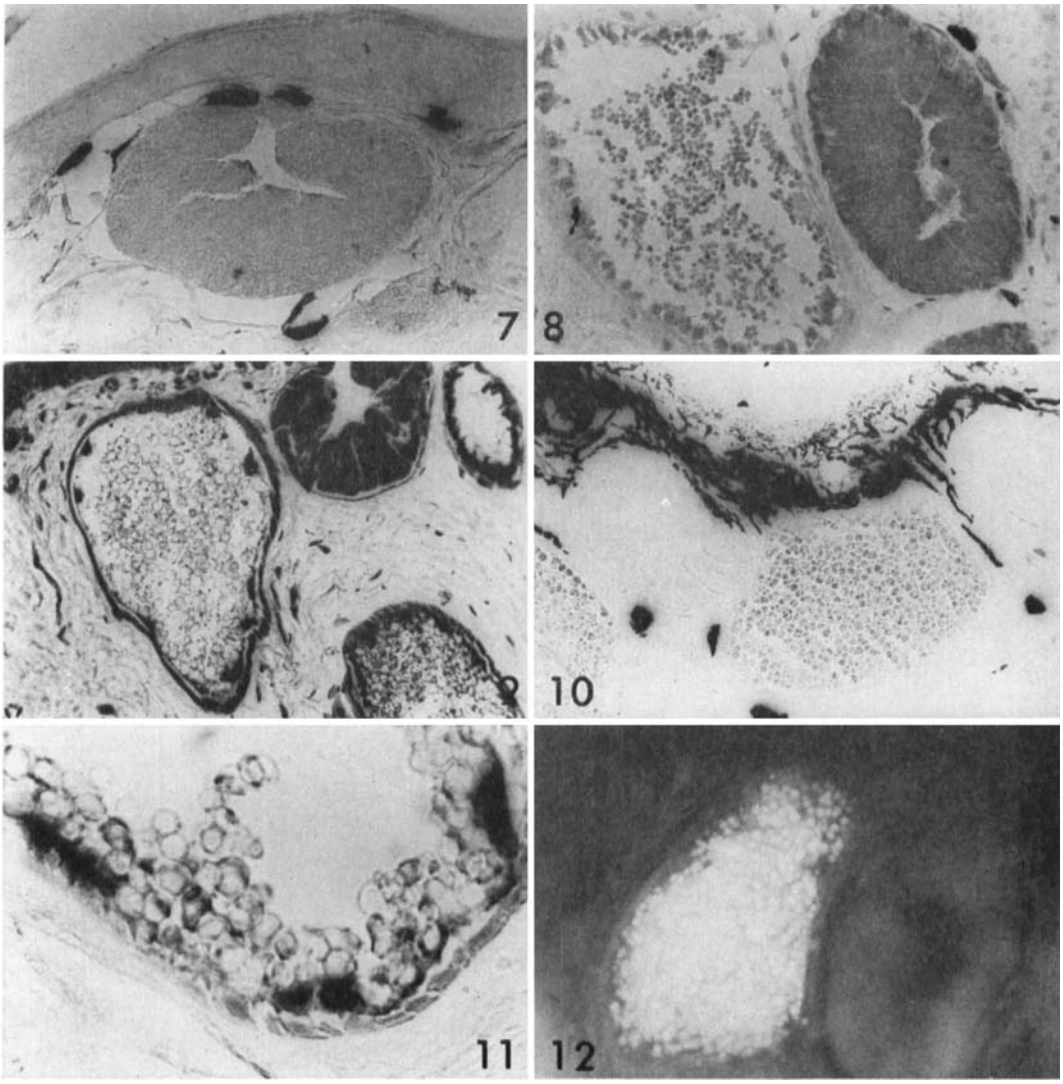
8 F-CPC fixation, thioglycolic acid reduction, mercury orange. All granular gland products orange. Note extracellular material in cellular gland (right). Perinuclear material and fine luminal precipitate can be seen in the luminal gland (left). Dark blue filter (Wratten 48). × 640.

9 Moller's fixative, diazosafranin. Luminal gland (left), dark cellular gland (upper center), and mucous gland (far upper right). Mucus, cellular secretion, perinuclear material and fine luminal precipitate positive; large luminal granules negative. Smooth muscle about the granular glands, and the epidermis (upper left) are also stained. Green filter. × 640.

10 Moller's fixative, chromaffin reaction. Cellular glands (upper left and right) negative; large luminal granules (center and lower left) golden brown. Dark blue filter (Wratten 48). × 640.

11 Moller's fixative, ferric-ferricyanide. Luminal gland, showing negative large granules and positive (blue) perinuclear cytoplasm and fine luminal precipitate. Red-orange filter (Wratten 23A). × 2,250.

12 Formalin-induced fluorescence. Cellular gland (right) negative; luminal granules (left) intensely positive (cream yellow). Perinuclear material negative. K430 barrier filter. × 640.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