Histochemistry of Mucus in the Skin of the Frog, *Rana pipiens*

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ABSTRACT Histochemical characteristics of the integumentary mucous glands of the leopard frog (*Rana pipiens*) are described so that their role in water balance can be better understood. Mucus is acidic, due to its content of sulfate and carboxylic acids. The carbohydrate moiety contains periodate-engendered groups which are not in close proximity to acid radicals. Protein was not demonstrated. Neuraminic acid is either absent or is not susceptible to neuraminidase digestion.

Despite considerable physiological research on amphibian skin (Deyrup, '64), little is known of its histology or the histochemistry of its secretions. Mucous glands produce an agranular, basophilic secretion; serous glands secrete a granular, acidophilic substance, which may be toxic to other species (Noble, '31). Details of glandular development in tadpoles were given by Bovbjerg ('63) and Verma ('65). Noble and Noble ('44) described the morphology of the glands during their secretory cycle.

Except for the pharmacological effects of several integumentary toxins (Licht, '67, for review), chemical properties of amphibian skin secretions are unknown. It is commonly assumed that mucus protects amphibians from excessive water loss or gain, but the underlying protective mechanisms are not yet understood. Friedman et al. ('67) reported that secretions aid water and electrolyte balance. Frog skin maintains an alkaline pH at its surface, presumably through the combined effects of respiration and secretion. The alkaline pH minimizes loss of electrolytes. These authors theorized that bicarbonate ions in tissue fluids and secretions produced this pH, but recognized that the lack of chemical information on skin secretions prevented deeper understanding of the problem.

My report provides histochemical data on frog mucus, and it is part of an effort to explain the role of integumentary secretions of amphibians living under divergent ecological conditions.

MATERIALS AND METHODS

A specimen of *Rana pipiens* was collected near Flint, Michigan, late in March and decapitated within 24 hours. Skin was excised from between the dorso-lateral folds, and fixed for 24 hours in 8% cetylpyridinium chloride (hexadecylpyridinium chloride) in 10% formalin (Conklin, '63). Tissues were embedded in Paraplast and sectioned at 6 μ. Unless otherwise noted, all histochemical tests were made on this specimen.

Azure A staining at controlled pH was used to study mucous basophilia. Unbuffered aqueous solutions of 0.05% azure A (C.I. 52005) were adjusted with HCl to pH of 1.2, 2.2, 3.2, 4.2 and 5.1. The pH of these solutions changed less than 0.1 unit during staining. Staining time was 4.5 minutes in all cases. Tissues were examined immediately after staining, before dehydration.

To support findings from the azure A staining, sections were subjected to mild methylation (0.8 ml conc. HCl, 99.2 ml absolute methanol, 37°, four hours; Fisher and Lillie, '54) and saponification (1% KOH in 70% ethanol, 25 minutes; Spicer and Lillie, '59). Subsequently, these sections were stained with unbuffered azure A (0.05%) at pH 4.0 for four and one-half minutes.

Alcian blue was used in a technique modified from Mowry ('63): a 0.5% solution of dye in 3% acetic acid (pH = 2.8) was made as directed, but staining was re-

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duced to ten minutes. The sections were immediately transferred directly to 3% acetic acid (2 minutes), instead of tap water. Both modifications decreased background staining of collagenous fibers and nuclei. The extinction pH for alcian blue was determined by staining 30 minutes in 1% solutions adjusted to pH 0.4 and 1.0 with HCl.

Mucous basophilia was also studied by the critical electrolyte concentration (CEC) method of Scott and Dorling ('65). Solutions of alcian blue were made with different concentrations of $MgCl_2$, ranging from 0.05 to 1.0 *M*.

Several procedures involving the periodic acid Schiff test were conducted. Lillie's cold Schiff reagent (Humason, '67) was made with rosaniline HCl (Eastman; C. I. 42510). Sections were oxidized with periodic acid for ten minutes and exposed to Schiff's reagent for five or ten minutes. Some sections were acetylated with acetic anhydride-pyridine (Lillie, '65, p. 178) before the PAS, to distinguish native ketones from periodate-engendered aldehydes. Control sections were acetylated and saponified before PAS staining. A combined alcian blue-PAS sequence was performed according to Mowry ('63). The proximity of acid radicals to periodate-engendered groups was determined by treating sections with periodic acid-phenylhydrazine (5%; 30 minutes) Schiff and periodic acid-diamine-azure A (0.05%; pH 3.6; 4.5 minutes) (Spicer, '61). The diamine was a 0.2% solution of N, N-diethyl-*meta*-phenylenediamine $(HCl)_2$ buffered to pH 5.0 with 0.2 *M* Na_2HPO_4.

Mercuric bromphenol blue was used as a general protein stain (Barka and Anderson, '65, p. 63). Sections were digested with trypsin (Lillie, '65, p. 265) or pepsin (Pearse, '61, p. 917), and stained with mercuric bromphenol blue to demonstrate loss of protein. Proteins sometimes mask some carbohydrate moieties (French and Benditt, '53; Quintarelli, '63a); therefore sections were treated with trypsin or pepsin and stained with PAS to show protein-masked carbohydrates. Control slides were incubated in buffer alone before staining.

Neuraminidase digestion was used to detect neuraminic acid (Spicer and Warren, '60), following the procedure of Hukill and Vidone ('67). Cetylpyridinium chloride presumably interacts with carbohydrate anions in mucus, and might interfere with enzymatic digestion. Therefore, neuraminidase treatments were effected on skin fixed in buffered neutral formalin (Lillie, '65, p. 38) as well as on material fixed in cetylpyridinium chloride-formalin. A 0.05% solution of purified *Vibrio cholerae* neuraminidase (Calbiochem; 500 units/ml) was made in a 0.1 N sodium actetate-acetic acid buffer (pH 5.5). After incubation for 21–24 hours in the buffered enzyme or plain buffer, sections were stained with unbuffered azure A (pH 4.0), alcian blue at pH 2.8, or PAS. Some slides were exposed to 1% alcoholic KOH for five minutes before treatment with neuraminidase, to render neuraminic acids more susceptible to the enzyme (Leppi and Spicer, '66).

RESULTS

Mucous glands had small, irregularly-shaped lumina containing fine, reticular precipitates of secretion (fig. 1). Intracellular mucus was more dense than the luminal secretion, but exhibited identical histochemical properties.

Mucous glands stained with unbuffered azure A were rose-red, rose-violet or pale violet at pH values of 4 and 5, 2 and 3 or 1, respectively (figs. 2, 3). After methylation, saponification and staining at pH 4, mucous glands were rose-violet, similar to the results of sections treated only with azure A at pH 3 (figs. 4–6).

Mucous glands stained with alcian blue at pH 2.8 were intensely aquamarine (fig. 7). This reaction was not greatly diminished by lowering the pH of the staining solution to 0.4 (fig. 8). At pH 5.7 and 0–0.6 *M* $MgCl_2$, mucous glands remained similarly and uniformly colored (fig. 9). Other tissue components were also stained at the lower concentrations of $MgCl_2$. At 0.8 *M* to 1.0 *M* $MgCl_2$, the alcian blue solution produced only scattered blue flecks and streaks in the mucous glands (fig. 10).

With PAS, mucous glands appeared pink while serous glands were magenta (fig. 11). After acetylation, the reaction was abolished in both glands (figs. 13–14), but was restored by acetylation and saponification (fig. 12). Alcian blue and PAS combined

produced a royal blue coloration in the mucous glands (fig. 15). Results were negative with periodic acid-phenylhydrazine-Schiff (figs. 17–18), and positive in the mucous glands with periodic acid-diamine-azure (fig. 16).

Mucus did not stain with mercuric bromphenol blue, either with or without previous treatment with trypsin or pepsin (figs. 19–20). PAS reactivity was unaltered by proteolytic digestion. Neuraminidase digestion, with or without prior exposure to KOH, did not alter the reactions of mucus. There were no differences between digested and control slides after azure A, alcian blue, or PAS (figs. 21–22), regardless of the fixative.

DISCUSSION

Individual histochemical tests concerning basophilia require cautious interpretation because of possible interaction of polyanions with basic proteins, removal of carboxyl groups by methylating agents, or other unexpected reactions (Quintarelli, Scott and Dellovo, '64; Quintarelli and Dellovo, '65). However, when several tests applied to the same tissue are in agreement, as in this study, more reliable conclusions may be drawn. Results of various tests with azure A and alcian blue indicate that carboxyl and sulfate groups contribute to the intense basophilia. Persistent metachromatic staining from pH 3 to 1 probably was due to sulfate groups alone, because carboxylic acids are practically undissociated below pH 3 (Quintarelli, Scott and Dellovo, '64). On the other hand, methylation removes sulfate groups (Fisher and Lillie, '54; Spicer, '60); thus, any staining at pH 4 after methylation and saponification probably was caused by carboxylic acids. Experiments with critical electrolyte concentration also indicate the presence of both types of acids. Strong alcianophilia caused by sulfate groups alone is not abolished by concentrations of $MgCl_2$ below 0.8 or 1.0 *M* (Scott and Dorling, '65). The critical electrolyte concentration for carboxylic acids is below 0.2 *M* $MgCl_2$ (Quintarelli and Dellovo, '65; Scott and Dorling, '65). Because staining of frog mucus diminished between these molarities of electrolytes, a mixture of carboxylic and sulfate acids is assumed.

Integumentary mucus of *Rana pipiens* has *vic* glycols or other periodate-engendered groups. Acidic and periodate-engendered portions of mucous molecules are not adjacent to one another. Phenylhydrazine ordinarily prevents condensation of aldehydes and Schiff's reagent except when acid groups near aldehydes block the reaction of phenylhydrazine (Spicer, '61). Negative results after periodic acid-phenylhydrazine-Schiff suggest that acid groups are not sufficiently near the aldehydes to prevent phenylhydrazine blockade. Positive results with periodic acid-diamine-azure support this conclusion. Diamine condensed on aldehydes would also combine with adjacent anions if the distance was short enough (Spicer, '61).

Protein in frog mucus may be absent, masked, or in quantities below the level of histochemical detection. Most mucins are thought to consist of short, oligosaccharide side chains off a proteinaceous core (Gottschalk, '63; Leppi and Spicer, '66), but protein has not always been histochemically demonstrated (Bélanger, '63).

Many unsulfated, acidic mucins are destroyed by neuraminidase (Spicer and Warren, '60; Hukill and Vidone, '67), but some resist digestion (Spicer and Warren, '60; Quintarelli, '63b; Leppi and Spicer, '66; Kent, '63). Spicer and Duvenci ('64) found that brief exposure to KOH rendered some of the mucins susceptible to neuraminidase. Resistance to digestion may be due to 0-acetylation of the neuraminic acid, certain acid-sugar linkages, or other factors which render the substrate incompatible with the enzyme (Leppi and Spicer, '66). Choice of fixative does not seem to be important. The carboxyl group responsible for some of the basophilia of frog mucus may either be some other uronic acid or it is an enzyme-resistant neuraminic acid.

Spicer, Leppi and Stoward ('65) and Stoward ('67) have used a notational system of naming and comparing mucopolysaccharides. Initials preceding the word mucin refer to the presence of *vic*-glycols (G), sulfate groups (S), and carboxyl groups (C). Omission of a symbol implies the absence of that component. After the

term mucin, various data are given. Reactions to basic dyes like azure and to alcian blue are symbolized by the letters B and A, respectively, followed by the lowest pH at which staining occurred. The highest molarity of $MgCl_2$ at which staining occurred with alcian blue is inserted parenthetically after the pH value. If the material is neuraminidase-sensitive, the letter S is appended. Mucus of *Rana pipiens* can, therefore, be described as follows:

CSG-mucin B1.0 A < 0.4(0.6*M* $MgCl_2$)

It is difficult to understand how this acidic mucus alone can maintain an alkaline pH at the surface of the skin. Comparable work on the serous secretion is needed before definite conclusions can be drawn. However, it is possible that this favorable pH is the result of the combined effects of acidic mucus and basic granular secretion.

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PLATE 1

EXPLANATION OF FIGURES

1 Tangential section through a curved piece of skin. M, mucous gland; S, serous gland with intracellular secretion granules ("cellular" stage); L, serous gland with luminal secretion granules ("luminal" stage); D, degenerating serous gland lacking secretion. Melanophores appear as irregular, black bodies. H + E × 100.

2 Azure A at pH 4. Nuclei blue, mucus rose-red (black in photomicrograph), serous secretions gray in section and photomicrograph. × 200.

3 Azure A at pH 1. Mucus pale violet; all other tissue components unstained. Yellow and green filters used to heighten contrast × 200.

4 Mild methylation + saponification + azure A, pH 4. Nuclei blue, mucus rose-violet. Yellow filter. × 200.

5 Mild methylation + Azure A, pH 4 (control section for slide shown in fig. 4). Totally unstained. See figure 6. × 200.

6 Same section as photographed in figure 5 but with darkfield condenser for better visualization of components. Two black mucous glands are in the upper right; one white "cellular" serous gland is in the upper left; and two "luminal" serous glands occupy the lower field. This method verifies the presence of unstained components not readily visible with brightfield microscopy. × 200.

7 Alcian blue, pH 2.8. Mucus intensely aquamarine, nuclei pale blue. × 200.

8 Alcian blue, pH 0.4. Mucus aquamarine; no other staining. × 200.

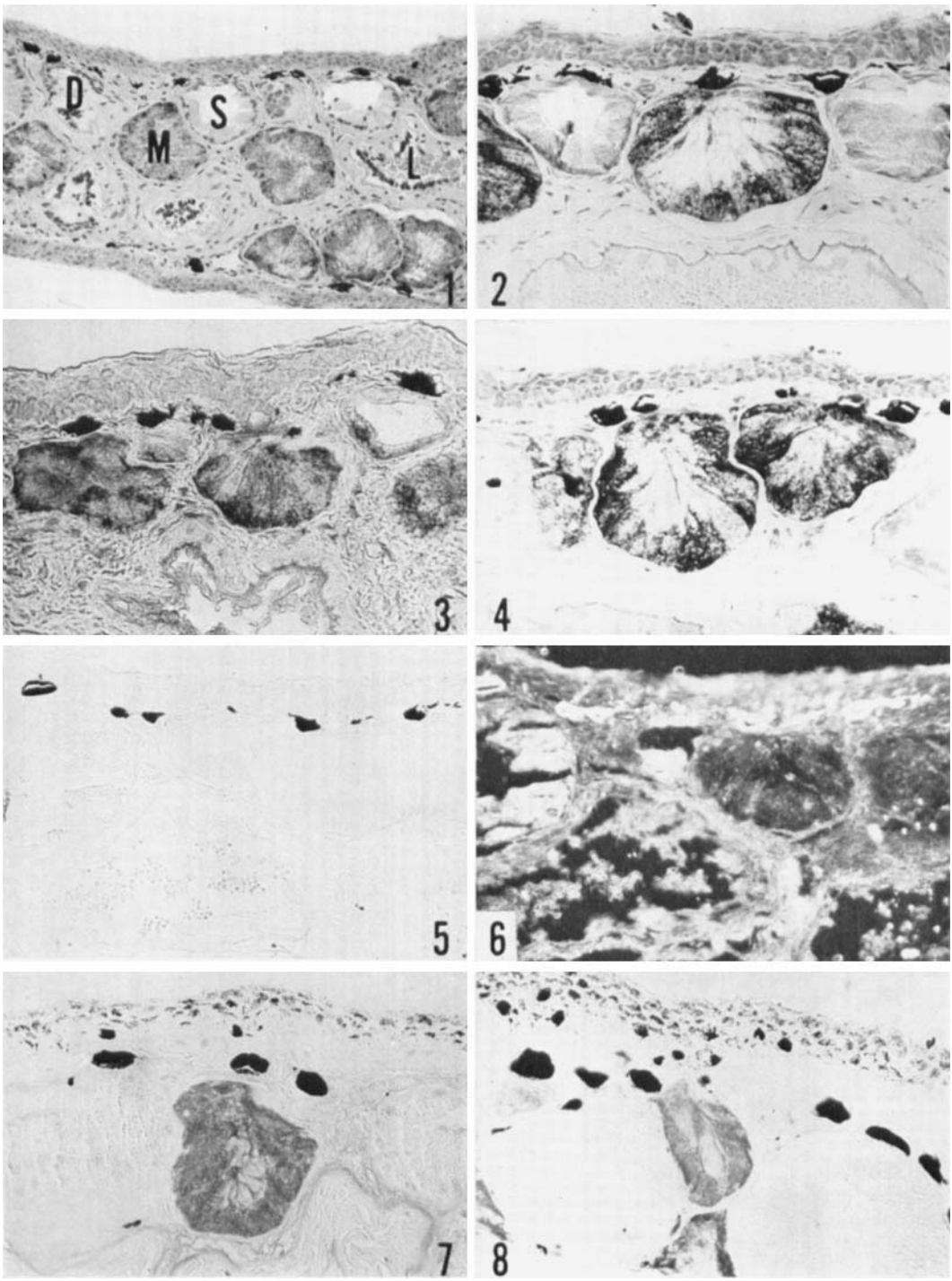
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PLATE 2

EXPLANATION OF FIGURES

9 Alcian blue, 0.6*M* $MgCl_2$. Mucus aquamarine. × 200.

10 Alcian blue, 1.0*M* $MgCl_2$. Flecks of pale blue appear in mucous glands. × 200.

11 PAS. Pink mucous glands appear lighter than magenta serous glands in this photomicrograph. A large, degenerating serous gland is present × 200.

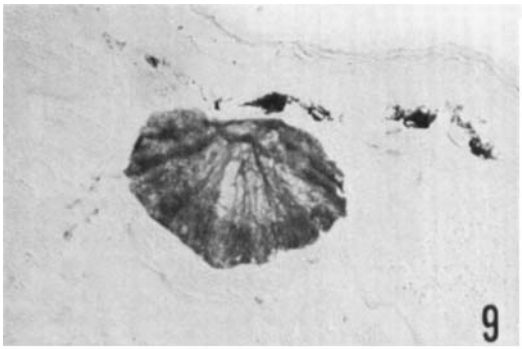
12 Acetylation + saponification + PAS. Mucous glands (center and far left) lightly colored; serous glands intensely stained. × 200.

13 Acetylation + PAS (control section for slide photographed in fig. 12). Secretions unstained. See figure 14. × 200.

14 Same section as photographed in figure 13, with darkfield, showing a dark mucous gland (center), two lighter, "cellular" serous glands (upper left and right), and part of an empty serous gland (bottom right). The brilliant white areas at the surface represent deposits of melanin. × 200.

15 Alcian blue + PAS. Mucous glands (right) royal blue; serous glands (left) magenta. × 200.

16 Periodic acid-diamine-azure. Nuclei blue, mucus rose-violet. Yellow filter. × 200.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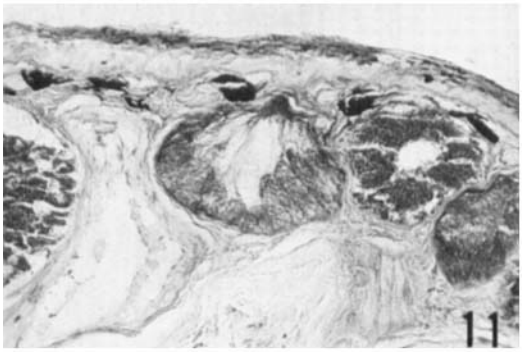
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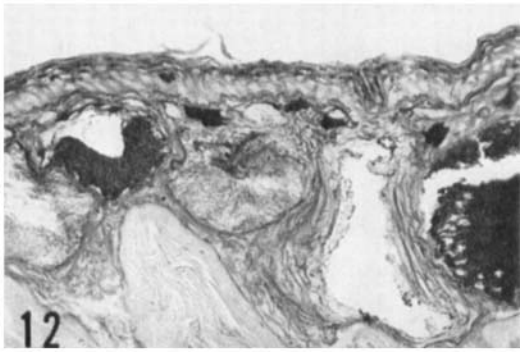
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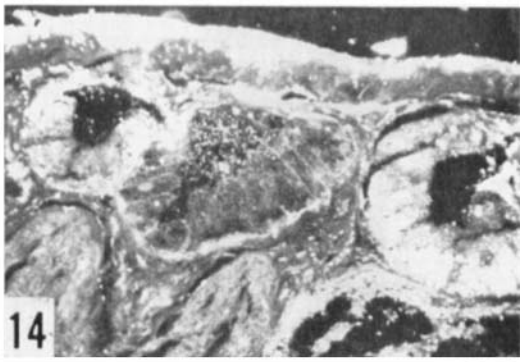
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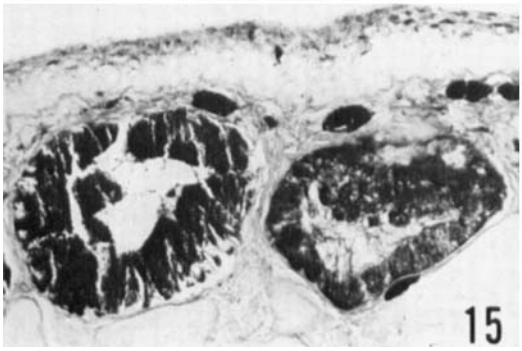
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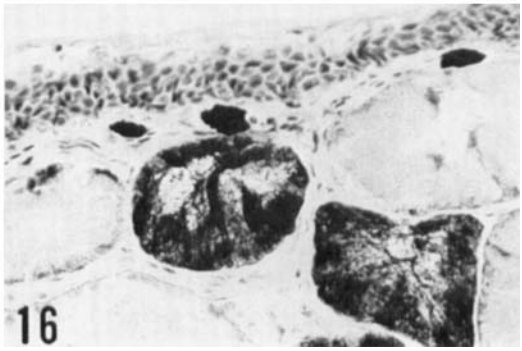
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PLATE 3

EXPLANATION OF FIGURES

17 Periodic acid-phenylhydrazine-Schiff. All tissue elements unstained. See figure 18. × 200.

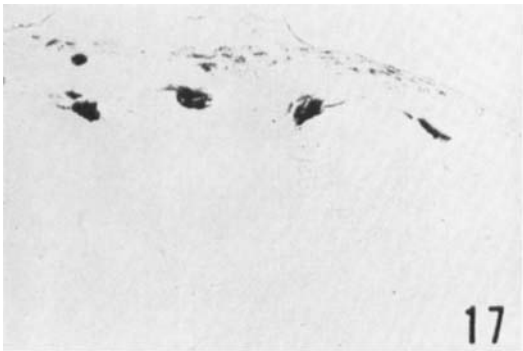
18 Same section as photographed in figure 17. Darkfield. Two mucous glands and an empty serous gland are dark; one serous gland is light. × 200.

19 Mercuric bromphenol blue. Certain nuclei, lipophores near surface and luminal serous granules stained blue; cellular serous granules and mucus are unstained. See figure 20. × 200.

20 Same section as photographed in figure 19. Darkfield. Dark mucous glands contrast well with serous glands. × 200.

21 Neuraminidase + alcian blue. Essentially the same as figure 7. Yellow filter. × 200.

22 Neuraminidase + PAS. Essentially the same as figure 11. × 200.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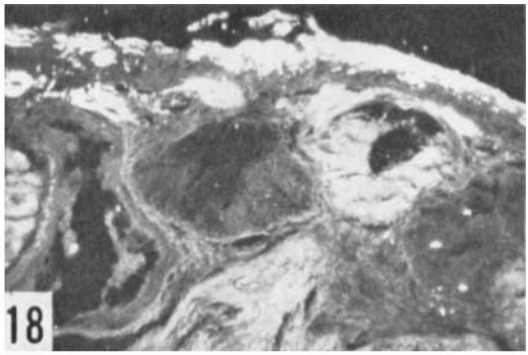
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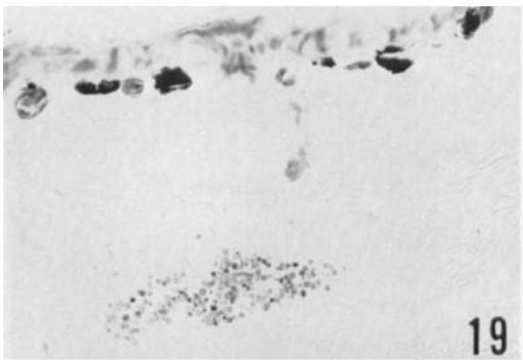
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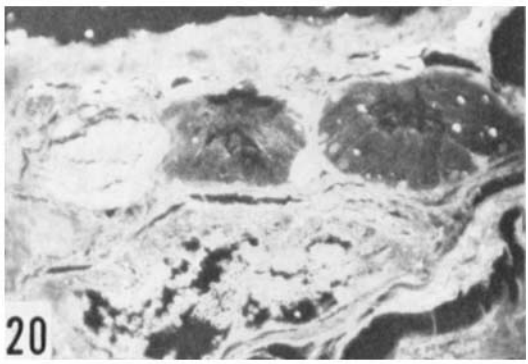
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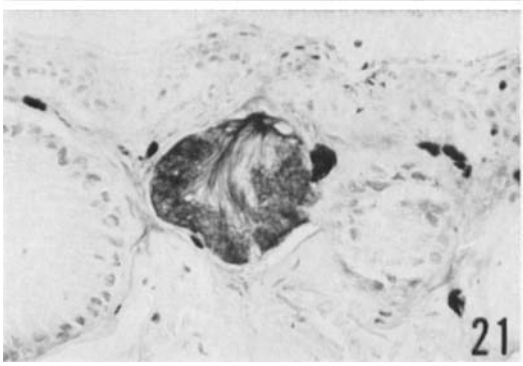
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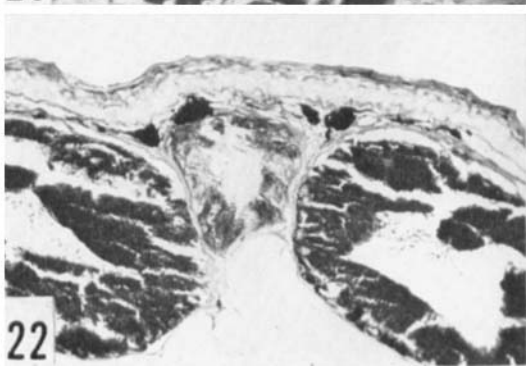
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