Histol Histopath (1990) 5: 343-348

Histology and Histopathology

Histological features and histochemistry of the mucous glands in ventral skin of the frog *(Rana fuscigula)*

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Summary. The glycoconjugate components of secretory granules were analyzed in cells of mucous glands in ventral skin from *Rana fuscigula*. The analysis was done with standard histochemical methods on semithin glycol methacrylate-embedded tissues. The staining patterns in semithin sections were comparable to those using paraffin-embedded tissue while the cytological detail was better preserved. The mucous glands contained at least two different types of secretory cells lining the lower two-thirds of the mature gland: a principal cell type filled with dense staining secretory granules and a solitary type containing paler staining, globular secretory granules. The principal type of cell contained variable amounts of acid glycoconjugates; predominantly carboxylated but also variably carboxylated and weakly sulfated glycoproteins. Other secretory cells contained mainly neutral glycoproteins. The results indicated that the mucus is a heterogeneous substance and that one cell type may produce different secretory products. We suggested that the variability in histochemical staining might be related to the sequence of biosynthesis of the secretory granule.

Key words: Mucous glands, Secretory granules, Histochemistry, Glycoconjugates, Plastic sections

Introduction

The abdominal amphibian skin has been extensively studied as a cellular model for transepithelial active ion transport in tight epithelia. There is also compelling evidence that the mucous glands of frog skin contribute to the transepithelial ion transport (Thompson and Mills, 1983; Bjerregaard, 1989) and that the mucus secretion may play an important role in osmoregulation (Friedman et al., 1967). Despite the obvious interest in the mucus glands of frog skin, very little is known about the role of the mucus secretion in ion regulatory mechanisms or in any other physiological function. Towards this goal, it is important to determine the cytochemical features of different secretory cells within the mucus secreting cells. Unfortunately, very little is known about the histochemical analysis of the mucous glands, a view also shared by others (Mills, 1985).

Frog skin commonly contains two types of exocrine glands; a granular or poison gland found mainly in dorsal skin and a mucous gland which is widely distributed but is most abundant in the ventral skin (Bovbjerg, 1963). While a certain amount of ambiguity exists regarding the structure of the mucous glands in frog skin, it has become clear that there are several different cell types within an acinus contributing to the mucus secretion (Mills and Prum, 1984). Significant variation in the morphology of the secretory granules may also exist in mucous cells in general (Harrison et al., 1987). These morphological differences amongst secretory granules could very well relate to biochemical and physiological differences of the secretory cells comprising the mucous glands. In this regard, it has become increasingly apparent that mucus is generally a heterogeneous substance derived from different populations of cells and also from different glycoconjugates within a single cell (Jones and Reid, 1978; Basbaum, 1984). It was, therefore, of special interest to us to determine the histological details of the mucous glands and to do a histochemical analysis of the secretory granules in the different acinar cells.

Glycoconjugates are prone to diffusion streaming which could obscure details of the cellular structure especially when using liquid fixatives and paraffin embedded sections (Tock and Pearse, 1965). Hence, we chose to do the investigation on semithin sections of plastic embedded tissues, a procedure which preserve the cytological detail particularly well and also allow valid histochemical analysis of macromolecules.

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Materials and methods

Mature frogs were collected throughout the year, sacrificed and the abdominal skin removed. For light microscopy small pieces (2 to 3 mm) of skin were fixed for either 12-24 hours in buffered formalin or for 5-6 hours in a solution of 0.1% glutaraldehyde, 6% $HgCl_2$ and 1% sodium acetate (Spicer et al., 1983). The specimens were washed in buffer (pH 8.1), dehydrated in a graded series of alcohols, infiltrated with and embedded in glycol methacrylate polymer (Historesin; LKB, Sweden). This is a water-miscible resin which did not require removal of the plastic from sections before staining. Semithin sections (1 μm thick) were cut, floated on distilled water, collected on slides and dried at 60° C.

1. Staining of semithin sections

Sections examined for their cytological detail were stained with either alkaline toluidine blue or haematoxylin and eosin. The following histochemical techniques were used to differentiate the glycoconjugates: the periodic acid-Schiff (PAS) reaction to demonstrate glycoconjugates containing 1,2-glycols, sometimes with prior incubation in 1% malt diastase to demonstrate glycogen (Cook, 1982); alcian blue-PAS (AB-PAS) according to Mowry (1963) to distinguish between acidic and neutral glycoproteins; acidic glycoproteins were differentiated by their reactions with alcian blue also confirmed by results from the tannic acid-uranyl acetate stinguish sulfated from carboxylated glycoproteins; neuraminidase (Sigma, USA) digestion technique (Spicer et al., 1962) followed by alcian blue (pH 2.6) to demonstrate sialoglycoconjugates. Staining times, as recommended by the suppliers of the resin (LKB, Sweden), were slightly longer than customary for paraffin sections. Thus, for example, we stained semithin sections for 30-45 min in alcian blue. Although the sections required no special treatment for staining, we did take cognizance of the methods for staining semithin plastic sections (Bock, 1984).

2. Control sections

To test the validity of the results we also compared the staining reactions in resin sections with those in sections prepared according to the freeze-dried, formaldehyde vapour fixed (FDFV) and paraffin embedded method as previously described by Els (1974). The appropriate control and test sections were performed with the methods involving enzyme digestion.

3. Electron microscopy

Small pieces were fixed in cold (4°C) 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2-6 hours, washed and postfixed in 1% osmium tetroxide buffered with sodium cacodylate for one hour. Tissues were subsequently rinsed, dehydrated, cleared and embedded in Araldite. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined after mounting on gold grids. The tannic acid-uranyl acetate technique for mucins (Sannes et al., 1978) was also applied to some ultrathin sections.

Results

In semithin sections the cytological details were clearly discernible, better than in control paraffin sections, and the histochemical nature of the secretory molecules were evident. The intensity of the staining reactions were satisfactory, especially so with those sections preserved with the fixative solution containing glutaraldehyde (Spicer et al., 1983). The staining pattern also generally corresponded to observations in paraffin sections and, in addition, the resin embedded sections were free from diffusion artifacts.

Throughout the year the skin contained a profusion of mucous glands beneath the dermis, apparently in different stages of differentiation. The larger, actively secreting glands were easily recognizable by their structure (Fig. 1). The acini of these larger glands were lined by tall, distinct secretory cell types which could be distinguished by their staining reactions and by differences in morphology of their intracellular secretory products. The acini contained two prominently distinct cell types lining the basal parts of the glands. The principal cells were characterized by distinctive, small but intense-staining secretory granules in apical areas of the cytoplasm. By electron microscopy these granules most often contained a core of high electron density, surrounded by a zone of electron-lucent material (Fig. 2). This type of secretory granule was absent in smaller, seemingly less active glands. Interspersed amongst the principal cell type was a second, solitary and generally paler type of cell. A distinctive feature of these cells was the larger, globular secretory granules in the apical cytoplasm. Electron microscopy showed that, in contrast, these secretory granules were uniformly composed of electron dense material.

The upper regions of the glands were lined by squamous epithelial cells. Also in this region, at the origin of the excretory duct, we sometimes observed one or two very conspicuous oval cells (Fig. 9). These cells were filled with strongly staining cytoplasmic granules, which by electron microscopy appeared uniformly dense and elliptical in shape. Mills and Prum (1984) also described a similar, morphologically distinct cell in this region of the mucous glands of *R. pipiens*.

The secretory granules within the cells of the mucous glands were PAS positive, indicating the presence of reactive hexoses or deoxyhexose rich glycoproteins (Fig. 4). While the globular secretory granules in the singular cells always stained intensely with PAS, secretory granules within the principal cell stained with variable intensity. The PAS reactivity of the granules was also confirmed by results from the tannic acid-uranyl acetate

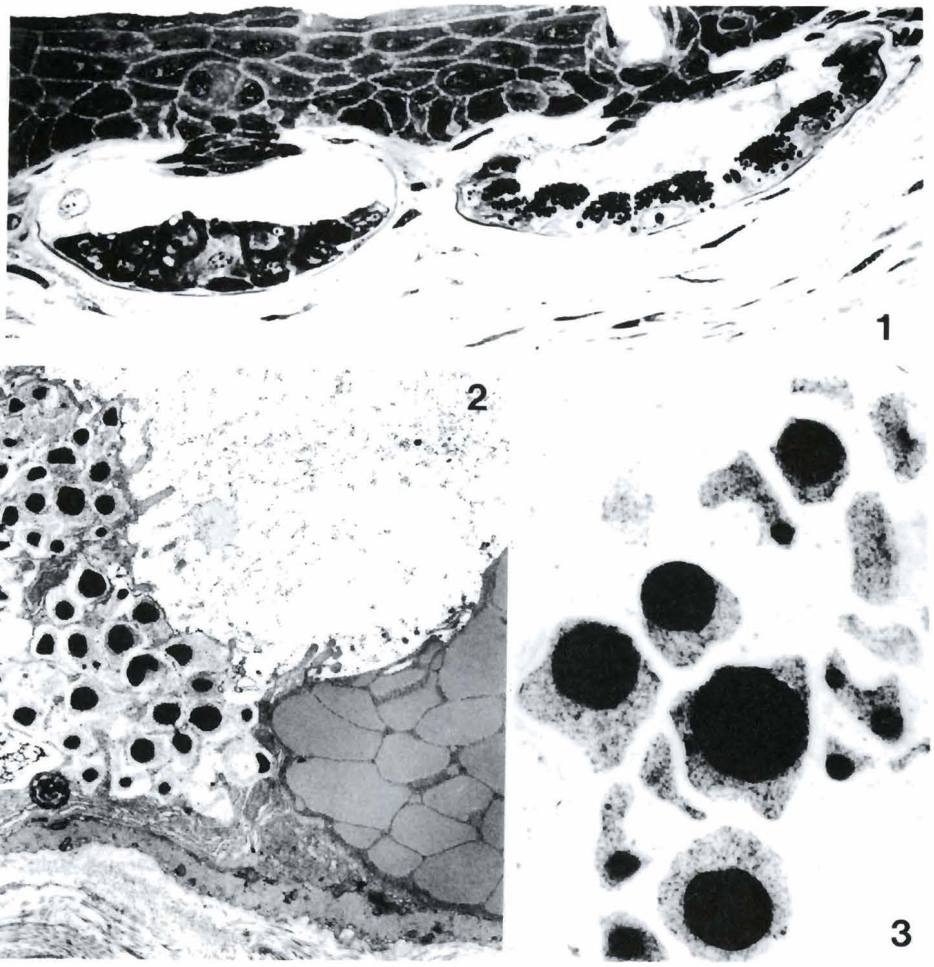


Fig. 1. Two forms of subepidermal mucous glands. The mature gland described in the text is on the right. 0.5 μm. Section stained with toluidine blue. × 500

Fig. 2. Portions of two different secretory cells. The secretory granules in the principal cell type on the left contained a dense core. The cell on the right contained uniformly dense secretory granules. × 5,500

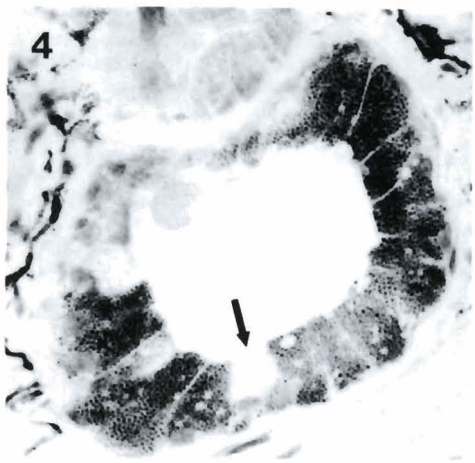
Fig. 3. An ultra-thin section stained with tannic acid-uranyl acetate showing a strong reaction in the secretory granules with the core of high electron density. × 16,500

technique on ultrathin sections (Fig. 3). The PAS reaction was unaffected by prior diastase digestion.

Further histochemical analysis revealed important differences in the histochemical nature of secretory granules within individual cell types. The principal cells generally contained secretory granules rich in acidic glycoproteins. Hence, the majority of the secretory granules stained strongly with alcian blue and also retained their alcianophilia with the AB-PAS combination, indicative of the acidic nature of the glycoproteins (Fig. 5). Occasionally we encountered a gland in which the cells contained secretory granules that stained only faintly with alcian blue (pH 2.6) and in serial sections stained reddish with AB-PAS combination. In fact, variations in the staining reactions of the secretory granules, without any clear pattern, were commonly encountered amongst the principal cells. The variations were most obvious amongst the principal cells of different glands but sometimes differences in staining reactions were observed even within the same population of cells in a given gland.

Additional histochemical differentiation of the acid glycoproteins suggested that the principal mucous cell type contained mostly less strongly ionized, carboxyl groups. Accordingly, nearly all the secretory granules in these cells stained strongly with alcian blue at pH 3.2 and 2.6 (Fig. 6) or with alcian blue in the presence of 0.05-0.1 M $MgCl_2$. Sometimes the cells also contained weakly sulfated glycoproteins. A smaller proportion of the secretory granules was variably stained with alcian blue at pH 1.0, when only weakly sulfated glycoproteins stain (Jones and Reid, 1973), but did not stain at pH 0.5 (Fig. 7). In duplicate sections the secretory granules similarly stained with alcian blue even in the presence of 0.3 M $MgCl_2$ but were unstained in the presence of 0.5 M $MgCl_2$ or higher, when only strongly sulfated glycoproteins stain (Scott and Dorling, 1965). These conclusions were confirmed by results with the HID method. The secretory granules were rarely stained with high iron-diamine but nearly always stained blue when high iron-diamine was followed by alcian blue staining.

Sialic acid- containing glycoproteins could also react with alcian blue at higher pH levels and at the lower electrolyte levels. Hence, we also tested for sialic acid by neuraminidase digestion (Spicer et al., 1962; Cook, 1982). Treating paraffin or semithin sections with neuraminidase for up to 24 hours had no effect on subsequent staining with alcian blue pH 2.6., indicating that sialic acid was not present in the molecules. However, since some sialic acid may be resistant to



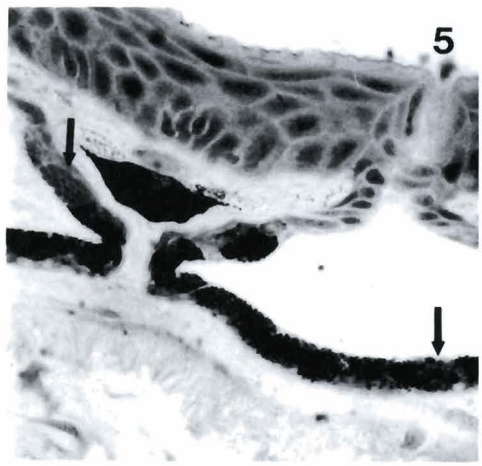
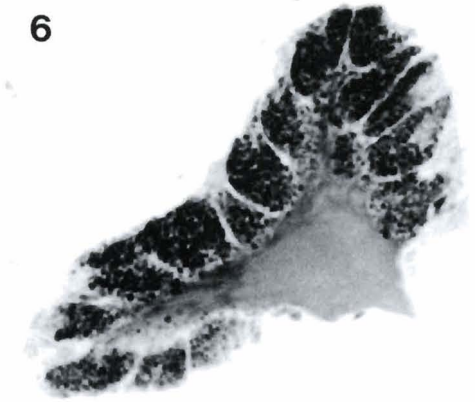
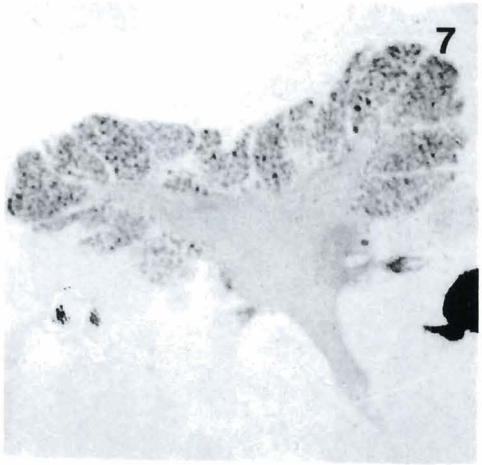


Fig. 4. A gland stained with PAS. All secretory stained positive except for the rare cell (arrow) that did not contain any PAS-positive secretory granules. × 520

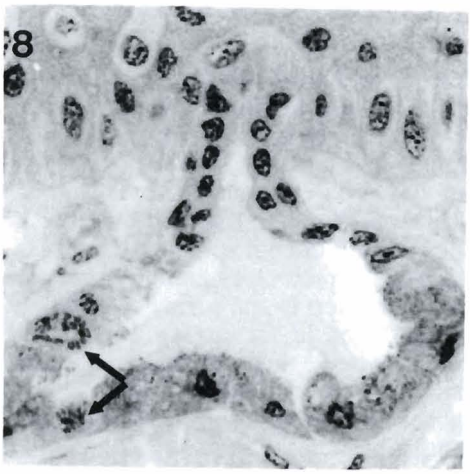
Fig. 5. The principal cell type contained secretory granules that stained mainly with AB (pH 2.6). Solitary cells contained secretory granules that stained with PAS (arrow). AB-PAS method. × 500





Figs. 6 and 7. Serial sections stained with AB at different pH levels. nearly all the secretory granules stained with AB (pH 2.6). in Fig. 6, while sometimes the cells also contained granules that, stained with AB (pH 1.0) as in Fig. 7. × 520

Fig. 8. Cells containing secretory granules that retain their PAS-positive reaction after deamination (arrows). The other granules stained with AB. × 525



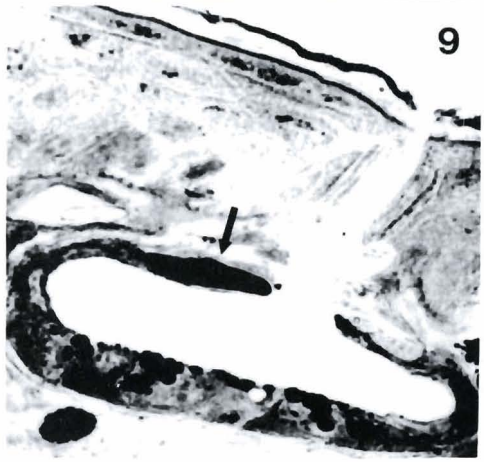


Fig. 9. A small gland which contained only PAS-positive secretory cells. Seen here is a conspicuous cell also with PAS-positive material, near the origin of the excretory duct. × 625

neuraminidase we also tested for sialic acid by examining the effect of acid hydrolysis on the basophilia of the granules (Lamb and Reid, 1969). Results were inconclusive as we encountered only variable loss of staining with alcian blue after 4 hours of hydrolysis with sulfuric acid.

The second cell type, with the globular secretory granules, contained only neutral glycoproteins. These granules stained strongly PAS-positive and had no affinity for alcian blue (Fig. 5). To rule out the possibility that acid glycoproteins were present but that their staining reactions were masked by protein associated with the carbohydrate (Spicer et al., 1965), some sections were first deaminated to eliminate possible blocking (Cook, 1982). However, prior deamination did not alter the PAS reactivity of the globular secretory granules, and in fact, the intensity of the reaction often increased slightly (Fig. 8).

Granules in the conspicuous, isolated cells sometimes

encountered near the origin of the excretory duct were similarly stained by PAS only (Fig. 9).

Discussion

We observed only mucous glands, apparently in different stages of development, in the sections of ventral skin. The larger, actively secreting glands had many features in common with the seromucous glands described by Mills and Prum (1984) who divided the mucous glands of frog skin into two morphological types; mucous and seromucous. We did not follow their division, since individual glands in *R. fuscigula* contained features corresponding to both these glands. Mills and Prum (1984) did acknowledge that their two forms of mucous glands could represent different stages in the development of the same gland.

The secretory granules within individual cells consisted of glycoproteins which were defined according to standard histochemical staining reactions. Collectively, the results indicated that the mucus secretion of the glands is a heterogeneous substance to which different cell types contribute. The secretory cells of the mucous glands contained combinations of neutral, carboxylated and sometimes carboxylated and sulfated glycoproteins. The bulk of the mucus consisted predominantly of acidic glycoproteins secreted by the principal cells. This finding supports earlier results by Dapson (1970) who identified acidic mucosubstances in the mucous glands of *R. pipiens*. Unlike Dapson (1970) we were also able to distinguish less strongly ionized, carboxyl rich and sulfated groups in the secretory granules.

Generally, acid glycoproteins may also include sialomucins and sulphomucins. While there was a suggestion that the principal cells may contain sialylated glycoproteins, results with specialized techniques to demonstrate sialomucins were inconclusive. Like Dapson (1970) for *R. pipiens*, we found that neuraminidase digestion did not alter the alcianophilia of the granules. On the other hand, neuraminidase resistant sialomucins may stain with alcian blue at pH 1.5, (Jones and Reid, 1973) a level at which we still encountered some staining. Unfortunately, after sulfuric acid hydrolysis to demonstrate possibly blocked sialic acid groups (Lamb and Reid, 1969), we encountered only variable loss of basophilia indicative of sialomucins. We have to consider the possibility that the loss of basophilia we encountered might well have been the result of nonspecific extraction of acid glycoproteins by acid hydrolysis (Cook, 1982). Therefore, at this stage it is unknown whether sialic acid may be present in the molecule.

Contrary to results of Dapson (1970) we also demonstrated cells with mainly neutral glycoproteins in the cytoplasm. These were primarily confined to the secretory granules in the cytoplasm of the few solitary cells in the basal parts of the glands and also to the distinct cell type near the origin of the excretory duct (Figs. 5, 9).

We also consistently encountered variations in the histochemistry of secretory granules within the same cell population. Thus, the principal cells with dense-core granules, occasionally contained secretory products with little affinity to alcian blue, mostly contained carboxylated acid glycoproteins and sometimes contained carboxylated and sulfated glycoproteins. Similar variations in histochemistry of mucin secretory granules within individual cells are becoming increasingly apparent (Jones and Reid, 1978; Spicer et al., 1978; Harrison et al., 1987).

According to a scheme proposed by Harrison et al. (1987), variability in staining within a given cell could be attributed to a temporal sequence of biosynthesis of the mucous secretory granule. Biosynthesis of mucin glycoconjugates include at least two post-transcriptional modifications to the secretory protein; firstly glycosylation of the protein followed by modifications to the sugar moiety (Phelps, 1978; Laboisse, 1986). Accordingly, those granules that did not stain with PAS contained only protein; secretory granules staining with PAS could be related to the stage when the cell was producing mainly glycoproteins; secretory granules would stain with alcian blue when the glycoproteins had been carboxylated and the presence of sulfated glycoproteins would coincide with the stage when sulfate groups had been conjugated to the glycoprotein.

Also consistent with the idea outlined above, was the observation that the first demonstrable glycoconjugates in smaller, apparently developing glands were always neutral glycoproteins (Fig. 9). Acid glycoproteins only appeared in the principal cells when the glands were larger and appeared more active. These observations also lend support for our suggestion that differences in the morphology of the mucous glands could represent different stages in development. Similarly, we also cannot rule out that the solitary cell type, with the globular secretory granules, portray a different stage in the development of a principal cell. Dapson (1970) did not observe any variability of staining in mucous glands of *R. pipiens*. Whereas we did our analysis on numerous specimens collected throughout the year, Dapson (1970) studied the mucous glands of one specimen only.

Without supporting biochemical and other experimental evidence it is difficult to assign a physiological role for the mucus secretion. Frog mucous glands are under adrenergic control (Thompson and Mills, 1983) but it is not clear whether stimulation has any direct effect on the type of secretion by the mucous cells (Mills and Prum, 1984). Many functions have been attributed to frog skin mucus, including thermoregulation (Lillywhite, 1971) and some kind of involvement with osmoregulation and water absorption (Friedman et al., 1967; Dapson, 1970). We have noted that the mucus secretion in the glands of *R. fuscigula* is composed of a mixture of glycoproteins. Mixtures of polymers, especially those containing a high concentration of glycoproteins, often separate into different phases (Edwards, 1978). Consequently the mucus covering the ventral frog skin could well exclude certain macromolecules while at the same time allowing smaller molecules, including

electrolytes, to pass through. In this way the ion transporting cells in ventral frog skin could conceivably be protected by the mucus from the environment and, by having access to ions, still be involved in osmoregulation.

Acknowledgements. We gratefully acknowledge the financial support of the Nellie Atkinson bequest and the Medical Research Council.

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Accepted March 1, 1990