HISTOCHEMICAL STUDIES OF THE SECRETORY PROCESSES IN BOVINE SALIVARY GLANDS

A thesis presented in partial fulfilment of
the requirements for the
Degree of Master of Science
at Massey University

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1981

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by Mervyn John Birtles

Salivary glands from 12 bovine animals were dissected, weighed and sampled for histological examination. The total salivary gland weight was positively correlated with body weight but there were not normally consistent differences between the weights of left and right glands. However, in animals that had chronic re-entrant cannulations of the left parotid and mandibular ducts, the ipsilateral glands were always lighter. The histological features of salivary glands and the histochemical reactivity of their secretory and duct cells were examined. Parotid gland secretory endpieces were elongated and their individual cells contained PAS+ve granules. These cells were shown by immunohistochemistry to be the site of protein secretion and thus were classified as proteoserous cells. Chronic parotid duct cannulation in association with duct obstruction caused dilation of the secretory endpiece lumens and degenerative changes within the endpiece cells. Intralobular duct cells contained PAS+ve granules which may be the secretory component that is associated

with secretory IgA. Variable numbers of intrastriated duct cells occured in the parotid glands of different animals and in retrospect, this was found to correlate positively with the animals known susceptibility to bloat. The parotid excretory duct contained many goblet cells which contribute a small amount of mucosubstance to the proteoserous secretion.

Secretory endpieces of the mandibular gland were composed of mucous cells which were PAS, AY and weakly AB+ve and demilune cells which were PAS and AB+ve as well as acidophilic and pyroninophilic. Clumps of plasma cells were observed in the intralobular connective tissue. The effect of obstruction of chronic duct cannulation on the mandibular gland was to dilate endpiece and intralobular duct lumens, cause degenerative changes in mucous and demilune cells and increase the numbers of small lymphocytes, PMN neutrophils and mast cells in the connective tissues of the gland. By contrast with the excretory duct of the parotid, that of the mandibular contained no goblet cells but simply a stratified columnar epithelium.

Mucous cells of the sublingual gland were PAS+ve, AY+ve and weakly AB+ve and arranged into long tubular endpieces. The demilune cells contained abundant PAS+ve, AB+ve, AY-ve granules. Many plasma cells were present in the connective tissue between the secretory endpieces and around the intralobular and interlobular ducts. In animals with chronic cannulations of parotid and mandibular glands the ispilateral sublingual gland weighed less than the contralateral gland.

The posterior tongue, soft palate, pharynx and the lingual

aspect of the epiglottis contained extensive areas of glandular tissue. The secretory endpieces consisted of a high proportion of mucous cells and a few scattered proteoserous demilune cells. The glandular tissue of the epiglottis contained abundant plasma cells in the intralobular connective tissue.

Based on their histochemical reactivity the demilune cells of the intermediate buccal glands produced a purely serous secretion. In addition, the intermediate and dorsal buccal glands contained many AB, AY and PAS+ve mucous producing cells.

The labial glands were small, scattered lobules of secretory tissue found at the labial commisures. The glandular lobules were composed of tubular secretory endpieces capped with large proteoserous demilune cells which were AY-ve, PAS+ve, strongly acidophilic and pyroninophilic. Large numbers of plasma cells were found in the connective tissues within and around the secretory tissue.

ACKNOWLEDGEMENT

My thanks are due to Dr D.H. Carr, who acting in the capacity of supervisor, provided much help and encouragement during the preparation of this thesis. I would also like to thank Professor R.E. Munford for his helpful criticism of the text, for making departmental facilities available for this study and generously allowing the use of his typewriter.

I wish to thank Dr W.T. Jones, Applied Biochemistry Division, DSIR, Palmerston North for supplying the salivary protein band 4 antibody and for permission to include the photograph (Figure 3.7) of gel electrophoresis of bovine salivary protein. I am indebted to Mr M.P. Gurnsey, Applied Biochemistry Division, DSIR, for valuable assistance in obtaining the tissue samples and for supplying background information regarding the animals used for this study.

My appreciation is extended to Ms Irene Hall for her assistance during tissue collection and in the histology laboratory.

For the sketch showing the gross anatomy of the bovine salivary glands (Figure 1.1), I gratefully acknowledge the artistry of Mr W.G. Keereweer.

Finally, my thanks are due to Miss F.M. Williams, who typed the draught copies of the manuscript and also my wife who typed the final copy and who supported my efforts during the term of this study.

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1. INTRODUCTION

The bovine salivary glands, although remote from the fore-stomachs of this ruminant species, form a secretion which provides an appropriate medium for fermentative digestion. No mammals, in fact few animals, with the exception of termites and a few species of wood borers, have the ability to produce enzymes for the breakdown or digestion of cellulose and hemicellulose; the high energy containing compounds of plant material (Church, 1976). As fore-gut fermenters ruminant species provide a capacious chamber, the reticulo-rumen, which accommodates a large population of micro-organisms capable of synthesizing the necessary enzymes for the digestion of plant material. Since the walls of the reticulo-rumen contain no exocrine tissue, salivary secretions assist in maintaining optimum conditions of the liquid environment of the chambers for the suspension of the ingesta and the microbial population (McDougall, 1948).

During microbial fermentation, large volumes of volatile fatty acids are produced. If an effective microbial population is to survive this production of acid, the rumen contents must be adequately buffered. Not withstanding this, volatile fatty acid production lowers the pH in the reticulo-rumen from about 7 in the unfed animal to approximately 5.8 depending on the amount of carbohydrate in the diet (Turner and Hodgetts, 1955). Secretions from the parotid, ventral buccal, palatine, pharyngeal and minor buccal glands are strongly buffered with bicarbonate and phosphate (Kay, 1966). The work of Turner and Hodgetts (1955) showed that bicarbonate and phosphate buffers are almost equally important

and account for most of the buffering within the range pH 5.8 - 7.0. However, although large amounts of bicarbonate and phosphate are produced in the saliva, they are not sufficient to buffer all the acid produced during fermentation. Fatty acids which are not neutralised by salivary buffers are mostly neutralised by bicarbonate buffers present in the extra-cellular fluid and blood after being absorbed through the rumen wall by active transport mechanism (Church, 1976, Bartley, 1976).

In addition to its buffering capacity saliva also assists the maintenance of the microflora by the provision of nitrogen (and minerals) (Bartley, 1976, Church, 1976). Nitrogen, from both urea and mucus (Phillipson and Mangan, 1959) helps promote an active microbial population in the reticulo-rumen. The recycling of urea through saliva is an important mechanism for conserving nitrogen (McDonald, 1948). Deamination with the consequent production and absorption of ammonia through the walls of the reticulo-rumen into the blood constitutes a potential loss of nitrogen for the animal. However, the return of part of the urea formed by the liver into the saliva makes at least some of this nitrogen once more available to the micro-organisms for possible protein synthesis. Data published by Somers (1957) has shown that, in sheep, 0.7 - 0.9 grams of nitrogen can enter the rumen through the saliva each day.

In addition to its role within the reticulo-rumen, saliva acts as an aid to mastication, taste and deglutition. Much of the normal diet of ruminants is coarse and each of these processes is aided by the moistening effect of saliva. Appropriately, the rate of salivary secretion is largely controlled reflexly by mechanical

and chemical stimulation of the mouth and by distention of the oesophagus and the reticulo-rumen (Kay, 1960). Somers (1957) confirmed the observation of several other authors that the physical properties of the food have an important bearing on the activity of the parotid glands; dry fibrous food stimulating an increased output of saliva compared with succulent green leafy material. Although both parotid glands are stimulated by the mastication of food the ipsilateral gland has a faster rate of secretion than the contralaterial gland during chewing (Kay, 1958). This differential in the rate of secretion of the two parotids provides a larger volume of saliva at the site of mastication and thus aids that process. Saliva also aids in oral hygiene by keeping the buccal cavity and teeth clean, moist and alkaline (Kay, 1966) and the secretions contain sufficient calcium ions to prevent the teeth from being drained of calcium (Schneyer and Schneyer, 1967). While in the mouth water soluble components of the food are dissolved in saliva and sapid substances gain access to the taste buds located in the circumvallate papillae found toward the posterior of the tongue.

Though it is probably the serous component of saliva that softens the food and dissolves sapid substances it is the mucus in saliva which confers slipperiness and thus facilitates deglutition. Kay (1960) noted that a mucous coat is a notable feature of boli collected from the cardia. The palatine and pharyngeal glands, which secrete a high proportion of mucus, may be an important source of this mucous coat.

A little discussed function of saliva is that of protecting the mucosal linings of the upper alimentary tract against

dessication, mechanical abrasion and bacterial invasion. In ruminants this begins in the mouth and continues into the pharynx oesophagus, reticulo-rumen and omasum and probable terminates a short distance distal to the omasal-abomasal junction (McDougall, 1948). This is subserved by the mucous, serous and immunoglobulin components of total salivary secretion.

Saliva is formed by a mumber of exocrine glands which are located near the buccal cavity and communicate with it by means of an excretory duct system of variable length. On the basis of their secretory product, these glands can be divided broadly into three classes :

1. Serous secreting
2. Mucus secreting
3. Mucoserous secreting

Despite much use of this classification it has been found, by the application of specific histochemical techniques and electron microscope studies, that bovine salivary glands do not fall discretely into the above mentioned classes.

The major paired salivary glands, by weight of secretory tissue, are :

1. Parotid
2. Mandibular
3. Sublingual

The minor paired salivary glands are :

1. Ventral buccal
2. Intermediate
3. Dorsal buccal
4. Labial

In addition, there exists substantial amounts of glandular tissue which is embedded in the submucosal connective tissue of the buccal cavity and from which short ducts open directly onto the epithelial surface. These exocrine glands secrete a saliva-like substance which is a component of the total saliva collected from oesophageal fistulae (Kay, 1960). Included in this group are the :

1. Palatine glands
2. Lingual glands
3. Pharyngeal glands

1.1 THE GROSS ANATOMY OF THE BOVINE SALIVARY GLANDS

All the salivary glands with the exception of the lingual and palatine glands are paired. The description that follows is based largely on the accounts of Habel (1970) and Getty (1975) and the general arrangement of the glands is shown in Figure 1.1.

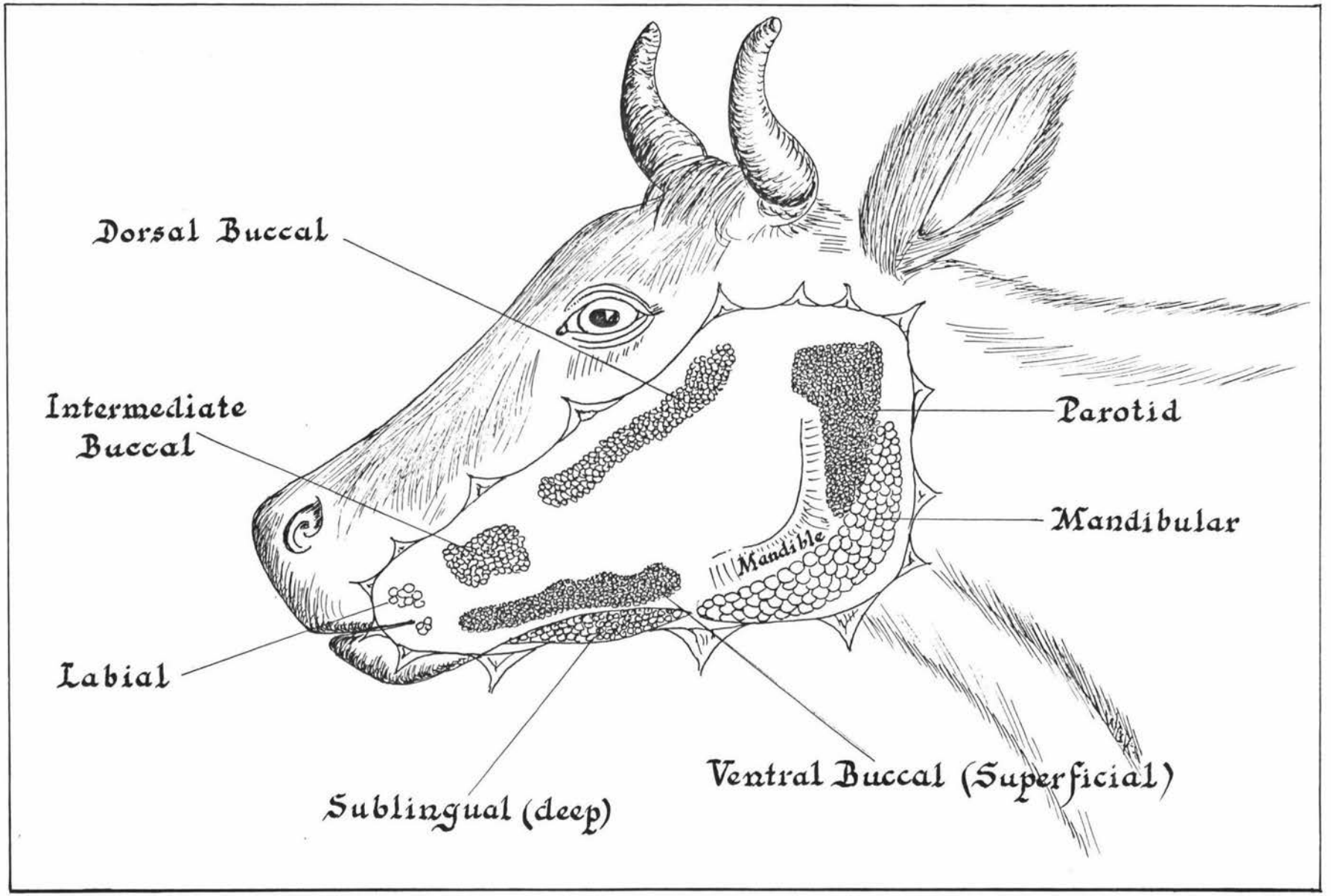
1.1.1 PAROTID GLAND

The parotid is pinkish brown in colour and lies along the caudal border of the masseter muscle from the zygomatic arch to the angle of the mandible. The confluence of numerous small excretory ducts gives rise to the parotid duct which empties into the buccal cavity via the parotid papilla opposite the upper fourth premolar or first molar.

1.1.2 MANDIBULAR (submaxillary, submandibular) GLAND

The mandibular gland is cream or pale yellow in colour and distinctly lobulated. The gland lies in a curve medial to the angle of the mandible extending from the atlantal fossa to the basihyoid bone so that it is covered partly by the parotid gland, partly by the mandible. The mandibular duct is formed by the

FIGURE 1.1 Anatomy of the bovine salivary glands.



anastomoses of small excretory ducts which leave the gland at about the middle of the rostal border. The main duct crosses the lateral surface of m. digastricus passing rostrally on the deep surface of the mylohyoideus to the sublingual caruncle. It ends just lateral to the caruncle so that the opening of the duct is concealed by it (Nickel, Schummer, Seiferle, 1979).

1.1.3 SUBLINGUAL GLAND

The sublingual gland has a distinctive colour which ranges in different individual animals from orange to pale yellow. This gland can be divided into two parts :

The monostomatic portion which is approximately 10 cm long and 1-2 cm thick. This is situated beneath the mucous membrane of the mouth, between the body of the tongue and the ramus of the mandible. The excretory duct of this portion opens under the caruncle, near the opening of the mandibular duct.

The polystomatic portion extends in a chain of lobules from the palatoglossal arch to the incisive part of the mandible. The ducts from this portion empty into the lateral sublingual recess.

1.1.4 VENTRAL BUCCAL (inferior molar) GLANDS

The ventral buccal is a compact gland of similar surface texture and colour to the parotid. It extends caudally from the angle of the mouth to a point about 1 cm under the masseter muscle. A few lobules of the extremity of the gland are often found embedded in the m. masseter.

1.1.5 INTERMEDIATE AND DORSAL GLANDS, LABIAL GLANDS

These minor salivary glands are formed by lobules of glandular tissue, which are sometimes quite diffuse. They have a distinctive colour which ranges in different animals from pale yellow to orange.

Lobules of the intermediate buccal glands are scattered within and deep to the buccinator muscle. The dorsal buccal glands extend in a chain from the angle of the mouth to the facial tuberosity where they are covered by a thin, superficial layer of the buccinator muscle.

At the labial commissures, particularly in the upper lip, compact aggregations of glandular tissue known as the labial glands occur. The openings of the labial gland ducts are found on the small papillae on the internal surface of the lip.

1.1.6 PALATINE GLAND

The smooth part of the hard palate and the soft palate contain extensive areas of glandular tissue embedded in the subepithelial connective tissue. Each lobule is drained by a short duct which opens directly on to the surface of the buccal cavity.

1.1.7 LINGUAL GLANDS

Small lobules of glandular tissue under the dorsal epithelium and embedded in the musculature of the tongue form the lingual glands. They are most numerous at the root but some glandular tissue extends rostrally along the margins of the tongue. The glands associated with the circumvallate papillae known as gustatory or von Ebner's glands are also included within the lingual group.

1.1.8 PHARYNGEAL GLANDS

Pharyngeal glands are small compact lobules of glandular tissue lying in the subepithelial connective tissue of the pharynx and extend into the epiglottis where they irrigate the lingual surface. The laryngeal surface of the epiglottis receives ducts

from glandular tissue typical of that found in the upper respiratory tract.

1.2 THE GENERAL ORGANISATION OF SALIVARY GLANDS

The secretory tissue of the major salivary glands is encapsulated by irregularly arranged fibrous connective tissue and divided into lobes by connective tissue septa which arise from the hilus and are continuous with the capsule. Each lobe is further subdivided into several lobules. The extent of the interlobular connective tissue varies from gland to gland; very little in the parotid and extensive with a high proportion of adipose tissue in the sublingual. The connective tissue of the hilus and septa contains nerves, lymphatics and the intrinsic vascular supply of the gland as well as the various orders of extra-lobular drainage ducts.

Each lobule in turn is made up of secretory units or end pieces, their intercalated ducts, intralobular or striated ducts, myoepithelial cells as well as fine nerves and blood capillaries. These structures are supported by a network of fine collagen and reticular fibres.

With the exception of the ventral buccal gland, the minor buccal and associated glands of the buccal cavity and pharynx are made up of lobules of secretory tissue in which each lobule is surrounded by connective tissue containing blood vessels and nerves. Secretory units, intercalated ducts and intralobular ducts are found within each lobule (Leeson, 1967).

1.3 THE HISTOLOGY OF SALIVARY GLANDS

In the bovine as well as many other mammalian species (cat, dog, sheep, pig and rodent), the secretory cells are pyramidal in shape and are arranged around an enlarged central lumen to form a secretory endpiece. In some glands, between adjacent endpiece cells, there are fine intercellular canals or secretory canaliculi which are continuous with the lumen. Each endpiece is bounded by a distinct basal lamina. In many glands myoepithelial cells found interposed between the basal laminae and the basal plasmalemma of the secretory endpiece cells. Myoepithelial cells are enlongated or star-shaped cells with long, branching cytoplasmic processes which embrace the endpieces and intercalated ducts.

The cells of the secretory endpiece are classified as either mucous or serous secreting depending on the nature of the secretion or the cells morphology, histochemistry and structure. The pattern of arrangement of serous and mucous cells within a mixed gland varies.

Many mixed glands contain endpieces which consist of both serous and mucous cells. In such endpieces the mucus producing cells are generally near the duct system and the serous cells are confined to the blind end where they form a cap or crescentic shaped aggregation of cells over the mucous elements. They are commonly called serous caps or serous demilunes. These serous cells may have direct access to the lumen of the secretory endpiece but more commonly they communicate with it via a system of secretory canaliculi. The secretory canaliculi pass between the mucous cells and branch among the serous cells of the demilune (Young and Van Lennep, 1978).

1.4 THE CYTOLOGY OF SALIVARY GLANDS

1.4.1 SEROUS CELLS

Serous cells are usually described as pyramidal although Young and van Lennep (1978) point out that this is an over simplification since none of the sides of a serous cell is flat and furthermore, their shapes vary quite considerably. The basal plasmalemma, which may either rest directly on the basal laminae or be separated from it by a myoepithelial cell process, is usually smooth or only slightly folded. The lateral plasma membranes are often plicated which, when the lateral intercellular spaces are narrow, results in an interdigitation with neighbouring cells. The apical plasmalemma, lining the lumen of the acinus, is usually irregular and commonly forms small irregular microvilli. Small micropinocytotic caveolae may sometimes be seen between the microvilli. At the apical plasma membrane of bovine parotid gland serous cells a brush border consisting of long and sometimes branched microvilli is often present (Shackleford and Wilborn, 1969).

In many species the position of the nucleus in a serous cell seems to be governed by the number of secretory granules present. In animals prior to feeding, the greater part of the cytoplasm may be occupied by secretory granules and the nucleus occupy the basal third of the cell. Conversely, during or following feeding, after discharge of these granules, the nucleus tends to occupy a more central position within the cell. The shape of the nucleus is generally spherical with clumped chromatin and one or two small nucleoli (Young and van Lennep, 1978).

In the serous cells of the parotid gland the secretory granules show a certain amount of variability in size and electron density.

van Lennep, Kennerson and Compton (1977) have called the smaller electron dense granules type 1 granules and the larger, less numerous and less electron dense ones type 2. Since intermediate forms are very common, there seems little doubt that the two types described form a single class of granule.

In bovine parotid acinar cells rough endoplasmic reticulum (RER), seen as parallel arrays of membranous endoplasmic reticulum cisternae studded with ribosomes, is present in small amounts. The RER is found predominately in the basal parts of the cell although a few scattered cisternae may be found in the lateral or apical cytoplasm. van Lennep et. al. (1977) found that the amount of RER varies considerably from one cell to another.

The Golgi apparatus of serous cells is found in the supranuclear position of the cell. Elongated mitochondria are abundant and randomly distributed throughout the cytoplasm (Shackleford and Wilborn, 1969).

1.4.2 MUCOUS CELLS

Although irregular in outline the shape of mucus producing cells is generally more cuboidal than pyramidal and their "apical surface" is consequently more extensive than serous cells. The apical plasma membrane is usually devoid of microvilli and secretory canaliculi are seldom seen. Basal and lateral plasma membranes are mostly smooth or only slightly plicated giving rise to some interdigitation between adjacent cells.

The nuclei of mucous cells stain darkly with basic dyes, are flattened and usually located immediately adjacent to the basal plasma membrane. The large volume of secretory or mucigen

droplets within the cell apparently force the nucleus into this shape and position as well as producing the characteristic "foamy" appearance of the cytoplasm (Ham, 1974).

The RER consists of closely packed cisternae in the basal part of the cell while Golgi complexes are generally large with several dilated cisternae on their concave border. Mitochondria are found mostly in the basal portions of the cell but a few may be observed, along with some endoplasmic reticulum, in the upper parts of the cell (Bloom and Carlsoo, 1974).

1.4.3 INTERCALATED DUCTS

The duct epithelium typically consists of closely packed low cuboidal epithelial cells surrounding a narrow lumen. Intercalated ducts vary greatly in length, diameter and cell height both in different species and in different glands within the one species. Myoepithelial cells are commonly found in association with intercalated ducts and may extend a process on to the first part of intralobular ducts.

The spherical shaped nuclei occupy the greater part of the cells due to the latters small size. Cytoplasmic organelles are present in moderate number: mitochondria, scattered RER cisternae, a few small Golgi complexes in the peri-nuclear region and some lysosomes. The basal and lateral plasma membranes of intercalated duct cells are almost smooth with few plications. The apical plasma membrane forms a number of irregular, short, thick microvilli. The transition from a secretory endpiece to intercalated duct to intralobular duct is fairly abrupt.

1.4.4 INTRALOBULAR DUCTS

These ducts are lined by cuboidal or low columnar epithlium and have, as a characteristic feature, a distinctive radial striation over the basal third of the cell. The presence of these striations has led to the alternative term "striated ducts". The extent to which the striated ducts are developed varies among the same gland in different species and also among different glands of the same species and apparently in the same gland under different physiological conditions. In addition van Lennep et. al., (1977) have related the development and differentiation of basal striations to the dietary sodium status of the animal.

Ultrastructural studies on striated intralobular ducts, first published by Pearse (1956) reveal the striations seen with the light microscope to the rows of mitochondria lying end to end within basal folds of plasma membrane. A very similar structure has been recognised in the basal region of the epithelium of distal convoluted tubules of the kidney (Pearse, 1955).

Two types of folding occur. In one, an infolding of the basal plasma membrane is observed, so that the cytoplasm is split into a number of basal processes. These processes contain most of the mitochondria which are elongated and lie in single rows. In the other, the predominant feature is an extensive plication of the lateral plasma membrane.

The apical plasma membrane of intralobular duct cells forms a few small microvilli and apical protusions or "blebs" have occasionally also been observed. The cytoplasm in the apical region of these cells contains a few scattered RER cisternae, some

mitochondria and a few lysosomes. Golgi complexes can be quite numerous. Vescicles of various diameters are commonly found in the apical cytoplasm.

Another type of intralobular duct besides striated ducts exists in certain species, notably rats and mice. This second type of intralobular duct cell contains large, eosinophilic granules, the morphology of which has been described by several authors (Tamarin and Sreebny, 1965, Radley, 1969, Dorey and Bhoola, 1972, Cutler and Chaudhry, 1973). No "granular" ducts have been reported in any of the bovine salivary glands.

As a general rule, striated intralobar ducts are most conspicuous in the parotid, next the mandibular and least so in the sublingual gland, (Leeson, 1967). Intralobular ducts within the minor buccal glands are either absent or poorly developed. In general, serous or mucoserous glands have well developed striated ducts by comparison with glands that produce predominantly mucus.

1.4.5 EXCRETORY DUCTS

The intralobular ducts drain into a number of orders of drainage vessels. The exact number of these varies between glands but at the most includes :

1. Interlobular ducts
2. Interlobar
3. Excretory or main duct.

In monostomatic glands, these excretory ducts unite to form a single main excretory duct. The interlobular ducts are lined by either simple columnar or striated cuboidal epithelium while interlobar ducts maybe lined by either stratified cuboidal

epithelum (several layers) or stratified columnar epithelium of two cell layers. Typically the main excretory duct is lined by either stratified columnar or pseudo stratified columnar epithelium although in this and the other drainage vessels occasional goblet cells are present. A short distance before the excretory duct opens into the buccal cavity, the duct epithelium changes to stratified squamous which becomes continuous with that of the buccal cavity.

1.5 THE COMPOSITION OF SALIVA - THE SECRETION OF WATER AND ELECTROLYTES

It has been estimated (Bailey, 1961, Kay, 1966) that up to 90% of all fluid which enters the reticulo-rumen is derived from salivary secretions. In ruminants, an important function of saliva is the regulation of volume and consistancy of the rumen contents. The volume of rumen fluid and particle size influences the outflow through the omasum and thus affects the rate at which undigested residues are removed from the reticulo-rumen (Church, 1976). Since the level and consistancy of fluid within the reticulo-rumen affects appetite and insufficient fluid volume causes rumination to cease it can be appreciated that an adequate volume of saliva is essential for the existence of the animal (Wilson, 1964).

A characteristic feature of salivary secretions in ruminants is the large volume produced relative to that of monogastric species. Somers (1959) has reviewed the measurements of salivary output by various ruminants. A large percentage of the saliva is supplied by the parotid glands which secrete rapidly and continuously while the mandibular glands secrete little except during feeding periods (Kay, 1960). In addition, a considerable quantity of saliva is

secreted continuously by the small glands of the mouth and the sublingual gland (Phillipson and Reid, 1958, Kay and Phillipson, 1959, Kay, 1960). Consequently, large amounts of water are secreted by the salivary glands, particularly the parotids. Bailey and Balch (1961) estimate the total volume of saliva secreted by cattle to be 98-190 litres per day.

An early suggestion that saliva is formed by ultrafiltration of blood plasma subsequently modified by the glands duct system was denied by Ludwig (1851)* who showed that the secretory pressure in the mandibular gland of the cat could exceed the systolic arterial pressure. It is generally agreed that there is no evidence for active transfer of water in any mammal and that water movement is a secondary osmotic consequence of electrolyte transfer occuring across a system permeable to water. The ionic composition of bovine saliva is summarised in Table 1.1.

The formation of saliva can be divided into two parts :

(a) the primary secretion which is formed by the endpieces or acini

(b) the secondary secretion or duct modified saliva

The primary secretion is formed by an active ion transport mechanism since it is associated with a transmembrane potential difference. The secretion can be formed against a pressure greater than arterial pressure or even in the absence of circulation but active transport stops when sodium is removed from the perfusion fluid. Ion transport also ceases and the transmembrane potential difference can be abolished after ouabain application which inhibits

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*cited by Leeson, (1967)

Constituents		Mixed Saliva	Parotid Saliva
Sodium	m.mol/L	161	157
Potassium	"	6.2	7.0
Chloride	"	7.1	7.4
Bicarbonate		126	127
Phosphate	"	26	23
Dry matter	%	1.02	1.05
Ash	%	0.89	0.91

Table 1.1 Mean composition of mixed and parotid saliva secreted during high rates of secretion in cows receiving adequate intakes of sodium chloride (Bailey and Balch, 1961).

sodium-potassium ATPase, the enzyme believed to be responsible for providing energy for active sodium transport (Young, Fromter, Schogel and Hamann, 1967).

The electrolyte composition of saliva differs considerably from that of blood plasma. An explanation is that an isotonic fluid with an electrolyte composition similar to that of blood plasma is produced by the secretory endpiece (primary secretion) and subsequently modified by absorption of electrolytes in excess of water during passage through the intralobular ducts (secondary secretion) (Yoshimura, 1967). The most direct evidence to confirm transfer processes occuring during passage down the duct system comes from micropuncture techniques and collection of luminal fluid from the ducts (Schlogel and Young, 1966). Changes in osmolarity, potassium, sodium and chloride ion concentration of the primary acinar secretion occurs between the intercalated ducts and the beginning of the main ducts, that is, the main transfer operations occur in the intralobular or striated ducts. No significant alteration occurs in the interlobular, interlobar or excretory ducts of an activity secreting gland, yet in the resting gland active transport may occur which alters the sodium-potassium ratio of the saliva. While this has been demonstrated in rat mandibular glands by Schlogel and Young (1966) it seems reasonable to extend these findings to other glands and species.

From the work of Yoshimura (1967) it appears that the formation of the primary secretion by the secretory endpiece is mainly due to the so-called cell 'drinking' mechanism or pinocytosis. Pinocytotic vesicles formed by infolding of the external basal

plasmalemma of the endpiece cell are transferred across the cell and emptied into the intercellular spaces or acinar lumen. The extracellular fluid thus transported through the cytoplasm may, in transit, dissolve or carry with it protein or potassium ions which are present in high concentration within cells. During periods of active secretion by the gland the protein and potassium ion concentration within the endpiece cells decrease as the rate of salivary flow increases; these cellular components are diluted by extracellular fluid passing through them. Sodium is probably transported through endpiece cells from the naturally high levels found in extracellular fluid. The mechanism of pinocytosis requires the productions of new cell membrane, an energy consuming process, the energy for which is supplied by ATP. The presence of strong ATPase activity has been clearly demonstrated histochemically along the basal and apical plasmalemma of the acinar cells (Yoshimura, 1967).

The histological structure of the glands seem to correlate with their ability to vary ionic composition. Secretions from those glands with a well developed striated duct system show a marked variation in ionic composition in relation to the sodium status of the animal. Although sodium is secreted by the secretory endpiece cells of sodium depleted animals the ion is reabsorbed from the primary secretion during its passage through the striated duct system (Kay, 1960). Miyoshi (1963) verified the role of the striated duct system in the mandibular gland of the dog by clamping the duct opening and stimulating the chorda tympani nerve for one

minute. In saliva which was within the duct system during stimulation sodium and chloride ion levels decreased and potassium ion concentration increased when compared with the control. A conclusion which can be drawn from this experiment is that sodium and chloride ions are reabsorbed and potassium ions are secreted within the duct system giving rise to duct modified saliva or secondary salivary secretion. Results from experiments conducted by Yoshimura, Okumura and Nishikawa (1958) support the view that bicarbonate in saliva originates from both acinus and intralobular duct. It would appear that the bicarbonate present in the acinar primary secretion originates from the extracellular fluid whereas the bicarbonate added in the striated duct as the secondary secretion is formed by the conversion of metabolically derived carbon dioxide to carbonic acid by carbonic anhydrase (Yoshimura et. al., 1958).

Urea, calcium and phosphate are probably added into the secondary secretion by the epithelial cells of the striated ducts. Seemingly, distinct loci or sites of selective permeability are present in the striated ducts which points to a considerable degree of specialisation and longitudinal organisation of the salivary gland striated ducts (Langley and Brown, 1960). The anions, bicarbonate and chloride enter the duct lumen at a site more distal than sodium and potassium. In the case of chloride, electroneutrality requires the entry of chloride ions into saliva be accompanied by an equivalent transfer of anions out of saliva. In most distal portions of the striated ducts it is probable that

there exists either a passive ion permeability in both directions across the epithelial cells or an anion exchange process (Burgen, 1967).

1.6 THE COMPOSITION OF SALIVA - THE SECRETION OF PROTEIN

Salivary proteins may be divided into three classes :

(a) Secretory proteins produced by the secretory cells of gland or proteins transferred from serum.

(b) Glycoproteins; protein conjugated with carbohydrate which forms a major component of mucus.

(c) Immunoglobulin; antibodies in saliva.

1.6.1 THE SECRETION OF NON-IMMUNOGLOBULIN PROTEINS

Saliva contains two types of protein :

(a) Serum proteins. Small amounts of blood plasma albumin, ceruloplasmin and lipoprotein are found in salivary secretions.

(b) Intrinsic secretory protein. Salivary amylase occurs in a number of species including man but is either absent or present in very samll amounts in ruminant saliva. Somers (1957) has briefly summarised the work of several authors whose observations appear to confirm the statement that salivary amylase activity is absent in ruminant species. Wegnes, Booth, Bohstedf and Hart (1940) observed that saliva from cattle does not contain any protease activity. More recent work by Ramsey (1962) has identified the formation of pregastric esterases and which Grosskopt (1965) called salivary lipase. Hamilton and Raven (1973) indicate that this enzyme preferentially acts on short chain triglycerides (typical of milk fat) containing butyric groups and releases butyric acid.

Its chief importance therefore is likely to be in the young suckling animal to aid in the digestion of a total colostrum or milk diet although a specific role of butyric acid in the mouth cannot be excluded.

1.6.2 FORMATION OF SALIVARY PROTEIN

As with other cell functions, the synthesis of particular proteins is controlled by information stored in the DNA molecule (Bloom and Fawcett, 1975). The order in which the bases (arginnine, cytosine, guanine and thyamine) appear on the DNA molecule prescribed the order in which amino acids are strung together to form a protein (polypeptide). The DNA code is transcribed onto a long, single-stranded molecule of messenger RNA which migrates from the nucleus and moves to the cytoplasm. The linkage of amino acids occurs at the sites of ribosomes where they are assembled in their proper sequences to form specific polypeptides by an intermediate molecule of transfer RNA. Ribosomes which produce protein for secretion must be attached to the cisternae of endoplasmic reticulum (ER) so that polypeptides, as they are synthesized, may be segregated within membranous vesicles to prevent them from mixing with those which the cell must retain. It is assumed that as the amino acids are linked to form polypeptide chains, the protein being synthesized is delivered through the membranous walls into the lumen of the cisternae of ER. Possibly there is a pore-like structure in the membrane through which the end of a newly forming macromolecule of protein could be pushed as rapidly as it was being lengthened by the ribosome (Ham, 1974).

Polypeptides formed in this way are transported within the cisternae of RER to a cytoplasmic site adjacent to the immature face of a Golgi complex. The way in which the products of RER reach the immature face of the Golgi stack is by means of transfer vesicles budding off the RER cisternae. Ribosomes are detached from the membrane as the transfer vesicles leave the RER and move to the immature face where they fuse with a Golgi saccule and empty their contents into its lumen. Three steps seem to be involved in the fusion and emptying process :

(a) The transfer vesicles outer lamellae of unit membrane fuses with the outer lamellae of the Golgi saccule. The fused lamellae become discontinuous at the point of fusion.

(b) The inner lamellae of the transfer vesicle and saccule fuse and then it too becomes discontinuous.

(c) The transfer vesicles contents are emptied into the Golgi saccule. The vesicles unit membrane thus becomes part of the membranous wall of the Golgi saccule and by this process, new membrane is supplied to the forming face.

Individual saccules of a Golgi stack are generally thin but those close to the mature face, particularly around the periphery become swollen and distended from within by their contents. The swollen portions of saccules bud off and assume the form of membranous globules which are called secretory vesicles or presecretory granules. They are filled with a protein containing fluid which becomes increasingly condensed. When the condensation process is complete the membrane bounded structures are termed

secretory granules or, if the protein exhibits enzymatic activity zymogen granules (Ham, 1974).

Before it was known that each zymogen granule was membrane bounded it was thought that their discharge through the cell's apex would be injurious to the cell membrane and that some cytoplasm was lost as the secretory granule broke free. Electron microscope studies by Jamieson and Palade (1967) have shown that the membrane surrounding a granule remains intact until it reaches and fuses with the apical cell membrane. Release takes place by the process of exocytosis. The membrane of the granule and the cell membrane remain fused around the site at which the rupture occurs so that the cytoplasm at all times is effectively sealed off from the secretory endpiece lumen and only the contents of the granule escape. The membrane which had surrounded the granule would thus provide additional membrane to the apical plasmalemma. Hence, the mechanism by which protein granules leave the apex of the cell does not cause a loss of cell membrane or cytoplasm but instead provides a constant supply of new membrane.

1.6.3 SECRETION OF GLYCOPROTEIN (MUCO-SUBSTANCE)

An important function of saliva is to keep the epithelial surfaces of the buccal cavity, tongue and oesophagus moist and thus protected against dessication and abrasion. The swallowed boli are also coated with mucus thus aiding deglutition and minimizing the possibility of mechanical damage of the epithelial linings from coarse components of the diet. These lubricant properties of saliva are largely due to glycoproteins

and mucopolysaccharides. It seems likely that the function of the lubricants in the saliva of ruminants extends beyond that in monogastrics to provide protection against abrasion of the reticulo-ruminal walls during strong sequential muscular contractions which both mix and triturate the chambers contents.

Evidence now available (Ham, 1974) indicates that most of the carbohydrate component of glycoproteins is being continually added to the protein formed by the RER during its transit through the Golgi saccules. Some sugar residues of the carbohydrate side chains of glycoproteins may already have been added soon after the polypeptides, synthesized at ribosomal sites are released into the ER cisternae. Usually N-acetylglucosamine is added first then mannose within the RER cisternae. Later in the Golgi apparatus galactose fucose and sialic acid are added which complete the carbohydrate side chains. These sequential additions are enzymatically controlled by sugar transferases which can be demonstrated to be abundant in the Golgi saccules. Sulphation of mucopolysaccharides also occurs in the Golgi apparatus.

Individual droplets of mucosubstance are formed by the budding off of small membrane bounded vesicles from the mature face of the Golgi stack. These mucigen droplets are stored within the cytoplasm and during nervous or chemical stimulation of the cell the contents are released through the apical cell membrane by exocytosis. Shortly after expulsion the cell soon resumes mucosynthesis which refills the apical cytoplasm with mucigen droplets (Ham, 1974).

1.6.4 IMMUNOGLOBULINS IN BOVINE SALIVARY SECRETIONS

The immunoglobulins of human secretions have been well characterised. It has been demonstrated that IgA is the most concentrated immunoglobulin found in external secretions (Tomasi and Bienenstock, 1968, Lamm, 1976). One molecule of secretory IgA consists of a dimer of serum IgA covalently bound to a protein specific for secretion; the secretory component (Tomasi, Tan, Solomon and Prendergast, 1965). Studies on the salivary and mammary secretions of the cow and sheep have established that there exists an immunoglobulin analogous to human secretory IgA (Lascelles and McDowell, 1974).

Immunoelectrophoretic studies on mandibular salivary secretion from several species including sheep and cattle were conducted by Hurlimann and Darling (1970) in which an anti-bovine gamma globulin and an anti-human IgA were used in parallel. The results indicated that the immunoglobulin revealed by the anti-human IgA corresponds in shape and mobility to that in bovine saliva. In the cow and sheep this fast migrating immunoglobulin synthesized by the mandibular glands cross-reacts with anti-human IgA. Such cross-reactivity is a good criterion for the existance of homology between immunoglobulins. Thus, according to Hurlimann and Darling (1970) it can be considered that the immunoglobulin from the mandibular glands of sheep, cattle and pigs is IgA. In addition, the IgA possesses antigenic determinants which do not exist on IgA synthesized by the lymph nodes (serum IgA). These antigenic determinants likely correspond to the secretory component (Hurliman and Darling, 1970).

IgA is the primary class of immunoglobulin synthesized by plasma cells of glands and mucous membranes exposed to the external environment. The polypeptide chains of the immunoglobulin molecule are synthesized by the ribosomes of the RER and the covalent assembly of the heavy and light chains takes place inside the cisternae. The Golgi apparatus packages the formed protein and secretory vescicles are released from the concave or upper surface of the Golgi stack. The secretory vescicles are released from the plasma cell by means of exocytosis (Bloom and Fawcett, 1975).

The attachment of carbohydrate occurs in stages (Lamm, 1976). the initial sugars e.g. mannose and N-acetyl glucosamine are incorporated while the polypeptide is still in the ribosome; additional sugars, galactose and N-acetyl galactosamine are added along the secretion pathway while the final sugars e.g. fucose and sialic and may be added during the process of exocytosis (Tomasi and Grey, 1972).

Molecules of IgA to be incorporated into secretory IgA must cross the basal lamina to enter the secretory endpiece cells, duct lining cells or the epithelial cells lining mucosal surfaces. The mechanism by which this transfer occurs is not understood nor is it known whether or not any selectivity is involved (Tomasi, 1976).

Plasma cells which synthesize IgA and are located in the connective tissue beneath epithelial surfaces of mucosal linings and within exocrine glands arise from the differentiation of B lymphocytes following antigenic stimulation (Fudenberg, Stites, Caldwell and Wells, 1976). Antigenic stimulation causes a number of cellular

transformations along the immunoblast series, large pyroninophilic cells, proplasmacyte, plasmacyte or plasma cell. During these developmental stages the cell forms the necessary cytoplasmic organelles for protein synthesis namely, abundant RER and well developed Golgi appartus. The mature plasma cell has a characteristic oval or elliptical shaped basophilic staining cytoplasm with an eccentrically placed nucleus in which the chromatin is clumped in a distinctive pattern around the nuclear membrane. It is postulated by Tomasi (1976) that the molecules migrate from their production site through the connective tissue, across the basal lamina and intercellular space and into the epithelial cell where molecules of IgA are coupled to the secretory component.

Secretory component is synthesized by the epithelial cells and can be demonstrated in the supranuclear Golgi zone of the cell. Lamm (1976) has listed three possible loci for assembly of the secretory IgA molecule :

(a) Extracellularly, either between the epithelial cells or in the lumen.

(b) At the apical plasma membrane of the epithelial lining cell.

(c) Within the interior of the epithelial cell.

It was reported by Lascelles and McDowell (1970) that IgA was difficult to detect in mixed saliva from sheep and they ventured the suggestion that this reflected the relative lack of significant local antigenic stimulation owing to the continuous and voluminous nature of the salivary flow. Watson and Lascelles (1971)

reported, however, that IgA was easily detected in non-parotid saliva from sheep and mixed saliva from cattle, though apparently absent from parotid saliva of sheep. From their results the conclusion could be drawn that the parotid gland in sheep does not secrete IgA, and that parotid saliva acts as a diluent of IgA secreted by the mandibular and sublingual glands. Histological study of the parotid glands from three ewes showed the virtual absence of lymphocyte-plasma cell series of cells using Alcian Blue and methyl green pyronin (Unna Pappenheim) stains, whereas cells of the lymphoid series and plasma cells were numerous in the interacinar areas of the mandibular gland. These findings were in agreement with the observation of Lee and Lascelles (1970) that the concentration of IgA specific cells in the parotid salivary gland of sheep was very low.

Other workers (Pahud and Mach, 1970, Mach and Pahud, 1971) have suggested that IgA is the predominant immunoglobulin in ruminant saliva. They state that IgA represents more than 85% of the total immunoglobulins present in saliva and that the levels of immunoglobulin are analogous to those found in human secretions by Tomasi and Bienenstock (1968). It should be noted however, that the saliva examined by these workers was collected directly from the buccal cavity and may have contained significant quantities of immunoglobulin derived from tissues other than the salivary glands.

An attempt to determine the relative importance of the IgA secretory system and the selective transport of IgG, into the secretion from sheep salivary glands was made by Watson and Lascelles,

(1973). This was accomplished by the collection of parotid saliva from sheep in which an indwelling cannula was inserted into a parotid duct. Non-parotid saliva was collected from the buccal cavity of the same sheep after ligation of the non-cannulated parotid duct. The levels of immunoglobulins and albumin in blood serum and in concentrated parotid and non-parotid saliva were measured using the reversed single radial immunodiffusion technique of Vaerman, Lebacq-Verheyden, Scolari and Heremans (1969). On the basis of the respective volume contributions for parotid and non-parotid saliva, the values obtained for immunoglobulin concentrations were computed to be similar to those reported by Mach and Pahud (1971) for sheep mixed saliva. The levels of all protein measured were much higher in non-parotid than in parotid saliva, with IgG, IgG_2, IgM and albumin each being 3.5 - 5.0 times higher in non-parotid saliva. A striking feature of the results of this study is the difference in the concentrations of IgA. It was found that in parotid saliva IgA comprised only 35% of the total immunoglobulin whereas IgA constituted 86% of the total immunoglobulin in non-parotid saliva.

The protective function of secretory IgA is likely not confined to the oral cavity epithelium, gingiva, tongue, teeth and oesophagus as it is in monogastric species. Since no secretory tissue exists in the rumen, reticulum or omasum, it would seem appropriate that the function of salivary secretory IgA in the buccal cavity would be extended into the first three chambers of the ruminant stomach. Secretory IgA antibodies have a viral

neutralising activity which can occur in the absence of complement fixation as well as the capacity to combine with oral bacteria in vivo. The exact mechanism by which these antibodies are protective is unknown. Lysis and killing of bacteria is generally thought to involve fixation of complement although there is ample evidence that IgA does not fix complement. IgA may be involved in opsonization: enhancement of the uptake and destruction of bacteria by phagocytic cells such as PMN neutrophils, and monocytes. Although parotid saliva promotes the ingestion of streptococci by leukocytes in vitro, there is no conclusive evidence that this activity is due to bacterial coating with salivary IgA (opsonization). However, IgA may function in limiting or inhibiting the adherence of bacteria to epithelial cell surfaces or, in the case of viruses, inhibit their entry into epithelial cells. The absorption or inactivation of nonviable antigens of potentially antigenic material which could be ingested may be limited by IgA (Tomasi and Grey, 1972).

1.7 SUMMARY

From the foregoing it can be appreciated that saliva is a complex secretion derived from several different glands located near the buccal cavity and pharynx but the function of which extends beyond the mouth and oesophagus into the fore-stomachs of the ruminant. The aim of this study is to investigate the histological features of the bovine glands which contribute to salivary secretion and to provide collective information regarding the histochemistry of the secretory cells involved. Possible relationships between gland structure, histochemistry, salivary composition and protective functions of salivary secretions will be discussed.

2. MATERIALS AND METHODS

2.1 ANIMALS

A total of twelve Jersey or Jersey x Ayrshire crosses were used in this study. The animals were numbered 1-12 and the group was subdivided according to body weight :

(a) Animals 1-6 were mature cows averaging 9 years of age with a mean live bodyweight of 429 kilograms.

(b) Animals 7-9 were immature cows with a mean live bodyweight of 300 kilograms.

(c) Animals 10-12 were mature steers with a mean live bodyweight of 500 kilograms.

Some additional information(Summarised in Table 2.1) was available for certain animals regarding susceptibility to bloat, % band 4 protein in salivary secretion and salivary duct cannulation. All animals were housed indoors and fed on a daily basis with either fresh grass or freshly cut lucerne. Water was available ad libitum until they were either anaesthetised using Pentobarb 500 (South Island Chemicals) or stunned with a captive bolt before exsanguination.

2.2 DISSECTION

The head was separated from the carcass at the level of the fourth or fifth cervical vertebrae taking care to ensure the upper oesophagus and epiglottis remained intact. The salivary glands on the right side of the head were then dissected in the following order :

Mandibular, parotid, sublingual, dorsal buccal, intermediate buccal

SUMMARY OF EXPERMENTAL ANIMALS

Table 2.1

Animal No.	Susceptibility to bloat	% B4 Protein in Saliva	Salivary Gland Duct Cannulation	Live Bodyweight Kg
1	High	24		425
2	Low	0		433
3	Low	0		465
4	High	14.8		403
5	Medium	5.6		425
6	Medium			425
7	Unknown		Left parotid and mandibular gland ducts cannulated for 23 days prior to slaughter cannulae ceased to function 5 days before death	280
8	Unknown		Left parotid and mandibular gland ducts cannulated for 21 days prior to slaughter mandibular cannulae ceased functioning 3 days prior to death. Parotid cannula was functioning when animal was slaughtered	330

Table 2.1 continued

Animal No.	Susceptibility to bloat	% B4 Protein in Saliva	Salivary Gland Duct Cannulation	Live Bodyweight Kg
9	Unknown	-	Left parotid and mandibular gland ducts cannulated for 21 days prior to slaughter Mandibular cannula was non functioning 9 days before slaughter. Parotid cannula still functioning at time of death.	288
10	Unknown	-	Parotid cannulation	500

and ventral buccal. The head was re-orientated on the dissection table and samples for histological examination were taken from the posterior tongue, epiglottis and soft palate. Glands from the left side were then dissected in the same order as for the right side.

2.3 SAMPLING AND WEIGHING

In a pilot study, involving one animal, samples were taken from ten different sites within each of the major salivary glands to assertain the uniformity of the glands histological appearance. On the basis of the results of this preliminary work it was decided that samples taken from about the middle of each gland could be regarded as representative of the histology of the entire gland.

As each gland was exposed samples for histological examination were taken immediately from about the middle of each gland weighed and placed in the appropriate fixative or quenched in iso-pentane cooled in liquid nitrogen. Following sampling the parotid, mandibular, sublingual and ventral buccal glands were dissected out entirely, stripped of adhering connective tissue and fat then weighed on a Mettler top loading balance (Watson, Victor Ltd). The weights of the previously taken histological samples were added to the entire gland weights and recorded.

2.4 FIXATION PROCEDURES

Four small samples, about 4 x 4 x 6mm were removed from each salivary gland with one sample being placed in each of the following fixatives :

(a) 10% neutral formol saline (Culling, 1974).

(b) Bouins fluid (Culling, 1974).

(c) Zenker formol (Culling, 1974).

The fourth sample was quenched in iso-pentane cooled in liquid nitrogen and stored in small plastic vials at -80°C. Tissues were left to fix in formol saline for 2-3 days. Bouins fluid fixation was complete within 24 hours when specimens were transferred into 70% alcohol. Tissue blocks were fixed in Zenker formol for 12-15 hours then washed in running tapwater for 6-8 hours before the commencement of paraffin wax processing.

2.5 PARAFFIN WAX PROCESSING AND SECTIONING

Following fixation and washing the tissues were loaded into a Shandon, Elliott automatic tissue processor (Watson, Victor Ltd). See Table 2.2 for processing schedule. Tissue blocks were embedded in 56°C melting point paraffin wax. Specimens were cut at 5-6um in thickness using a Reichert Sliding Microtome (Selby-Wilton Scientific Ltd). Sections were floated on warm water, picked up onto lightly albumenised 3" x 1" glass slides and air dried at 60°C overnight.

2.6 STAINING AND HISTOCHEMICAL METHODS

The following methods or combination of methods were applied to sections of salivary gland tissue :

1. Haematoxylin and Eosin (H&E)
2. Alcian blue (AB)
3. Toluidine blue
4. Alcian yellow (AY)
5. Periodic acid Schiff
6. Alcian blue, Haematoxylin and Eosin (AB/H&E)

PARAFFIN WAX PROCESSING SCHEDULE

Table 2.2

PROCESS	REAGENT	TIME
Dehydration	70% ethyl alcohol	1 hour
	95% " "	1 hour
	100% " "	1 hour
	100% " "	1 hour
	100% " "	2 hours
Clearing	Chloroform	1 hour
	Xylene	1 hour
	Xylene	1 hour
Impregnation	Paraffin Wax 56^{o}C M.P.	2 hours
	Paraffin Wax 56^{o}C M.P.	2 hours

7. Alcian blue, Haematoxylin, Periodic acid Schiff (AB/H/PAS)
8. Alcian blue, Alcian yellow, Haematoxylin and Eosin (AB/AY/H&E)
9. Alcian blue, Alcian yellow, Haematoxylin, Periodic acid Schiff (AB/AY/H/PAS)
10. Alcian yellow, Haematoxylin, Periodic acid Schiff (AY/H/PAS)
11. Methyl green Pyronin (Unna Pappenheim)

Step by step description of the methods used may be found in appendix 3.

2.7 IMMUNOHISTOCHEMISTRY

Paraffin wax sections 5 µm in thickness were cut from tissue blocks fixed in Bouins fluid, floated on warm water and picked up onto clean glass slides.

Method for anti-bovine salivary protein band 4 antibody

1. Dewax sections and bring to water.
2. Inhibit endogenous peroxidase activity by treating sections in a freshly prepared solution of 0.3% hydrogen peroxide in absolute methanol for 30 minutes.
3. Wash slides in at least 3 changes of phosphate buffered saline (PBS) pH7.1 for 15 minutes.

Solution Preparation

Sodium Chloride	8.5 grams
Disodium hydrogen phosphate (anhyd.)	1.07 grams
Sodium dihydrogen phosphate ($2H_2O$)	0.039 grams
Distilled water	to 1 litre

4. React sections with (a) anti-bovine band 4 antibody and (b) control sections with the antibody absorbed by its own antigen in a moist chamber for 30 minutes.

Antigen and Antibody Preparation

The antigen, band 4 salivary protein was isolated and purified by Dr W.T. Jones, Applied Biochemistry Division, D.S.I.R. Palmerston North. Antibodies to Band 4 protein were raised in rabbits following the method published by Harboe and Ingild (1975) and prepared by Dr W.T. Jones. For use the antibody was diluted 1: 1600 in normal bovine serum albumin.

5. Wash well in 3 changes of PBS.
6. React with horseradish peroxidase conjugated goat anti-rabbit IgG (supplied by Miles-Yeda Ltd) in a moist chamber for 30 minutes.
7. Wash 3 times in PBS.
8. Incubate sections at 22^{0}C in diaminobenzidine solution for 10-30 minutes.

Solution Preparation

3.3 diaminobenzidine	75 mg
0.001% hydrogen peroxide in	
0.05M Tris buffer pH7.6	100 ml

9. Wash in 0.5M Tris buffer

Solution Preparation

0.2M Tris	25 ml
0.1M Hydrochloric acid	37.5 ml
Distilled water	to 100 ml

10. Wash in distilled water
11. Counterstain lightly with 1% light green for a few seconds.

12. Dehydrate, clear and mount in D.P.X.

2.8 MICROSCOPY AND PHOTOGRAPHIC EQUIPMENT

A minimum of nine slides were examined for each salivary gland (three differently fixed tissue blocks, three slides from each block). In most cases the section area examined under the microscope was approximately 16mm^2. The slides were examined with a Leitz Ortholux microscope (Medical Supplies (NZ) Ltd). Photomicrographs were taken on Agfachrome 50L film using a Leica 35 mm camera. The exposure times were measured and calibrated with a Micro-six L exposure meter (Medical Supplies (NZ) Ltd). Colour prints used in this thesis were reproduced from 35 mm transparencies by Kodak N.Z. Ltd.

3. RESULTS

3.1 SALIVARY GLAND WEIGHTS

The parotid, mandibular, sublingual and ventral buccal glands were weighed and the mean weight for each gland, total mean gland weight and the mean live bodyweight for each of the three groups of animals which have been segregated on the basis of sex, maturity and salivary gland cannulation are summarised in Table 3.1. Substantial variability between the weights of left and right salivary glands both within and between animals was apparent. Figure 3.1. demonstrates that the variability of gland weights between animals may be related to the variability of individual animal bodyweights. Except for animals with chronic cannulations of salivary ducts other treatments appear to have been without influence on gland weights. Figures 3.2 and 3.3 emphasize the variation that existed between left and right parotid and mandibular glands of individual animals. Only small differences were observed between left and right sublingual glands (Figure 3.4). The ventral buccal glands (Figure 3.5) followed no regular pattern in the non-cannulated animals; the right or left gland being the heavier of the pair. Of the four animals with parotid and mandibular duct cannulations three showed greater ventral buccal gland weights on the operated side (Figure 3.5).

3.2 HISTOLOGY AND HISTOCHEMISTRY

3.2.1 PAROTID GLAND

The bovine parotid gland was found to be composed of secretory endpieces which were long and narrow and did not conform to the

TABLE 3.1 SALIVARY GLAND WEIGHTS

	NON-CANNULATED MATURE COWS		CANNULATED IMMATURE COWS		MATURE STEERS	
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Body weight*	429.3	20.2	299.3	26.8	500	26.0
Left Parotid	120.9	19.0	71.0	4.6	123.1	3.8
Right Parotid	190.8	27.2	84.1	7.9	135	17.9
Left Mandibular	147.5	22.6	84.9	10.6	158.5	10.6
Right Mandibular	147.8	23.0	94.9	9.4	159.5	5.9
Left Sublingual	16.5	4.6	11.5	2.7	15.1	3.1
Right Sublingual	15.5	4.8	10.4	2.7	15.8	3.4
Left Ventral Buccal	21.7	3.8	18.8	2.2	24.1	4.0
Right Ventral Buccal	22.2	2.6	17.3	2.4	24.5	3.2
All left glands	306.7	42.0	186.3	6.4	320.9	12.2
All right glands	295.4	51.3	206.7	15.0	334.8	18.0
All paired glands	602.1	93.4	393.1	21.5	655.7	27.7

*The weight of all glands is expressed as g. Body weight is expressed as kg.

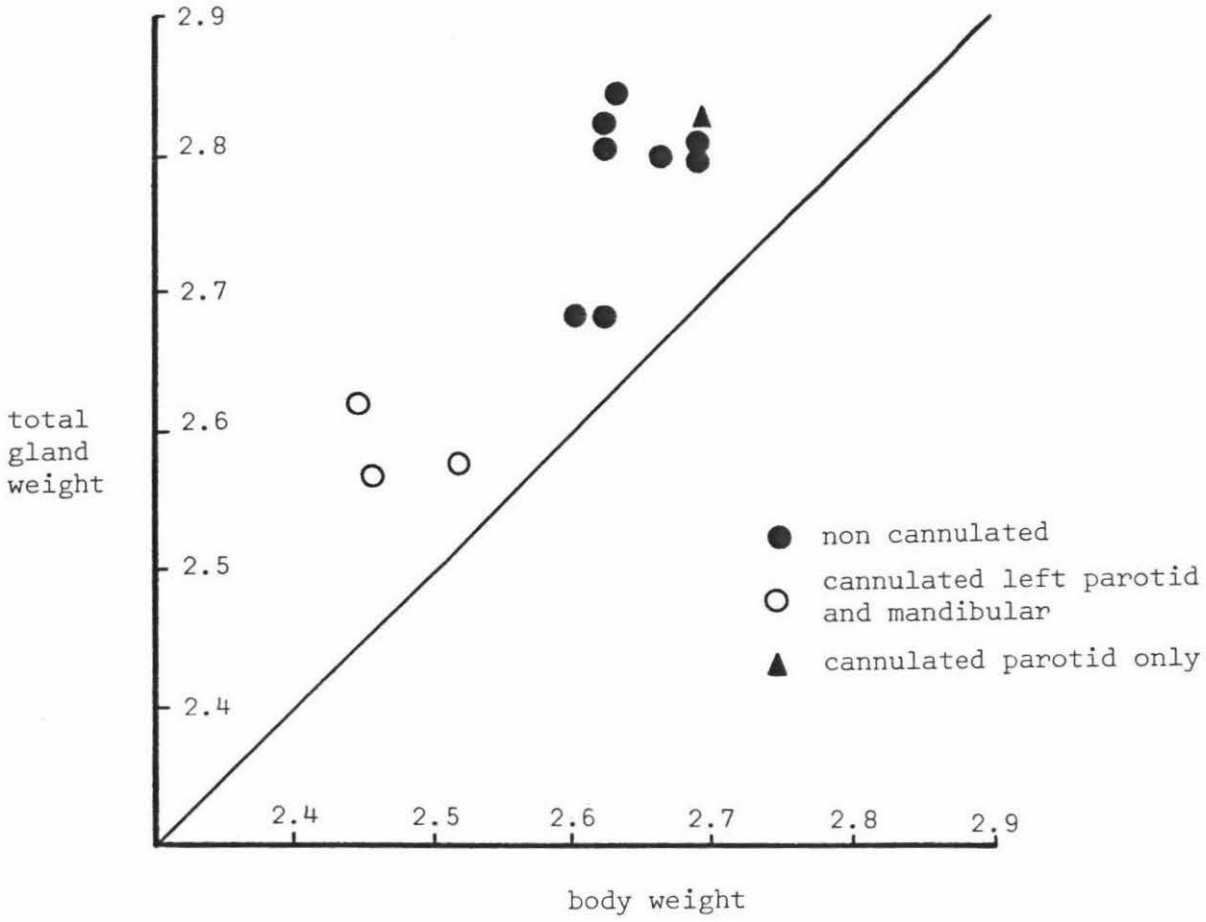


FIGURE 3.1

Allometric* diagram of total salivary gland weight (grams) and body weight (kilograms) expressed as logarithms.

*The term allometric is used in the manner defined by Huxley (1950). The straight line shown has a gradient of 1 and represents the isometric situation where the two glands have equal specific growth rates.

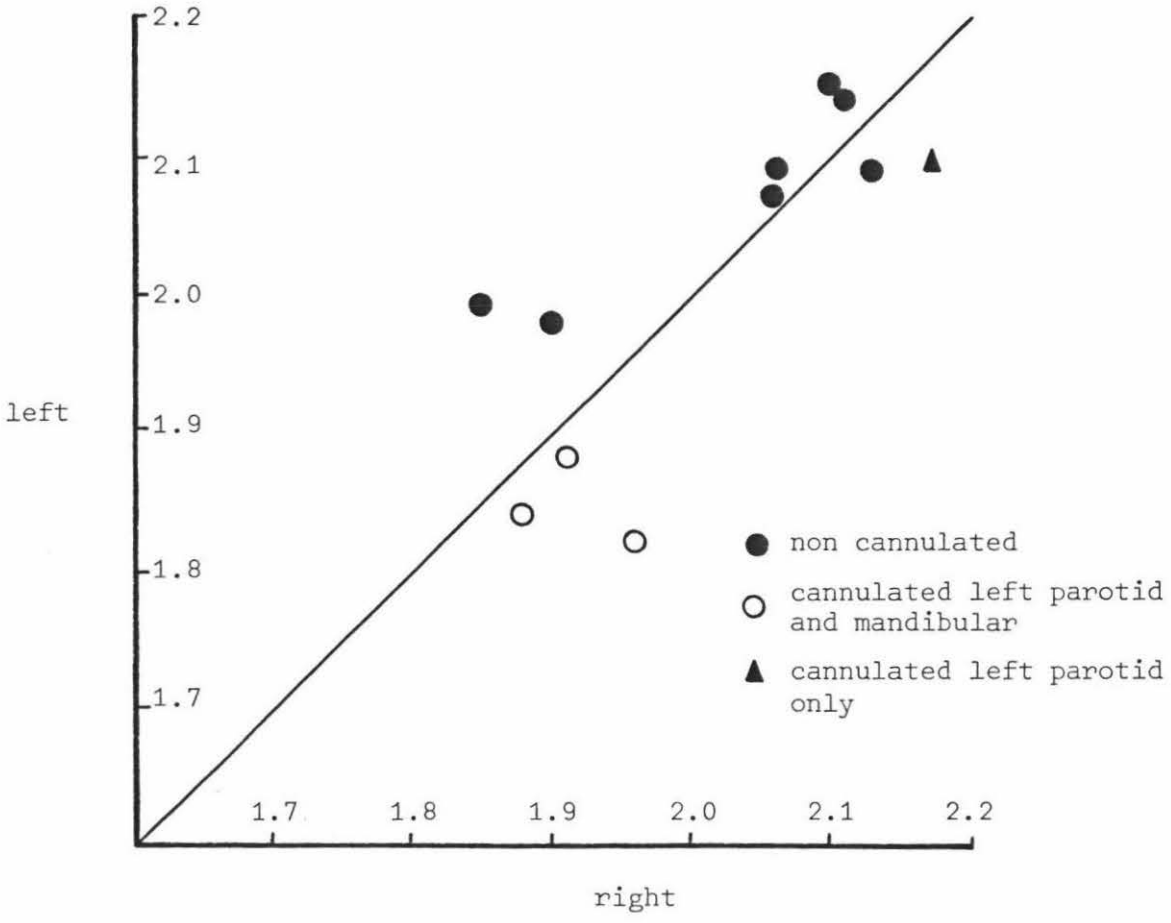


FIGURE 3.2

Allometric diagram of left and right parotid glands expressed as logarithms (grams).

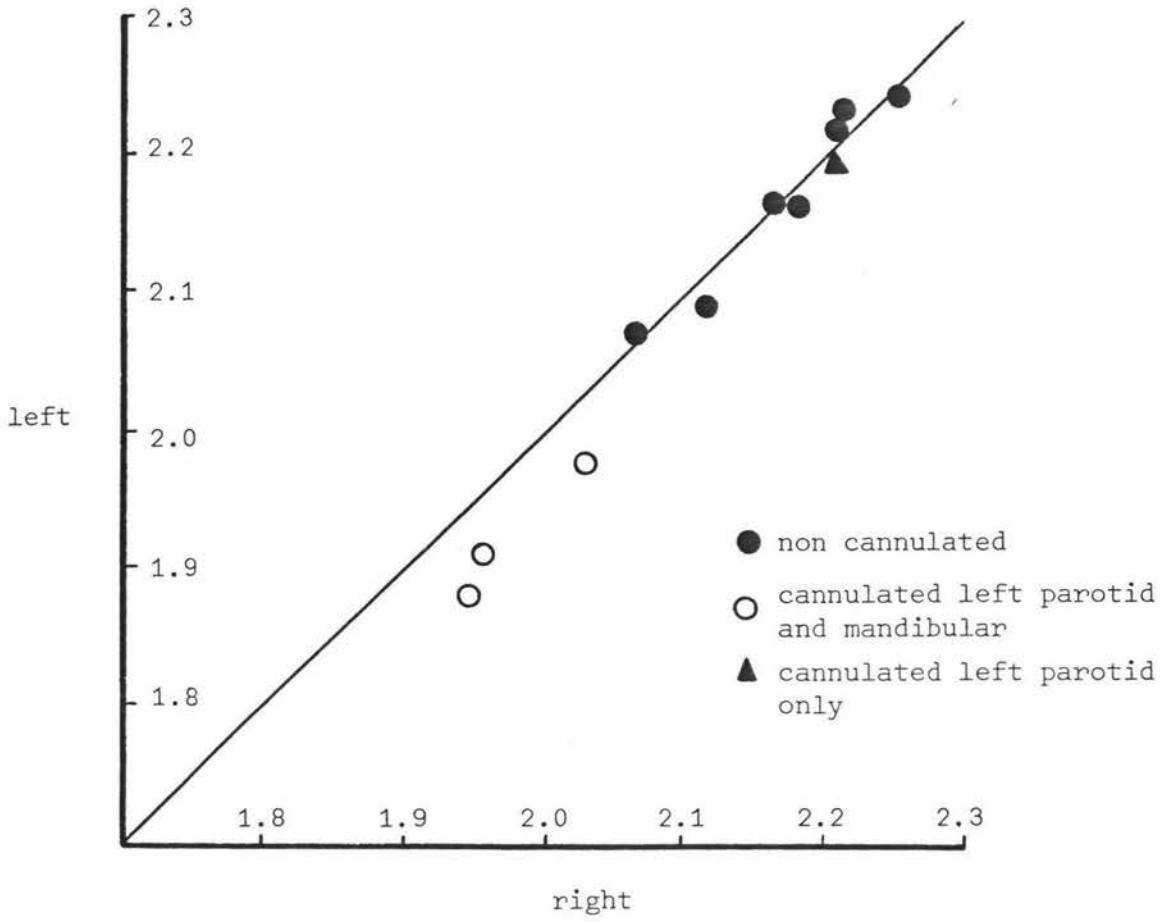


FIGURE 3.3

Allometric diagram of left and right mandibular glands expressed as logarithms (grams).

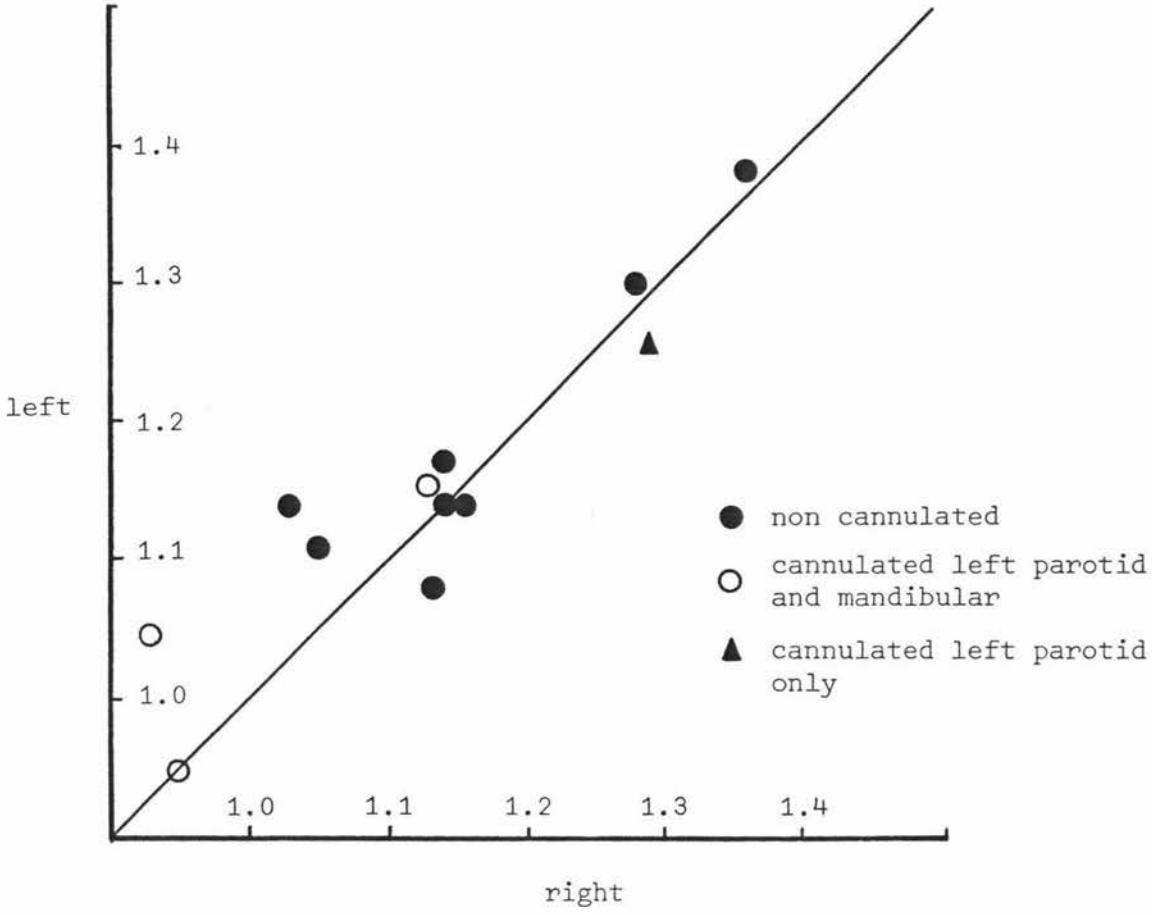


FIGURE 3.4

Allometric diagram of left and right sublingual glands expressed as logarithms (grams).

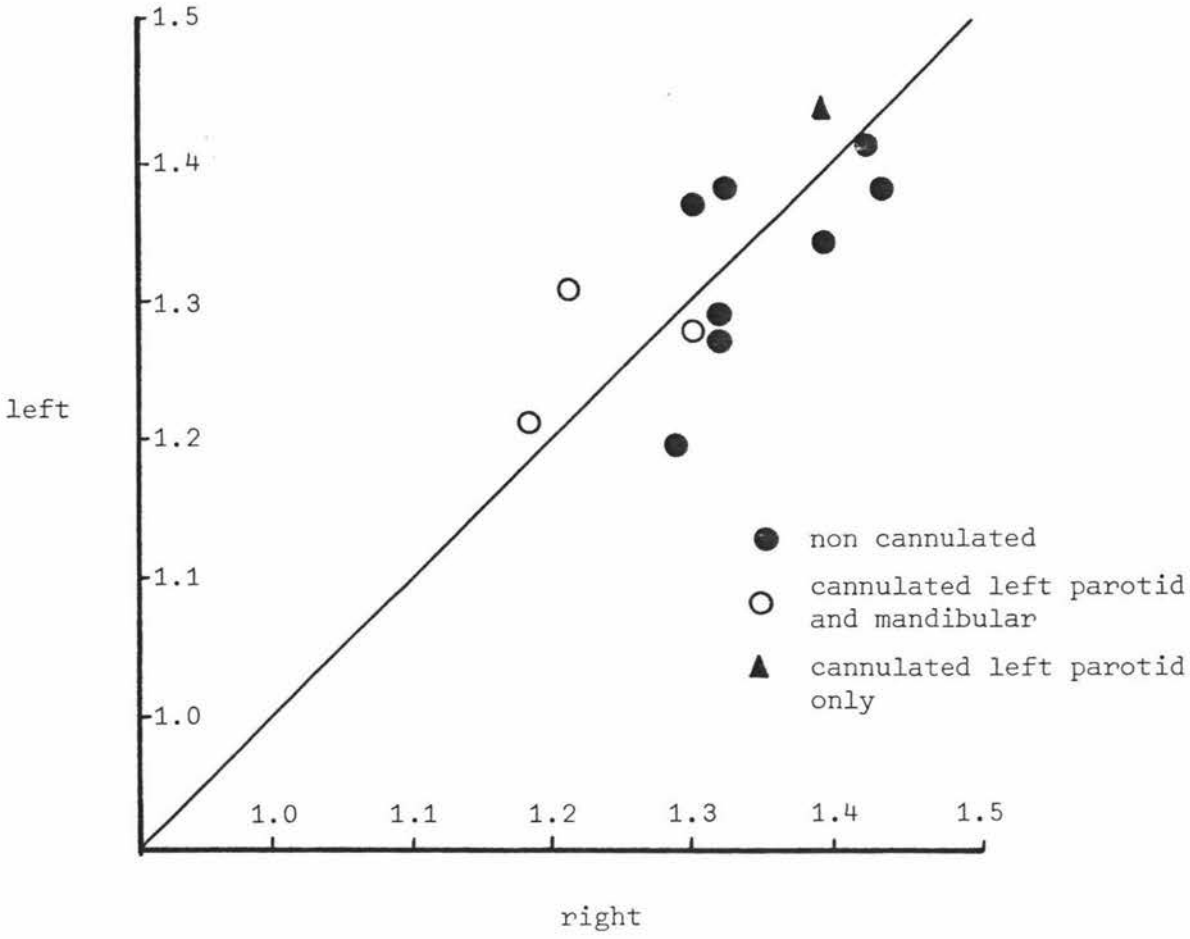


FIGURE 3.5

Allometric diagram of left and right ventral buccal glands expressed as logarithms (grams).

"classical" concept of a spherical acinus. The cells of the secretory endpieces were AB negative, PAS positive. The intensity of PAS staining varied between animals. No light microscopic evidence of cytoplasmic basophilia was observed in secretory endpiece cells stained with H&E nor did they react positively with methyl green pyronin. However, biochemical analysis of parotid saliva and immunohistochemistry has confirmed that the secretory cells of the parotid gland synthesize and release proteins (band 4) (Figure 3.6). The PAS positive granulation in secretory endpiece cells is likely evidence of band 5 which is a glycoprotein isolated from parotid saliva by gel electrophoresis (Figure 3.7).

The intercalated ducts were long and branched and in a single section as many as three intercalated ducts were seen to anastomose before joining an intralobular duct. The degree of development of intralobular ducts, judged by numbers of duct sections per unit area of glandular tissue, varied between left and right glands of some animals and also varied from animal to animal. The number of intralobular duct sections varied between 2 - 6 per $1.2mm^2$ of tissue section. No consistant correlation between numbers of duct sections and treatment could be found.

Epithelial cells lining intralobular ducts often contained PAS positive granules which were highly variable in intensity and concentration from one cell to the next. The plasmalemma at the luminal surface of these epithelial cells frequently has cytoplasmic protrusions resembling the "apical blebs" of an apocrine secretory mechanism* (Figure 3.8).

*Montagna and Parakkal, 1974

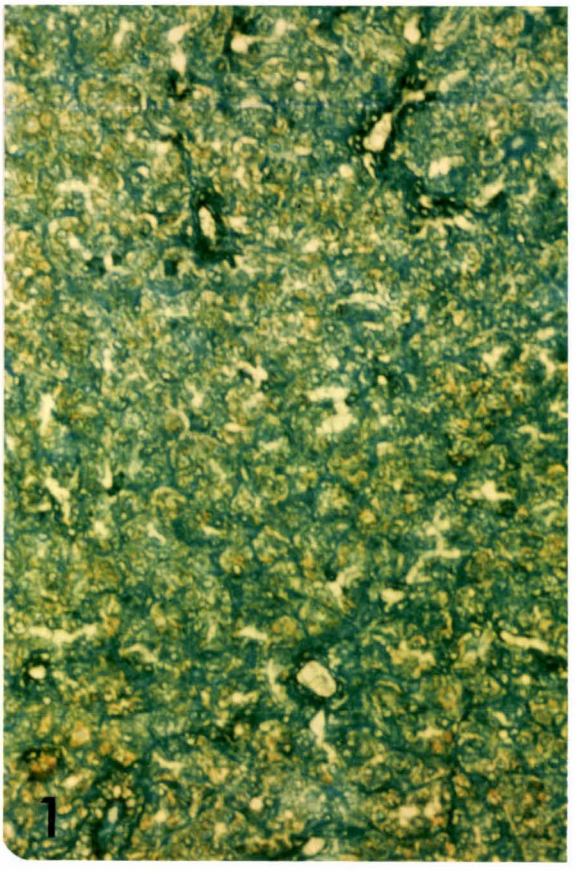
FIGURE 3.6

3.6.1 Parotid salivary gland. Anti-bovine salivary protein band 4 antibody control. Counterstained with light green. Magnification: X260.

3.6.2 Parotid salivary gland. Section reacted with anti-bovine salivary protein band 4. Light green counterstain. Magnification: X260.

3.6.3 Parotid salivary gland. Stain: PAS/H/Tartrazine. Note that PAS positive granules are not present in secretory endpiece cells. Red blood cells within capillaries are stained yellow. Magnification: X650.

3.6.4 Parotid salivary gland. Similar section reacted with anti-bovine salivary protein band 4 antibody. Only certain secretory endpiece cells react positively with the antibody. Magnification: X650.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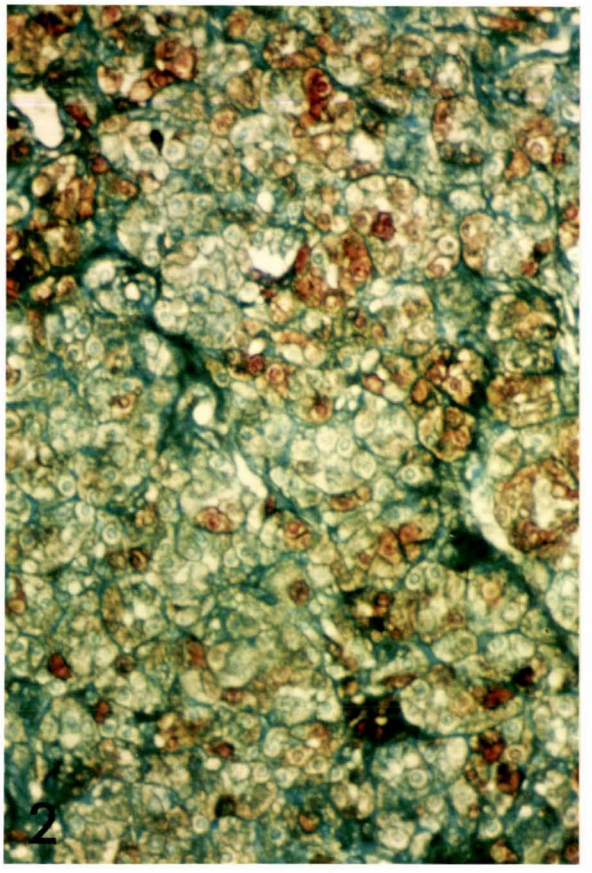
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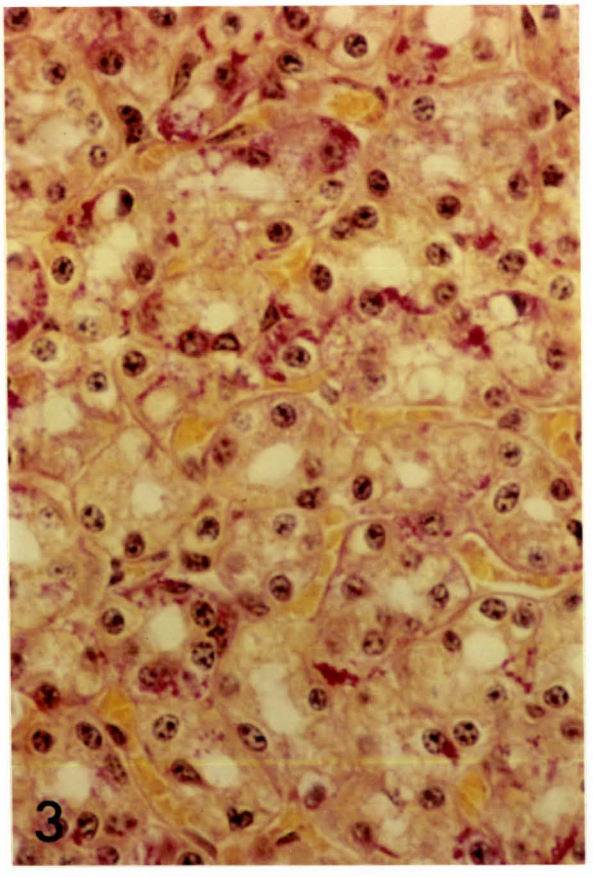
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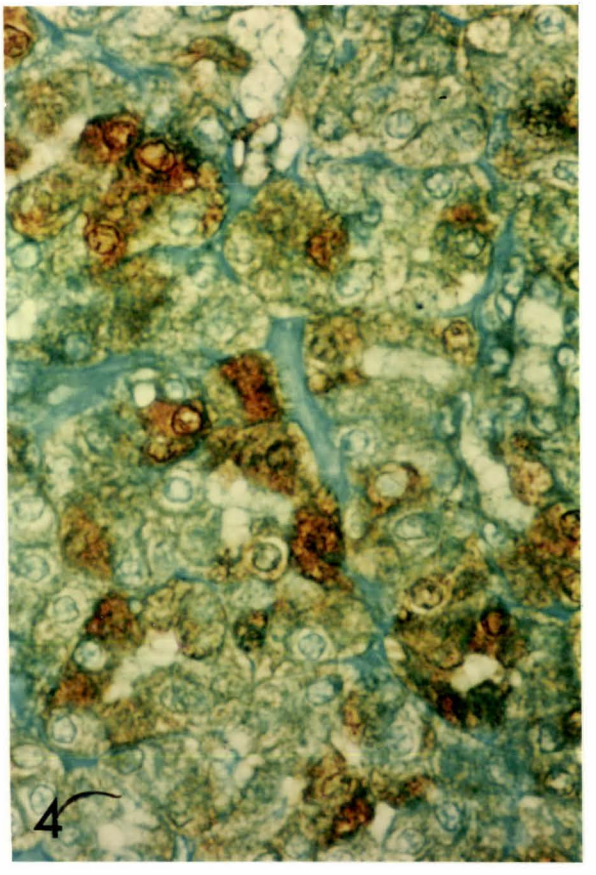
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FIGURE 3.7

Electrophoresis of bovine salivary protein on 7.5% polyacrylamide gel showing separation into numbered bands. Bands 1, 2, 3 and 5 contain a high proportion of carbohydrate.

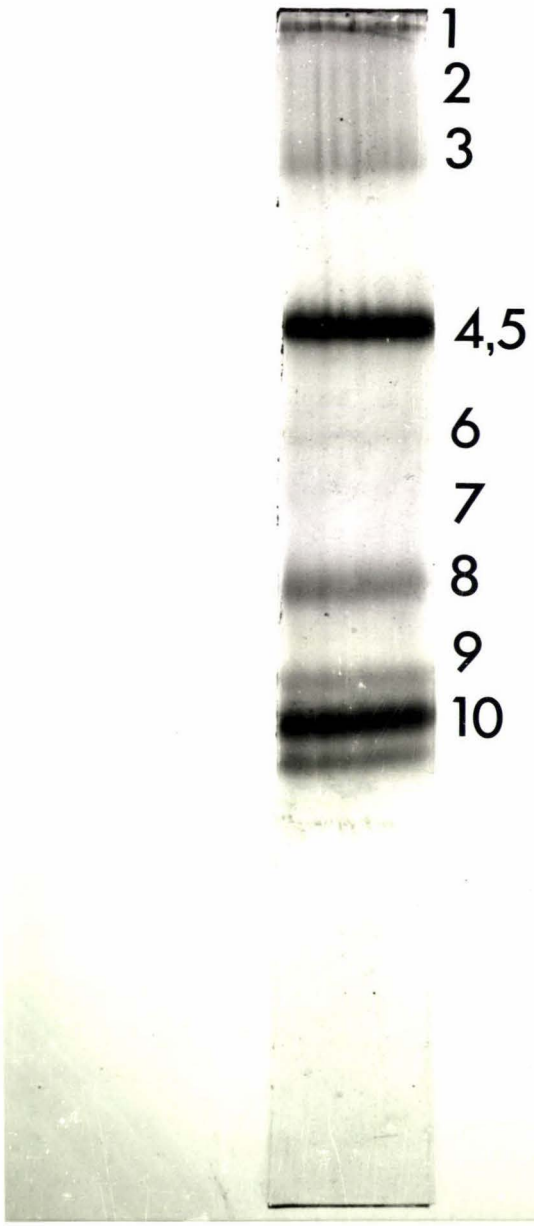
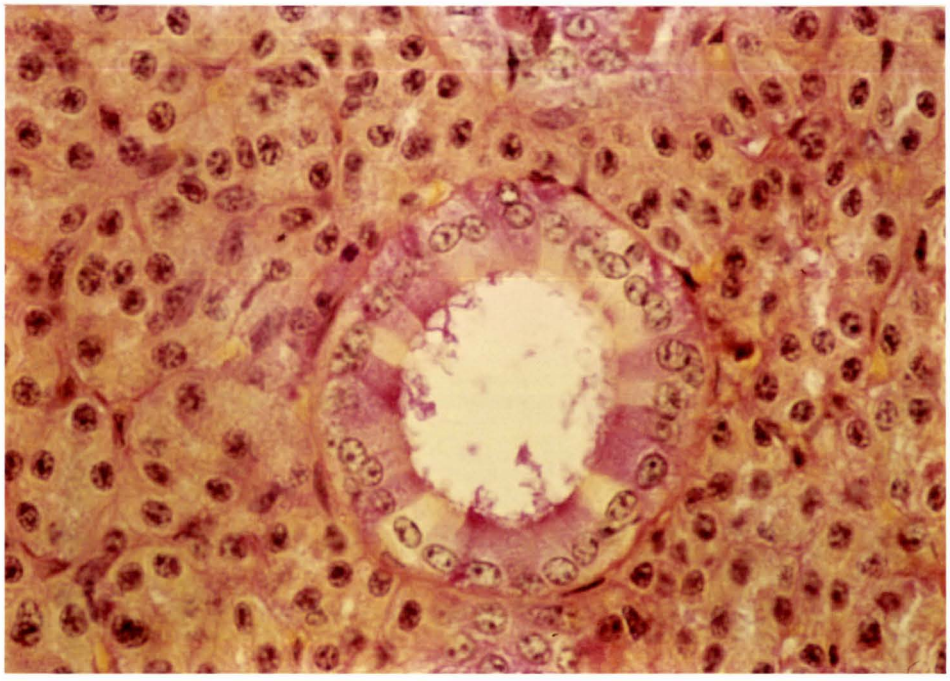
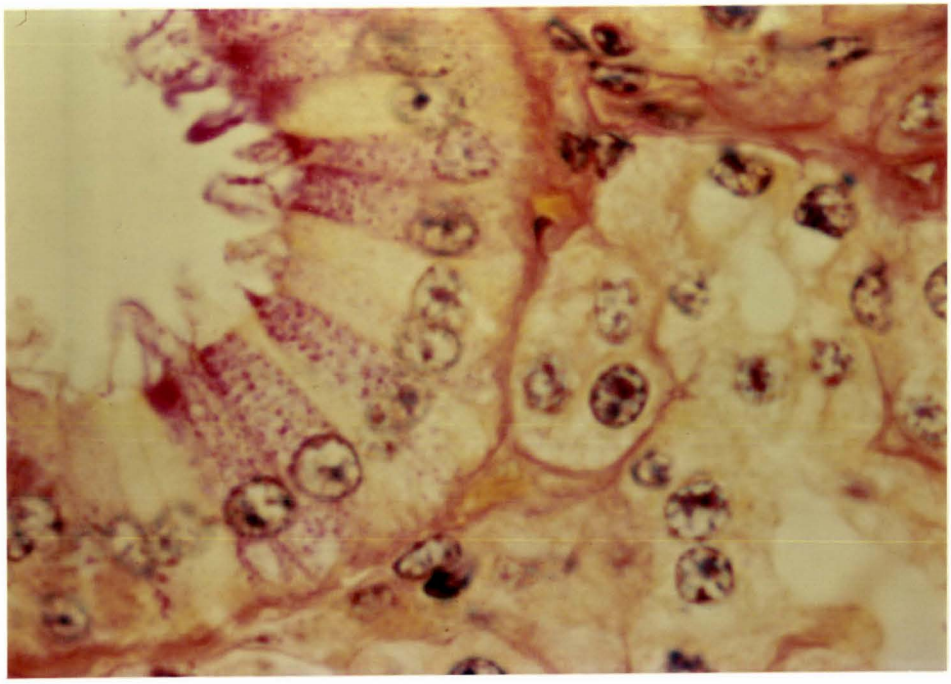

1
2
3
4,5
6
7
8
9
10

FIGURE 3.8

3.8.1 Intralobular duct of parotid gland. Stain PAS/H/ Tartrazine. The variable reactivity of these cells to PAS is characteristic of this gland and the ventral buccal. Magnification: X650.

3.8.2 Oil immersion photomicrograph of part of an intralobular duct. Stain: PAS/H/Tartrazine. Distinctive PAS positive granules occur particularly in the apical part of certain cells. The luminal surface shows "apical blebs" resembling an apocrine secretory mechanism. Magnification: X1650.





Basal striations were observed in the cuboidal cells of the intralobular ducts although variation was noted both between left and right glands from the same animal and between different animals.

Presumptive plasma cells of variable frequency between animals (Table 3.2), were observed within the basal lamina of intralobular ducts. These were round to oval in shape with a spherical nucleus in which the chromatin was sometimes clumped and sometimes more dispersed. Though the nuclei of these cell were occassionally centrally placed, the more usual site was eccentric. The cytoplasm contained granules which were eosinophilic, PAS and AB positive and strongly pyroninophilic (Figure 3.9). Some of the cells resembled plasma cells because of their shape, nuclear position and nuclear chromation pattern (clock-face) while others were more like mast cells particularly those cells reacting strongly to AB. Consequently, the morphological and histochemical features are most consistant with these intra-striated duct cells being considered, plasma cells. No other salivary gland, with the exception of the ventral buccal gland, examined in this study contained these cells within the intralobular duct epithelium.

Interlobular ducts, lined by stratified cuboidal epithelium, were surrounded by irregularly arranged fibrous connective tissue containing nerves and blood vessels. Interlobar and the main excretory ducts were also lined by stratified columnar epithelium but these had a high proportion of goblet cells which were PAS, AY and weakly AB positive (Figure 3.10).

Animal No.	Sex	Known Bloat Susceptibility	Frequency of intra-striated duct granular cells
1	F	HS	none
2	F	LS	abundant
3	F	LS	abundant
4	F	HS	very few
5	F	MS	some
6	F	MS	some
10	M	LS	abundant
11	M	HS	none
12	M	HS	none

Table 3.2 Apparent correlation between bloat susceptibility and the numbers of granular intra-striated duct cells found in bovine parotid and ventral buccal glands.

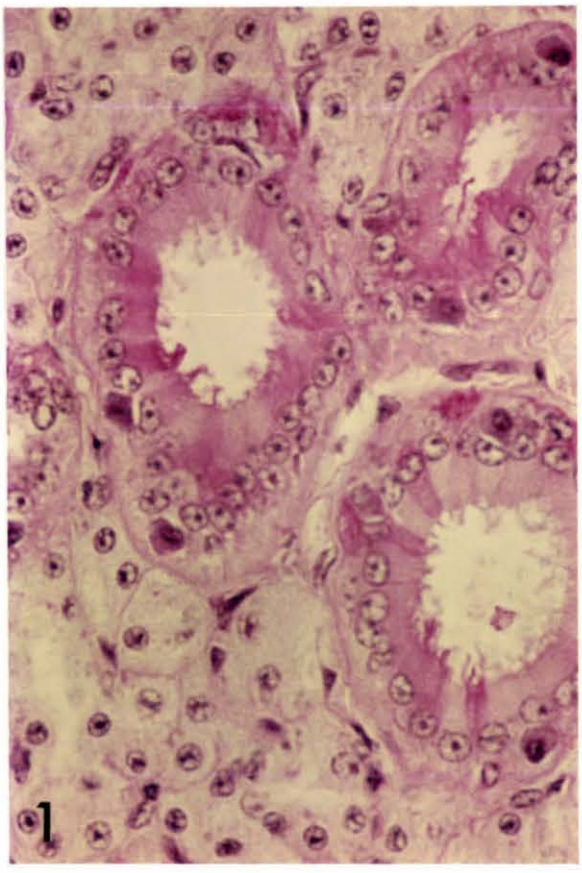
FIGURE 3.9

3.9.1 Intralobular ducts, parotid salivary gland. Stain: AB/H/PAS. The purple staining cells within the duct wall are intra-striated duct cells. Magnification: X650.

3.9.2 Intralobular ducts, parotid salivary gland. Stain: Methyl green pyronin. The intra-striated duct cells are strongly pyroninophilic (pink). Magnification: X650.

3.9.3 Intralobular ducts, parotid salivary gland. Stain: diastase digested PAS/H/Tartrazine. PAS staining of the duct cells is reduced (see Figure 3.9.1) in most cells. Notice the intra-striated duct cells. Magnification; X650.

3.9.4 High power photomicrograph of intralobular duct in Figure 3.9.3. This intra-striated duct cell displays all the features of a plasma cell. Magnification: X1650.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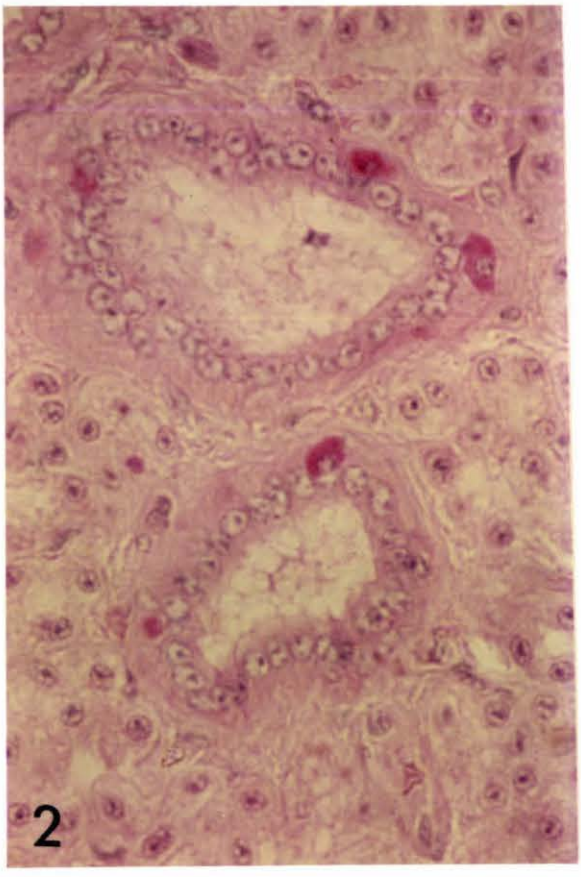
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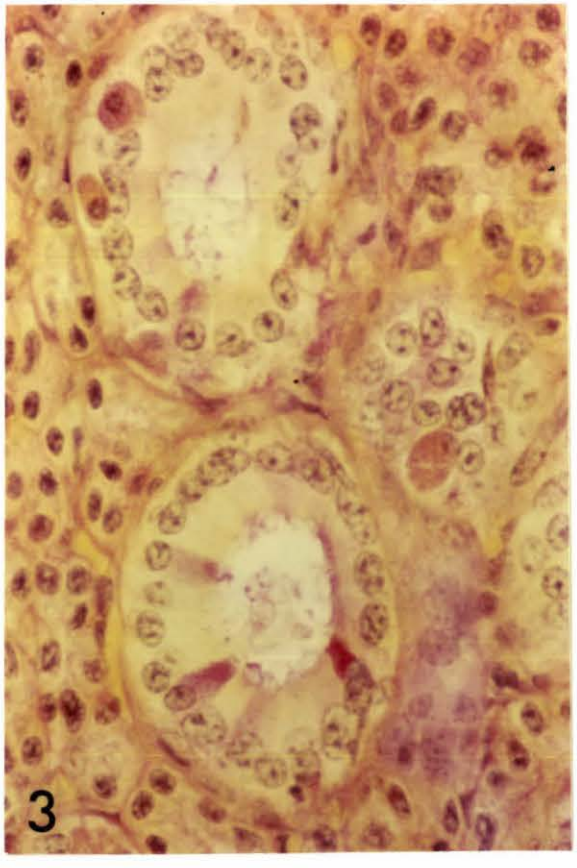
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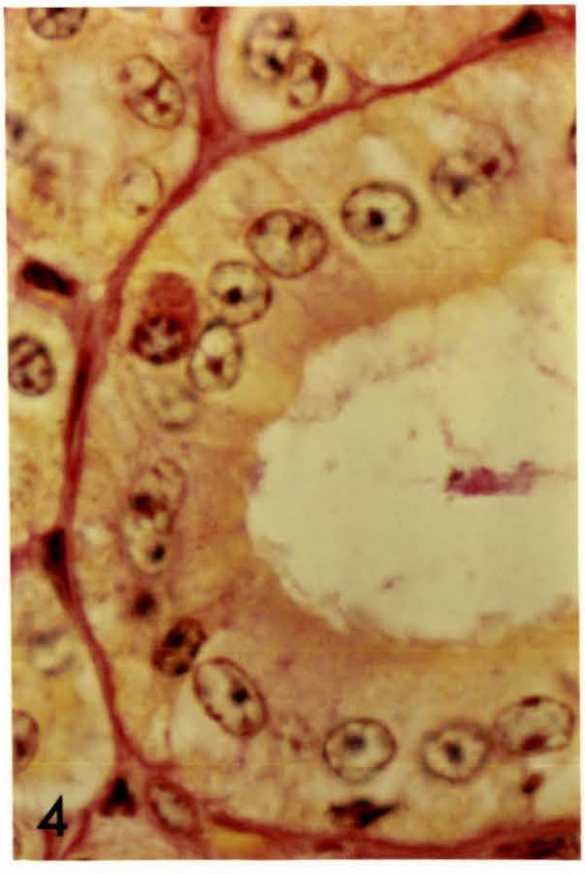
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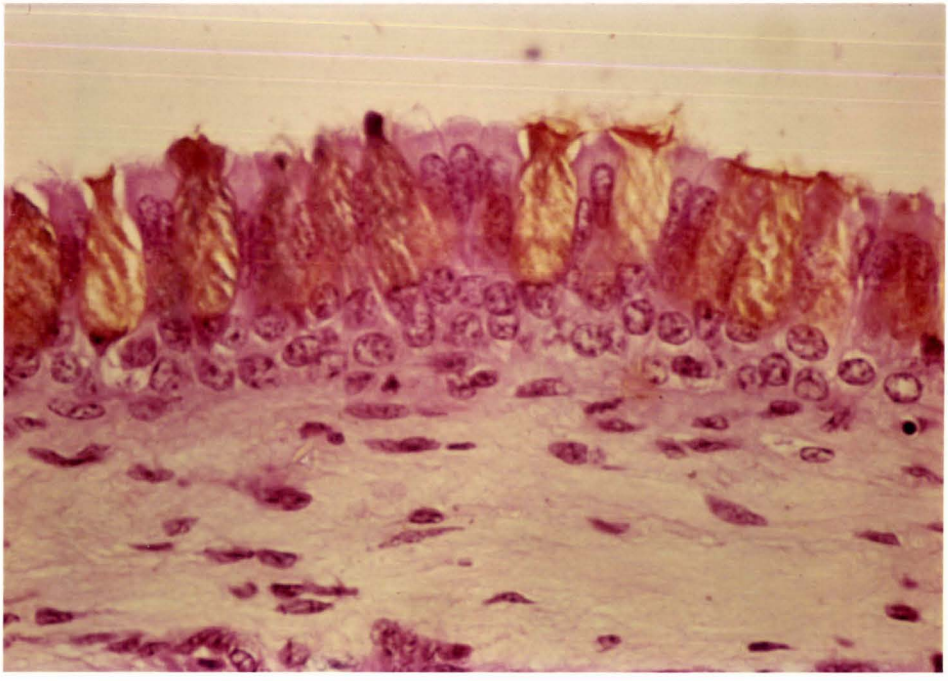
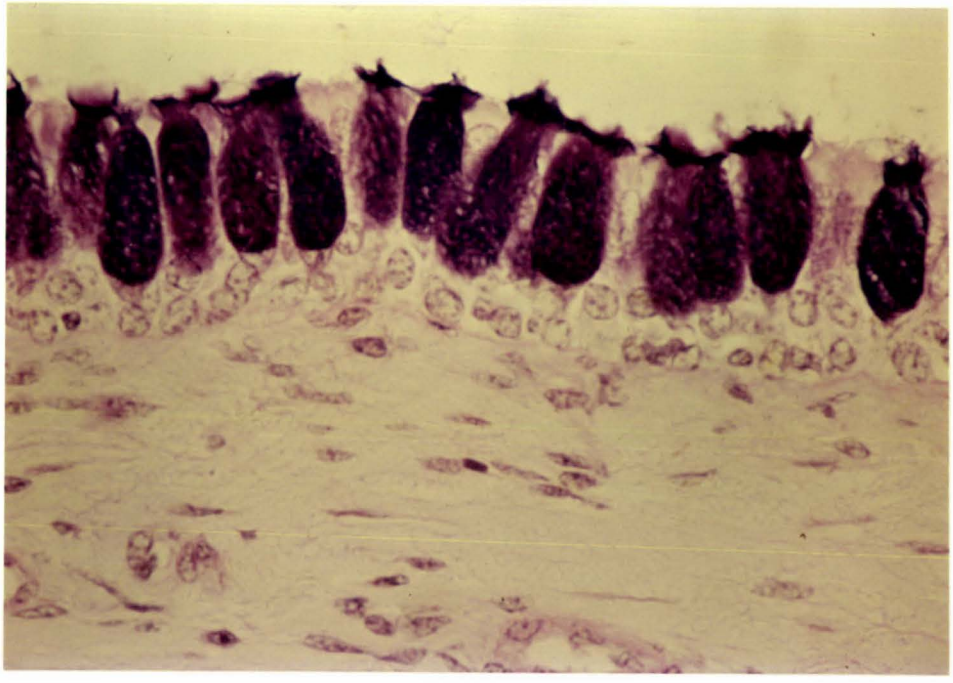
FIGURE 3.10

3.10.1 Excretory duct of the parotid salivary gland. This duct is lined by stratified columnar epithelium with goblet cells (stained yellow). Note the high concentration of goblet cells. Stain: AB/AY/H&E. Magnification: X650.

3.10.2 Corresponding section of excretory duct showing the reaction of the goblet cells to AB/H/PAS. The excretory ducts of the ventral buccal glands are lined by a similar epithelium. Magnification: X650.





3.2.2 MANDIBULAR GLAND

This gland contained secretory cells of two types arranged in endpieces which were elongated with a cap or demilune at their blind ends. The tubular endpiece was made up of secretory cells, each with a dark staining, flattened, basal nucleus and a "foamy" appearance of the cytoplasm (Figure 3.11). The cytoplasm reacted positively with toluidine blue, PAS, AY and weakly with AB (Figure 3.13). The demilune cells were large and rounded and contained a spherical nucleus which was positioned toward the base of the cell. the cytoplasm contained granules which were PAS and AB positive (Figure 3.12). When stained with H&E the granules were eosinophilic and perinuclear cytoplasmic basophilia was observed. The same basal regions of the cell were pyroninophilic.

The development and degree of basal striations of intralobular ducts varied between mandibular glands of the same animal and between glands from different animals. In general they were not as well developed as in the parotid or ventral buccal glands (Figure 3.11).

Plasma cells were found in large numbers in the intralobular connective tissue between secretory endpieces and around intralobular ducts. Unlike in the parotid gland, plasma cells were not found within the epithelium of the ducts (Figure 3.11 and 3.12).

The large ducts of the mandibular gland did not contain goblet cells in their walls (Figure 3.13). The epithelium consisted of stratified cuboidal which may have up to four or five cell layers.

3.3.3 SUBLINGUAL GLAND

The sublingual glands were highly lobulated and diffuse within

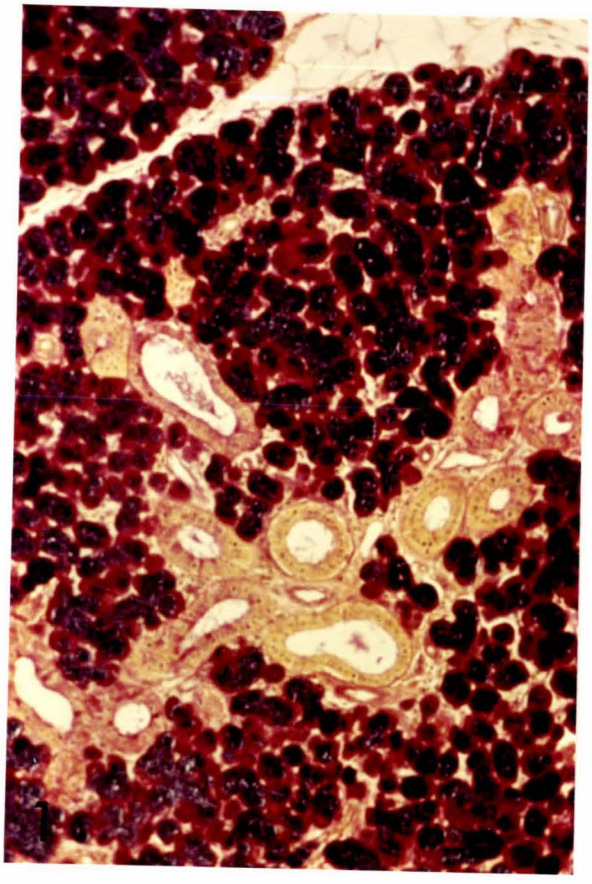
FIGURE 3.11

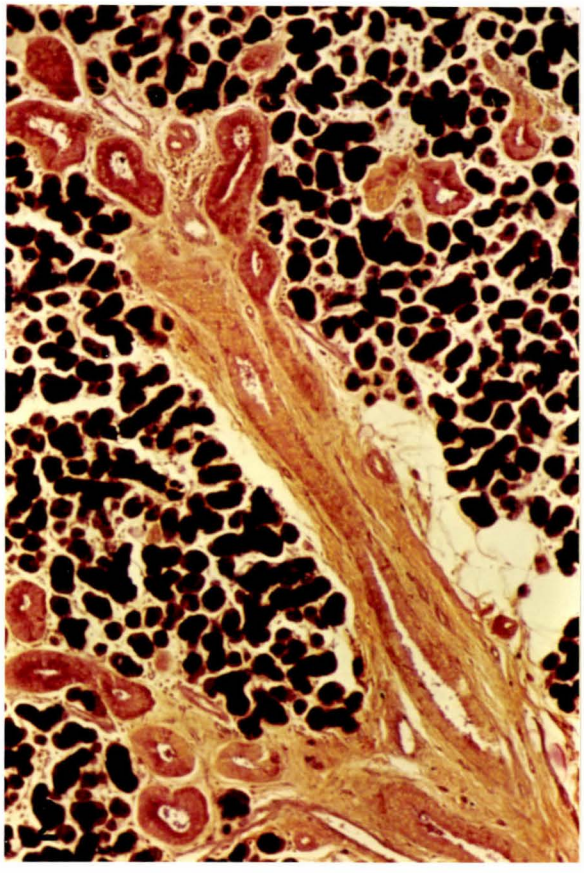
3.11.1 Low power photomicrograph showing part of one lobule of the mandibular gland. Stain: AB/PAS/H/Tartrazine. Magnification: X100.

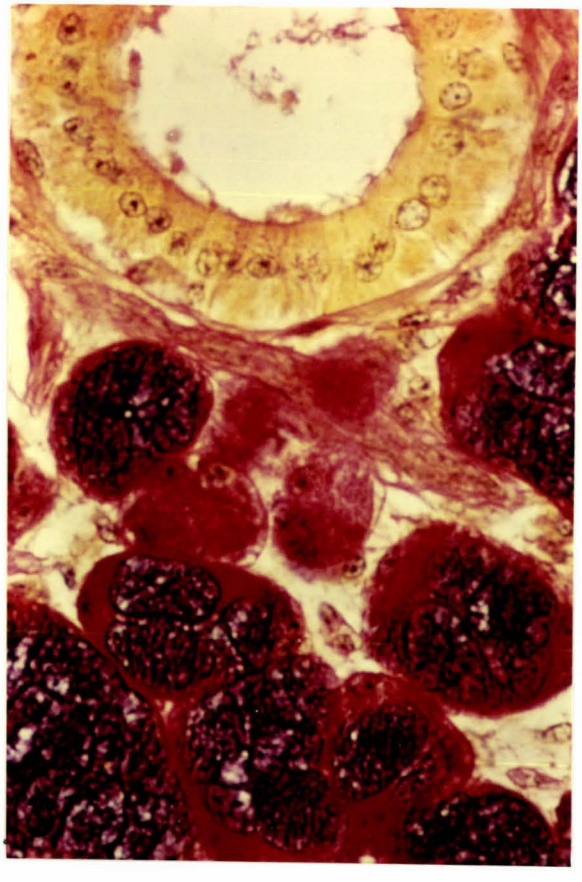
3.11.2 Mandibular salivary gland showing longitudinal relationship between intralobular and interlobular ducts. Magnigication: X100.

3.11.3 Higher magnification of Figure 3.11.1. Note the intense staining of the demilune cells with PAS. Magnification: X650.

3.11.4 Mandibular salivary gland. Stain: PAS/H/Tartrazine. The interlobular duct cells in this gland stain positively with PAS. No intra-striated duct cells were observed in this gland. Magnification: X650.







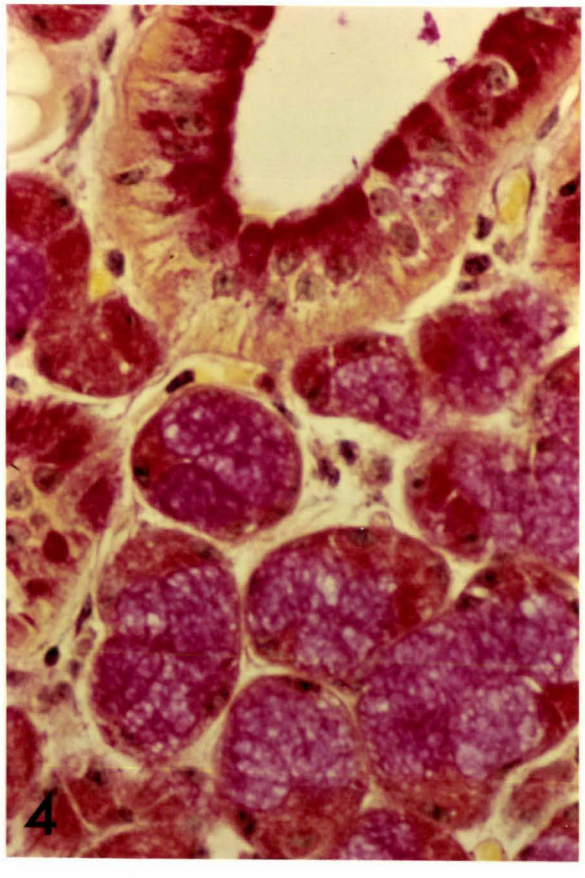

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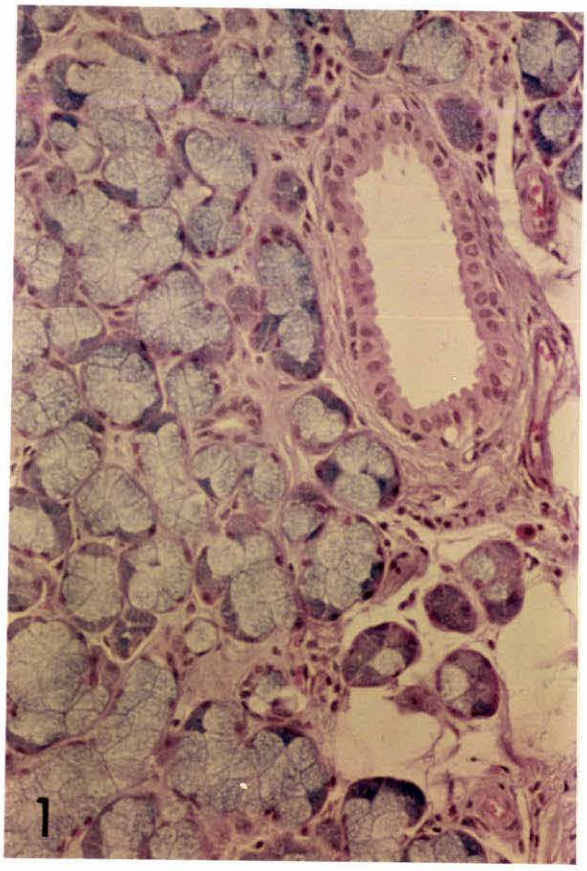
FIGURE 3.12

3.12.1 Mandibular salivary gland. Stain: AB/H&E. Magnification: X260.

3.12.2 Mandibular salivary gland. Stain: AB/H&E. Intralobular ducts show basal striations. Demilune cells contain AB positive granules. Magnification: X650.

3.13.3 Mandibular salivary gland. Oil immersion photomicrograph of part of an intralobular duct and secretory endpiece showing mucous and demilune cells. Stain: AB/H&E. Magnification: X1650.

3.12.4 Mandibular salivary gland. Stain: AB/PAS. The demilune cells react strongly to PAS. Mucous cells stain purple and show the characteristic 'foamy' appearance of the cytoplasm. Magnification: X650.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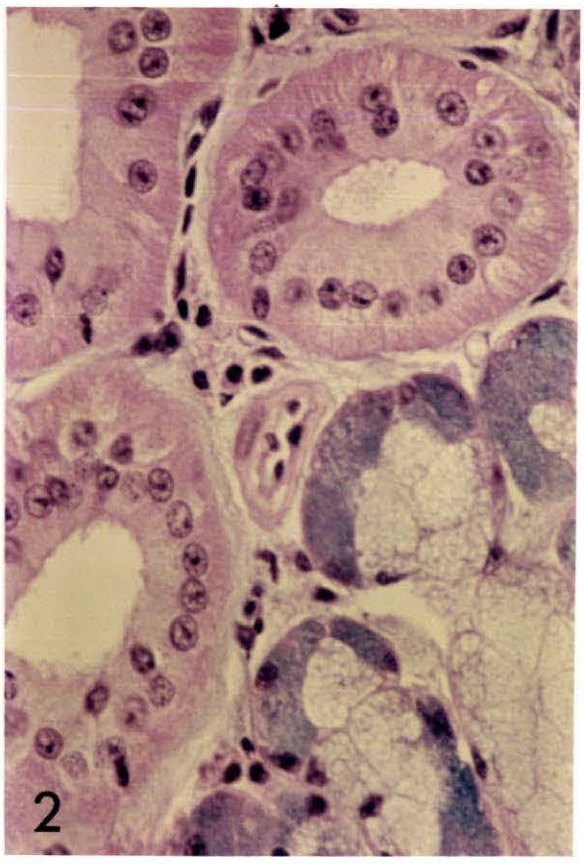
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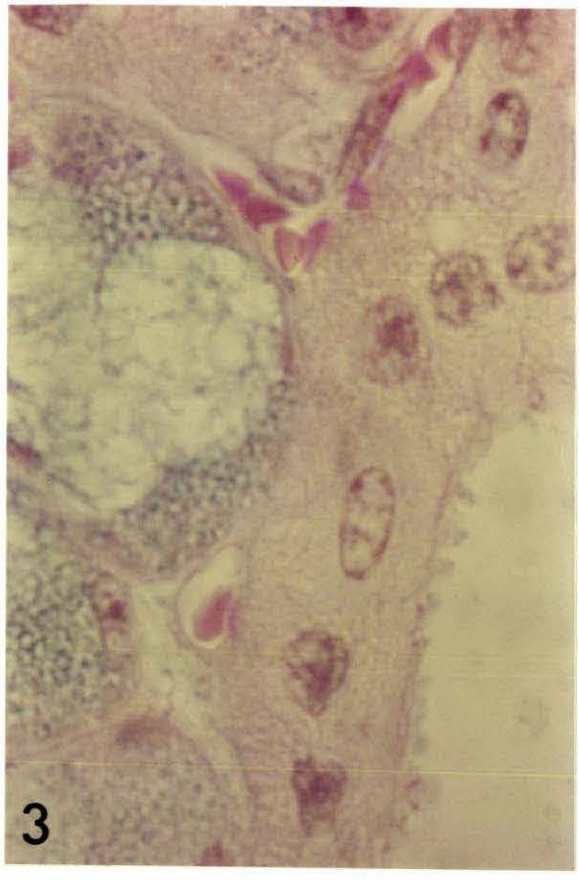
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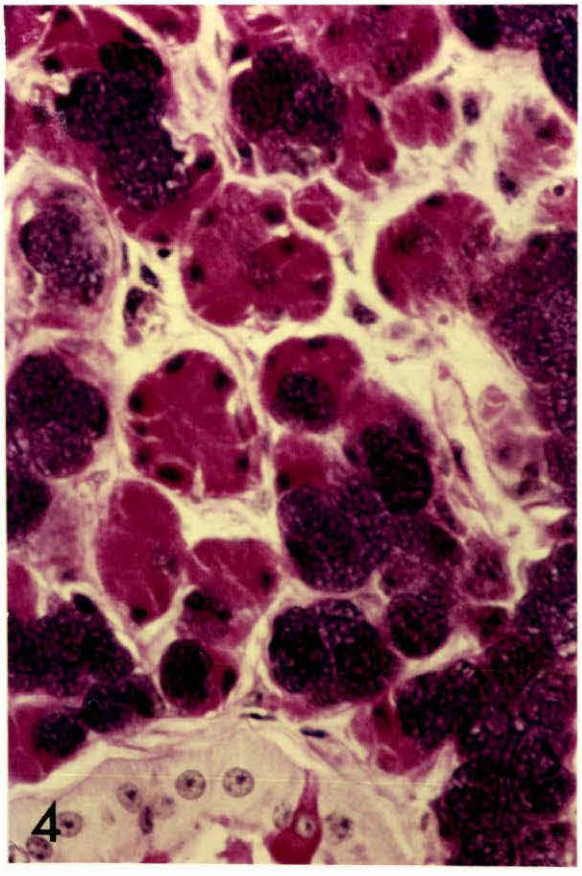
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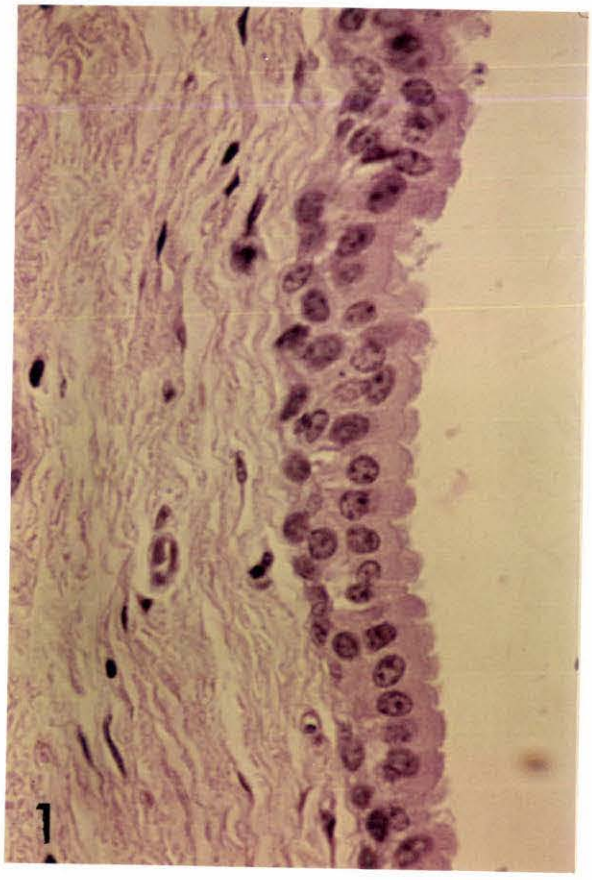
FIGURE 3.13

3.13.1 Mandibular excretory duct wall. The epithelium is stratified or pseudostratified columnar. Note the absence of goblet cells. Stain: AB/H/PAS. Magnification: X650.

3.13.2 Mandibular salivary gland. Intralobular duct and secretory endpieces. Stain: AB/AY/H&E. Magnification: X650.

3.13.3 Similar area to Figure 3.13.2. Stain: Methyl green pyronin. Plasma cell cytoplasm and the basal areas of the demilune cells are strongly pyroninophilic (dark pink). Magnification: X650.

3.13.4 Secretory endpiece cells and the wall of an interlobular duct. Stain: AB/H&E. Magnification: X650.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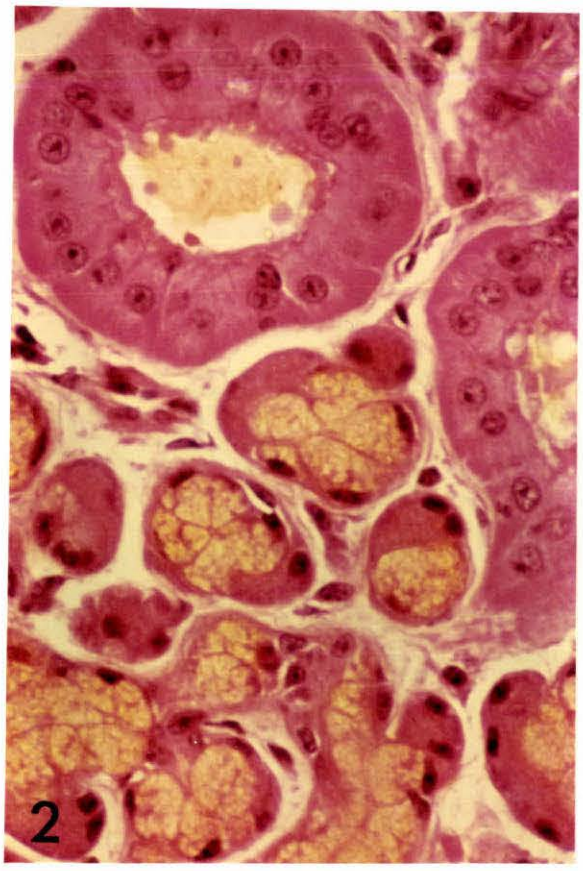
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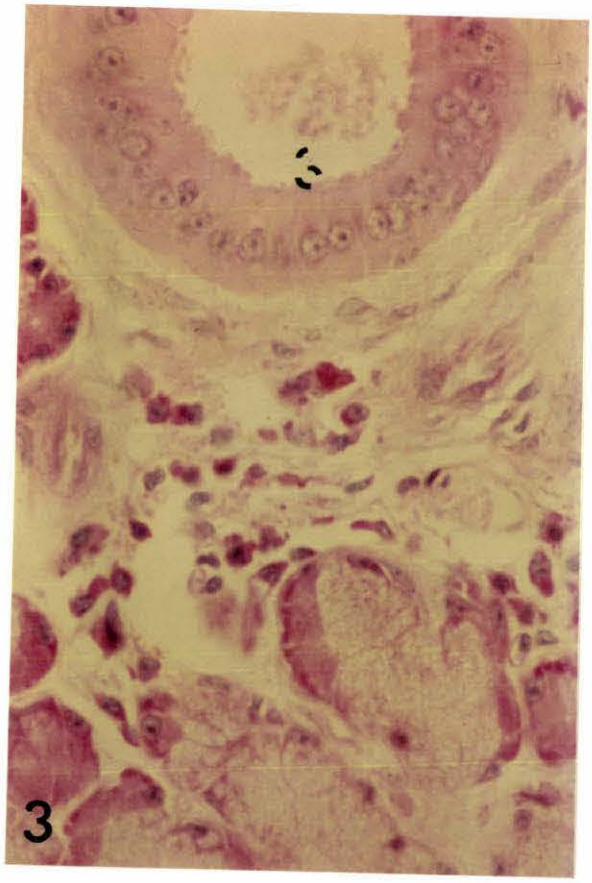
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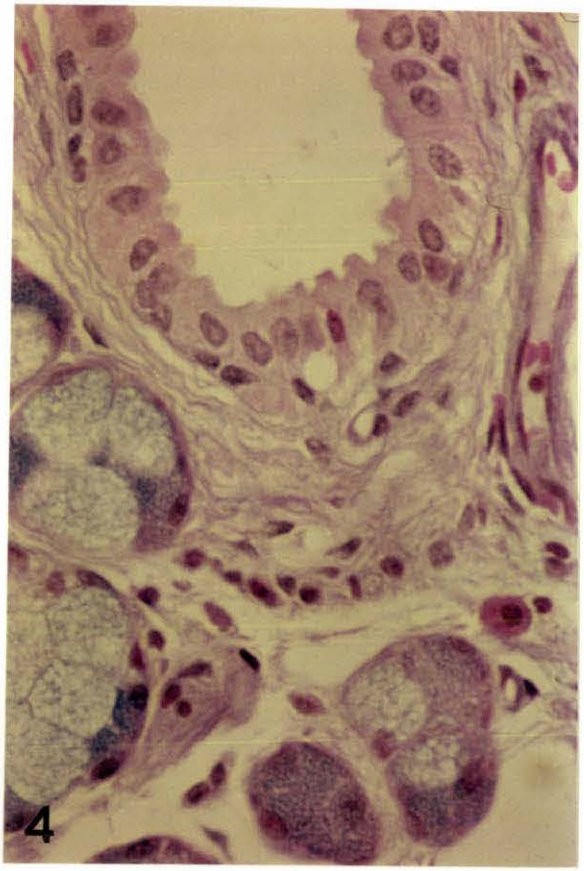
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loose connective tissue and adipose tissue (Figure 3.14). Cells of the secretory endpiece were arranged as long mucous tubes which had demilune cells associated with them (Figure 3.15). The demilune cells of the sublingual gland differed from those of the mandibular gland in both staining properties and size. In this gland the cells were slender in shape with the nucleus occupying the widest part of the cell. PAS positive, AB negative granules occur in the cytoplasm (Figure 3.14 and 3.16). The numbers of demilune cells and their development or size varied considerably from left to right gland as well as between different animals (Figure 3.15).

By comparison with the parotid and mandibular glands the duct system of the sublingual was poorly developed and the intralobular duct cells showed only slight basal striations (Figure 3.15).

Plasma cells were often seen in clumps between secretory endpieces and also scattered in the adipose tissue surrounding lobules of secretory tissue and the extra-lobular duct system.

3.3.4 POSTERIOR TONGUE AND SOFT PALATE

Glands beneath the epithelium of the dorsal surface of the tongue (Figure 3.17) and the soft palate (Figure 3.18) consisted of large diameter mucous end-pieces with a few demilune cells, intercalated and intralobular ducts. The glands drain onto the lining epithelium through long extralobular ducts lined by stratified cuboidal epithelium. The cells of the mucous endpieces stained positively with AY, PAS and weakly with AB. The demilune cells which were small and flattened reacted positively with PAS but were AB and AY negative (Figure 3.17 and 3.18).

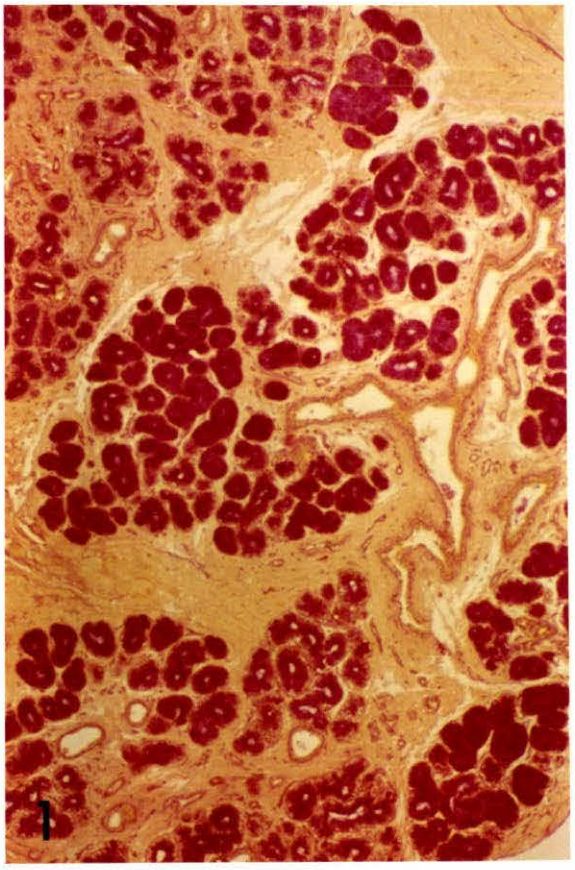
FIGURE 3.14

3.14.1 Low power photomicrograph showing the diffuse nature of the sublingual gland. Stain: PAS/H/ Tartrazine. Magnification: X90.

3.14.2 This shows a lobule of secretory tissue from the polystomatic portion of the sublingual gland in close proximity to the epithelium of the buccal cavity. The two pink stained, round structures are sections through excretory ducts. Stain: AY/H&E. Magnification: X90.

3.14.3 Sublingual salivary gland. Stain Ab/PAS/H/Tartrazine. Intralobular ducts and secretory endpiece arrangement can be seen. Magnification: X260.

3.14.4 Higher magnification of Figure 3.14.3. The intralobular duct shows basal striations and the demilune cells contain PAS positive granules. The mucous endpiece cells appear almost black. Magnification: X650.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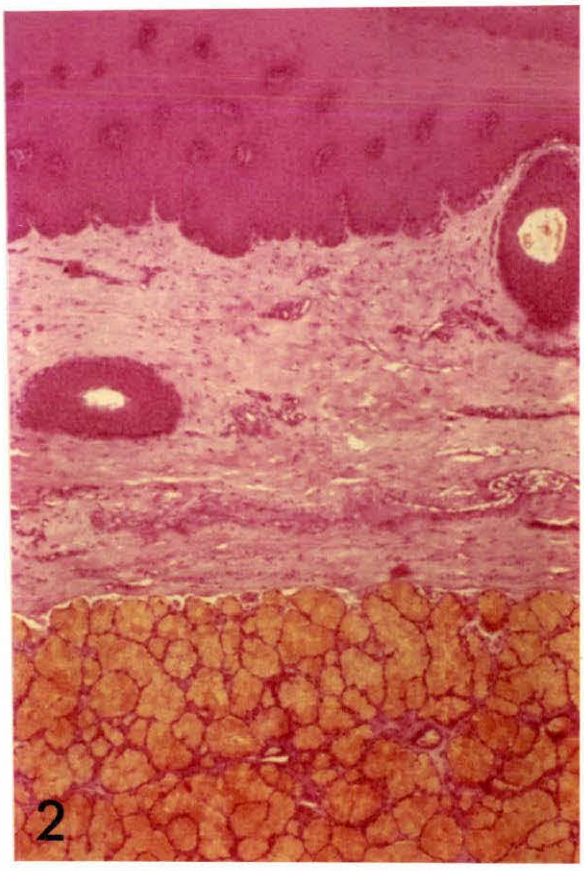
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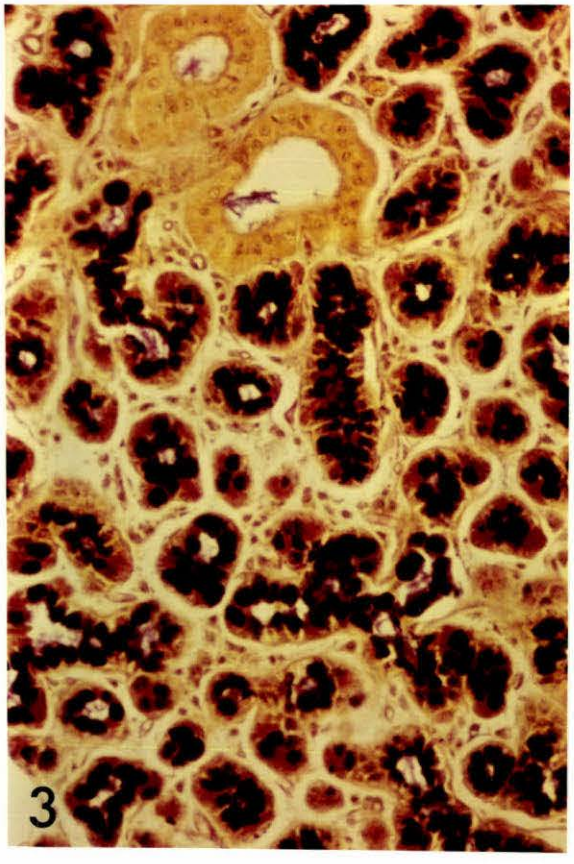
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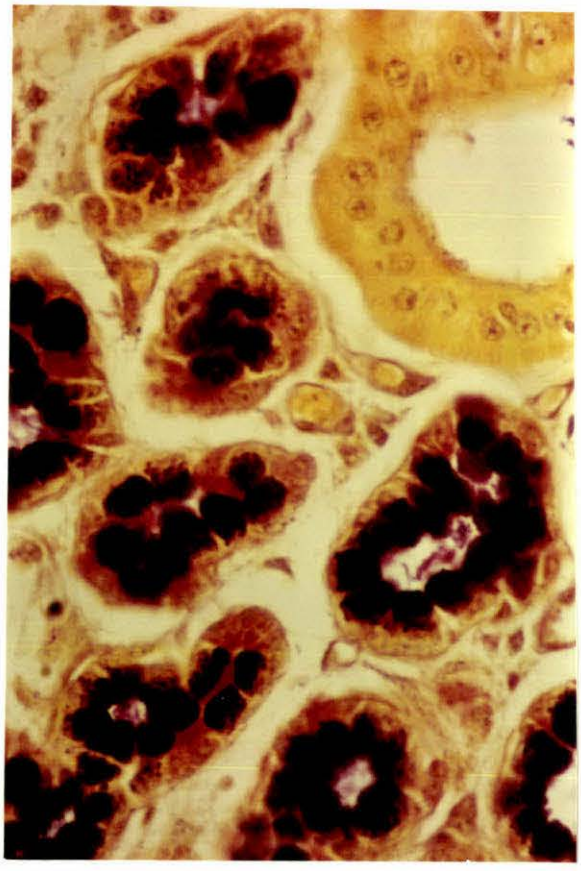


FIGURE 3.15

3.15.1 Sublingual salivary gland. Stain: AB/H&E. The long, branched tubular nature of the secretory endpieces can be seen. Magnification: X260.

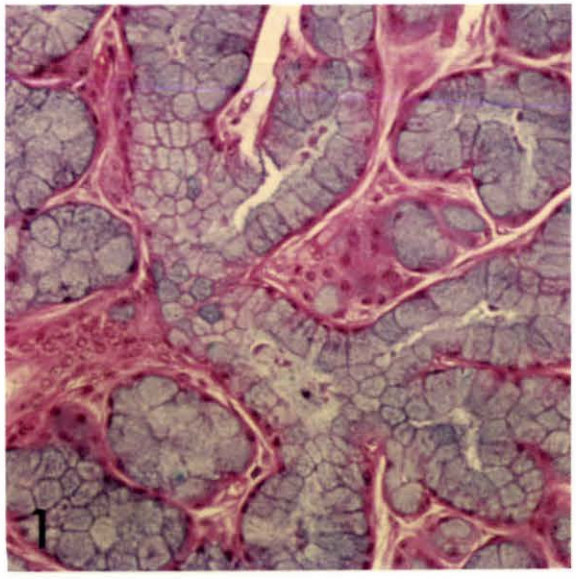
3.15.2 Cross sections through tubular endpieces. Sublingual salivary gland. Stain: AB/H&E. Magnification: X650.

3.15.3 Area within the sublingual gland where the endpieces are modified or contains a convoluted form of intralobular duct. Stain: PAS/H/Tartrazine. Magnification: X260.

3.15.4 Similar area to Figure 10.3. Stain: AB/H&E. Magnification: X260.

3.15.5 The sublingual gland, in some animals or one gland in an individual animal, show a lack of AB staining. Compare with Figure 10.2. Stain: AB/H&E. Magnification: X650.

3.15.6 Similar area to Figure 10.5. Although these mucous endpiece cells are AB negative, they react strongly with PAS. Stain: PAS/H/Tartrazine. Magnification: X650.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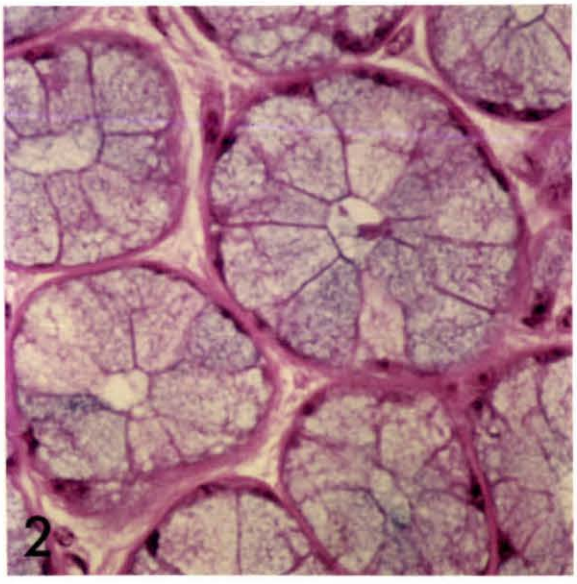
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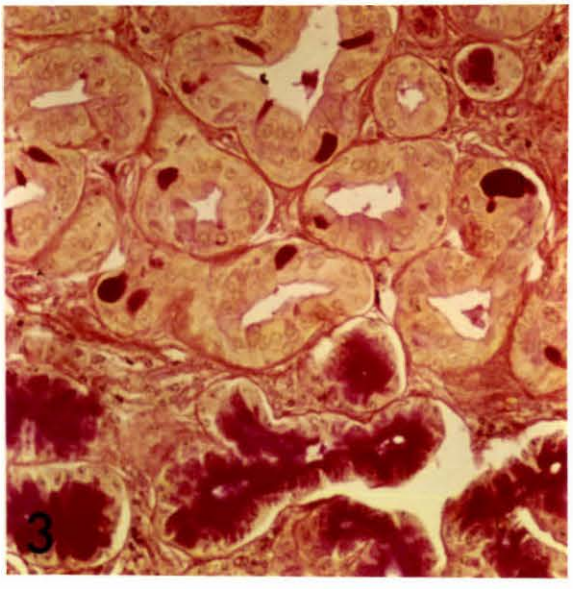
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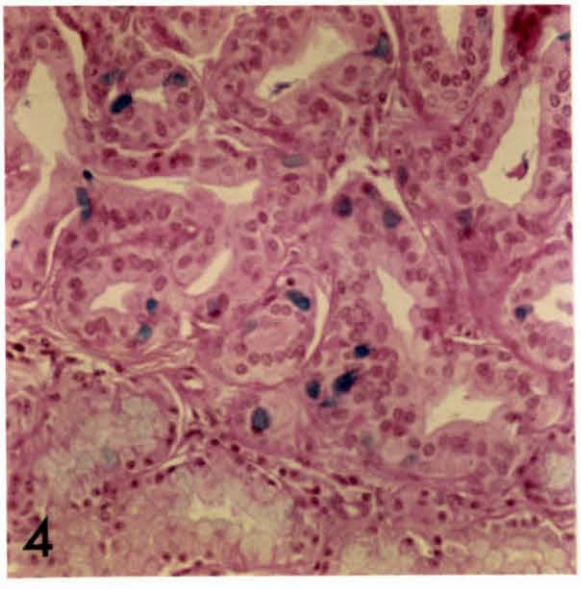
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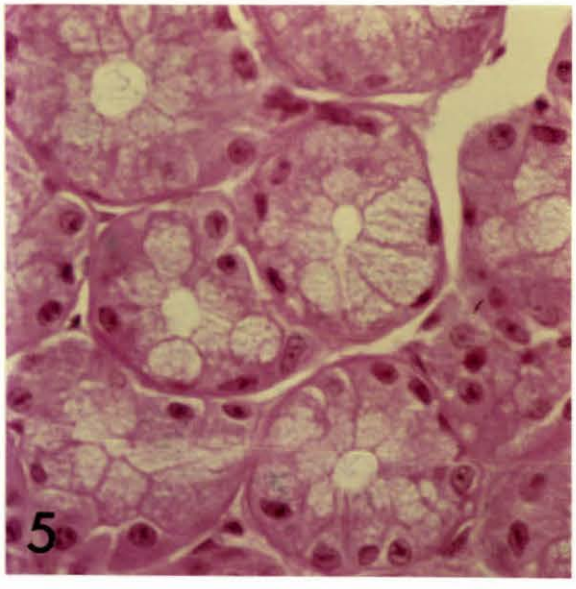
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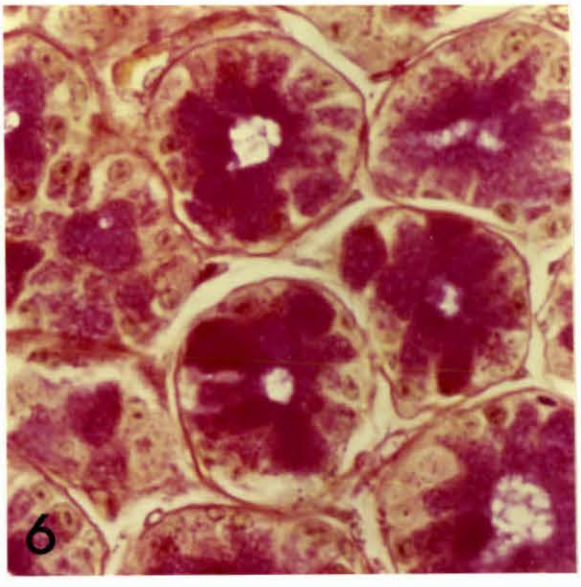
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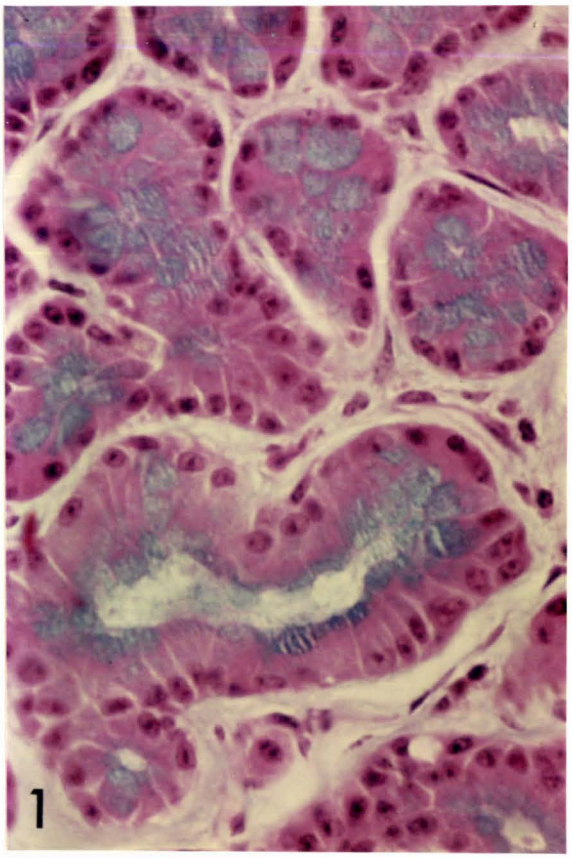
FIGURE 3.16

3.16.1 Sublingual salivary gland. Stain: AB/H&E. Magnification: X650.

3.16.2 Sublingual salivary gland. Stain: PAS/H/Tartrazine. Magnification: X650.

3.16.3 Sublingual salivary gland. Cross section through tubular endpiece. Stain: PAS/H/Tartrazine. Magnification: X1650.

3.16.4 Sublingual salivary gland. Part of intralobular duct wall. Stain: PAS/H/Tartrazine. Magnification: X1650.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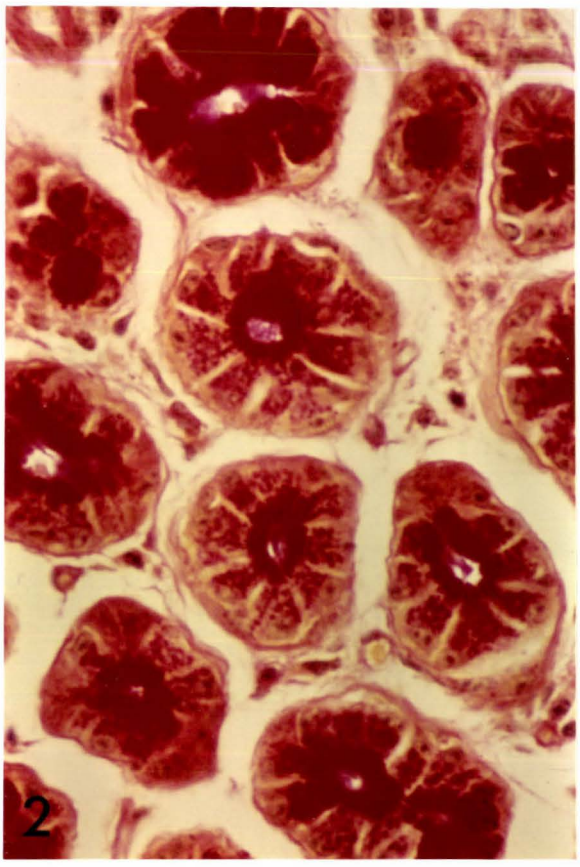
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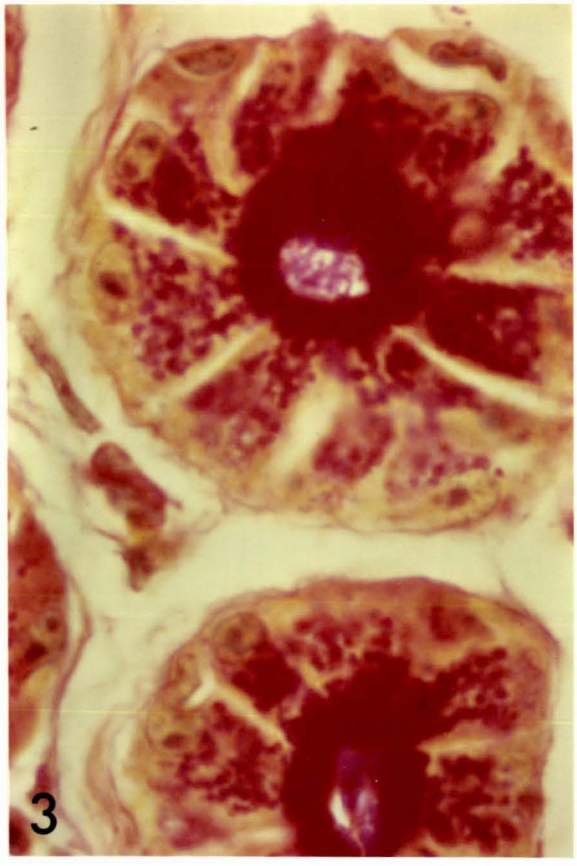
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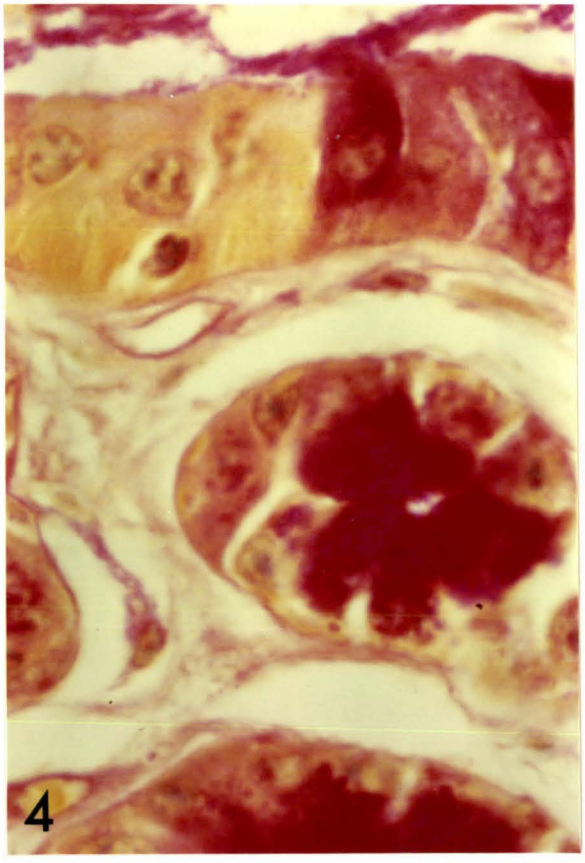
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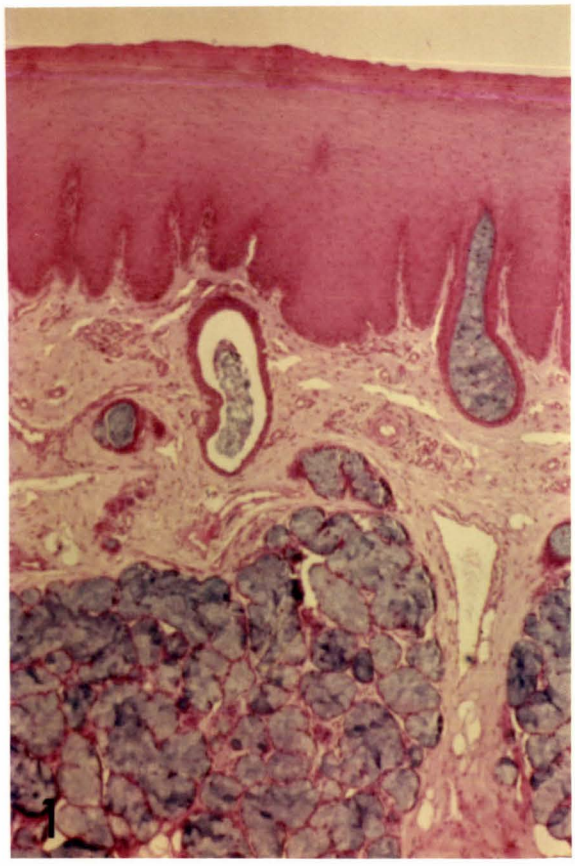
FIGURE 3.17

3.17.1 Dorsal surface of the tongue. Notice the large ducts leading to the surface. Stain: AB/H&E. Magnification: X60.

3.17.2 Deep within the tongue. Lobules of secretory tissue are found within adipose tissue and skeletal muscle. Stain: AB/H&E. Magnification: X60.

3.17.3 Posterior tongue. The long tubular form of the secretory endpieces is well demonstrated. Stain: AB/H&E. Magnification: X100.

3.17.4 Posterior tongue. Secretory endpieces. Stain: AY/H&E. Magnification: X260.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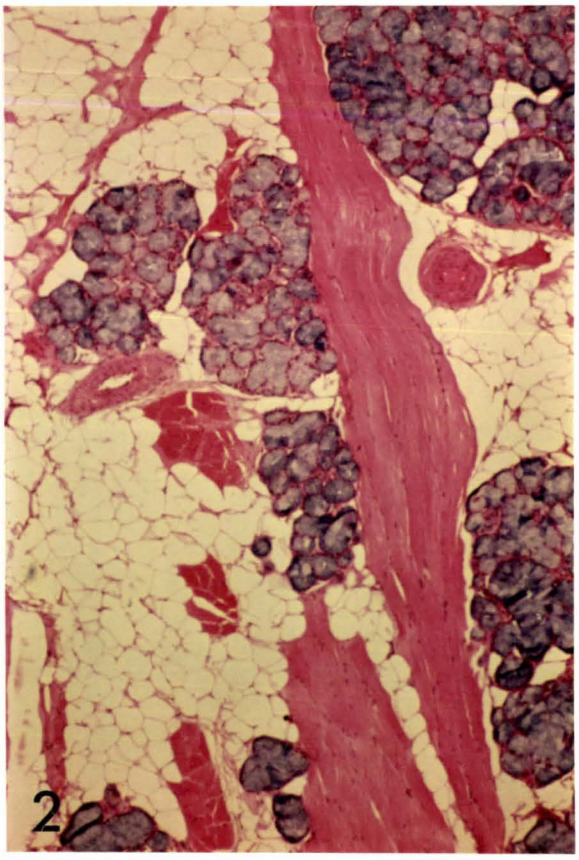
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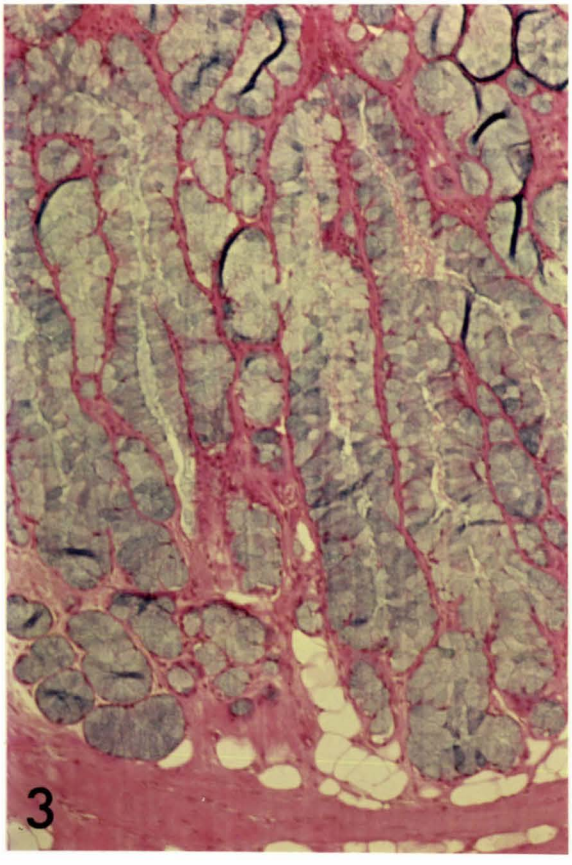
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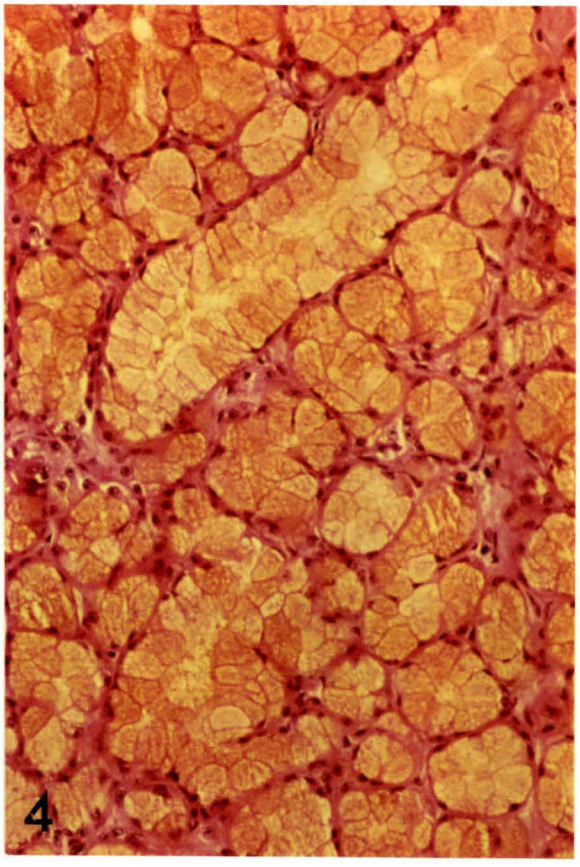
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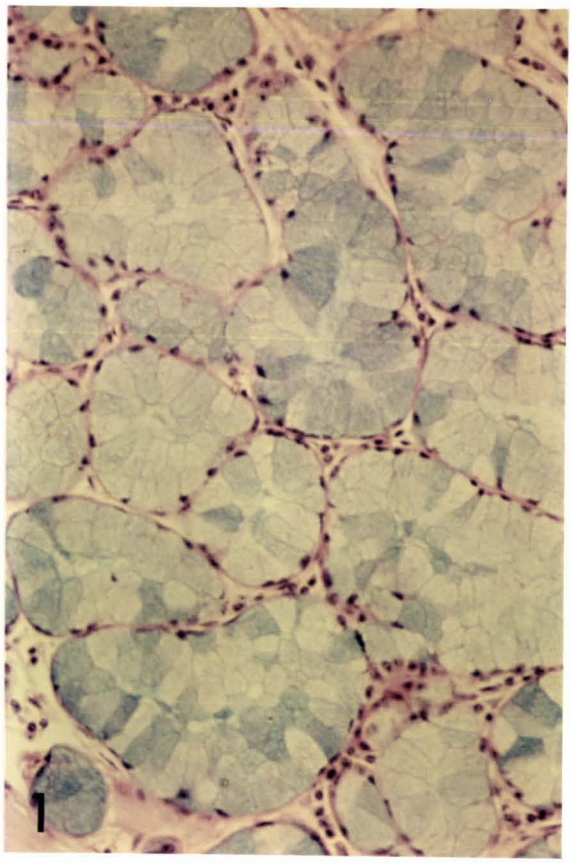
FIGURE 3.18

3.18.1 Posterior tongue. Secretory endpieces. Stain: AB/H&E. Magnification: X260.

3.18.2 Posterior tongue. Secretory endpieces. Stain: PAS/H/Tartrazine. Magnification: X260.

3.18.3 Secretory endpieces found in the glands of the soft palate. Stain: AB/H&E. Magnification: X260.

3.18.4 Soft palate. Secretory endpieces. Stain: PAS/H/ Tartrazine. Magnification: X260.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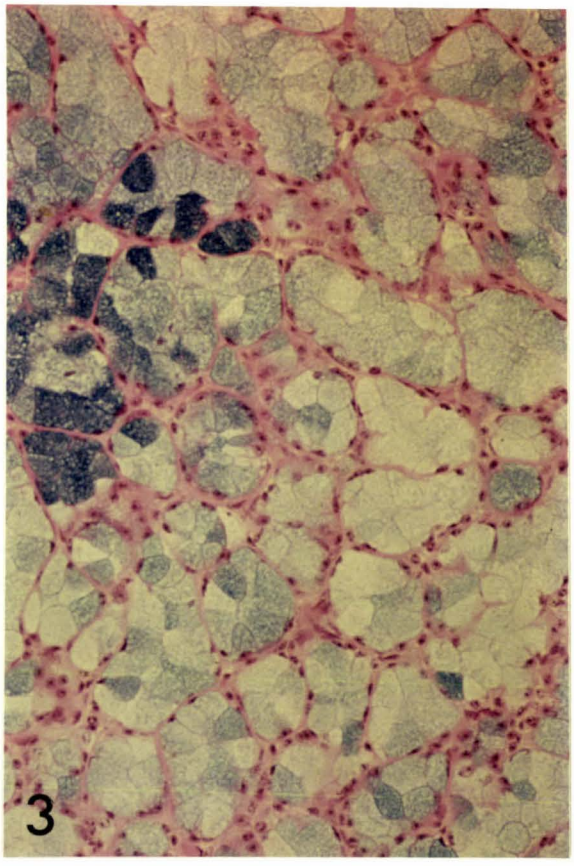
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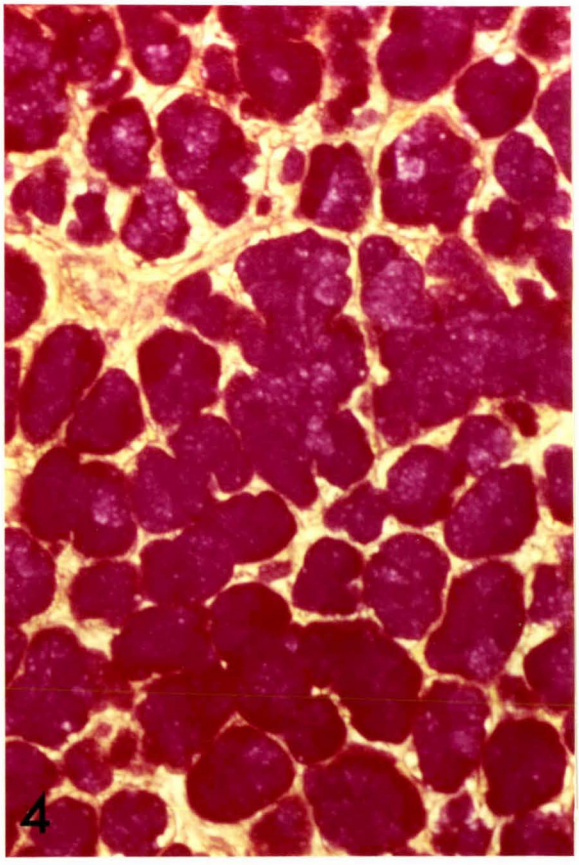
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3.3.5 EPIGLOTTIS

The glands of the lingual surface of the epiglottis were scattered within the subepithelial connective tissue of the structure and communicated with the surface via several short excretory ducts (Figure 3.19). The secretory endpieces consisted of mucus producing cells, which reacted positively with AB, AY and PAS, and demilune cells. The demilune cells were large and contained abundant cytoplasmic granules which were eosinophilic, AB and PAS positive (Figure 3.20). Numerous plasma cells were observed within the intralobular connective tissue surrounding the secretory endpieces.

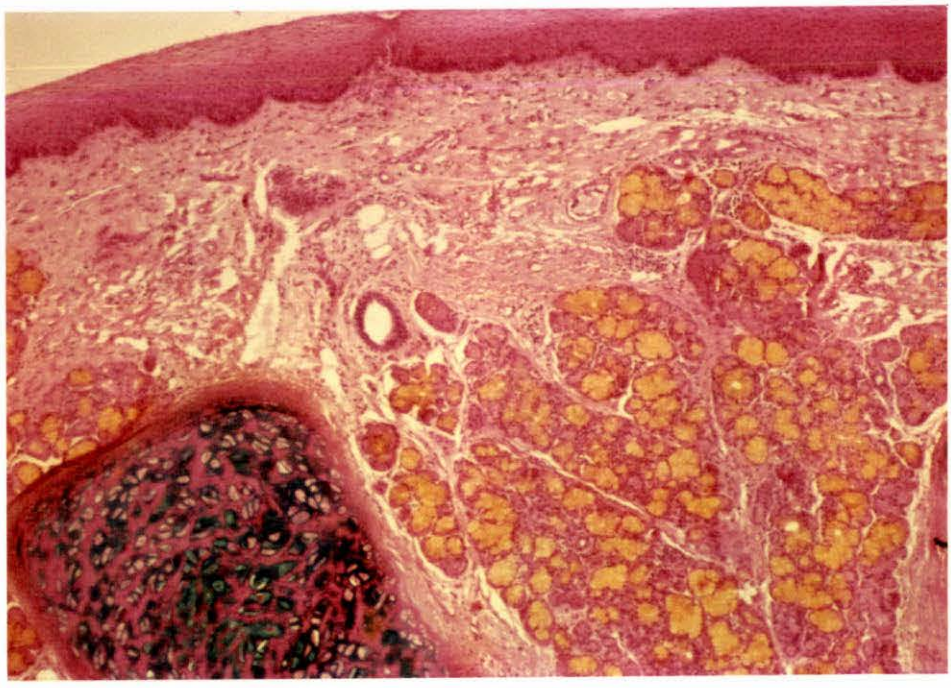
3.3.6 DORSAL, INTERMEDIATE BUCCAL GLANDS

Considerable variability was found between left and right glands and also those of different animals (Figure 3.21). These glands were predominantly mucus producing with variable arrangement and extent of the serous component. The mucous cells of the secretory endpieces were PAS, AY and weakly AB positive whereas the demilune cells were AB, PAS and AY negative (Figure 3.22 and 3.23). The lumen contents of some secretory endpieces and intralobular ducts within these glands contained an atypical mucus and included necrotic nuclei and cytoplasmic debris (Figure 3.24) which resembled a holocrine type of secretory mechanism. These were the only glands which, according to histochemical reactions, could be classified as containing truly serous demilune cells (Figure 3.21). In some buccal glands small islets or pockets of entirely serous endpieces were observed which appeared to have their own intercalated and interlobular duct systems (Figure 3.22).

FIGURE 3.19

3.19.1 Lingual surface of the epiglottis. Lobules of secretory tissue are found in the subepithelial connective tissue. Stain: AB/AY/H&E. Magnification: X60.

3.19.2 Epiglottis. The extensive secretory tissue extends deep into the structure. Elastic cartilage can be seen on the right. Stain: AB/PAS/H/Tartrazine. Magnification: X100.



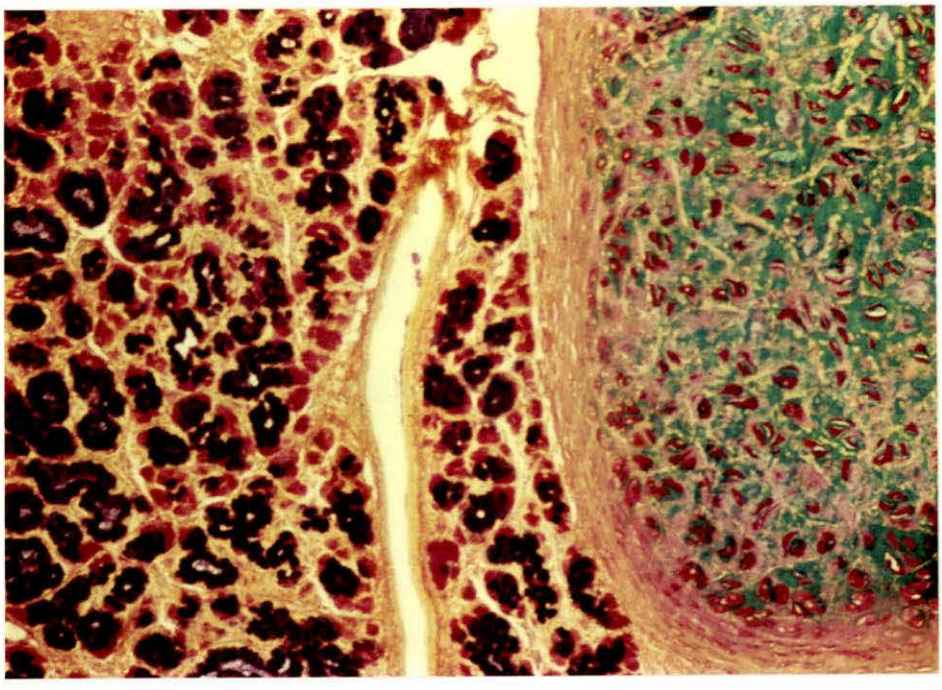


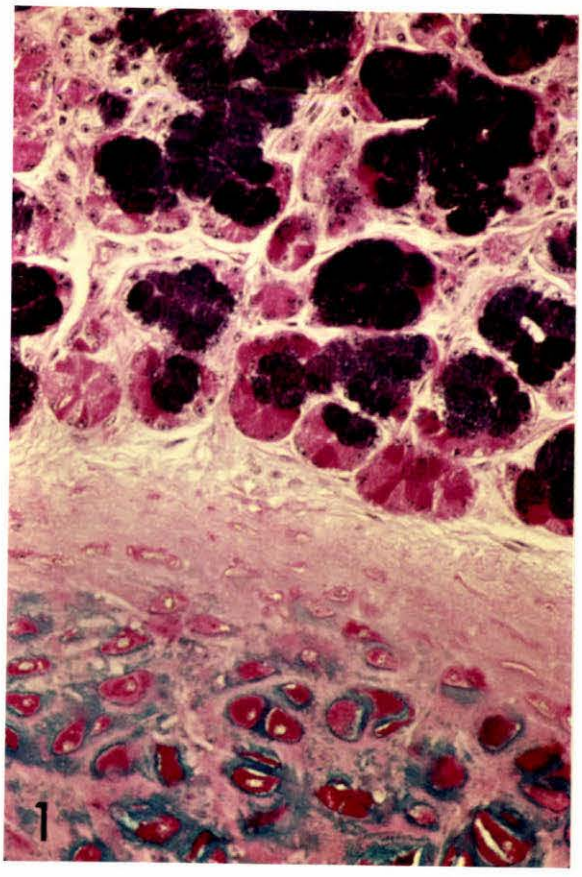
FIGURE 3.20

3.20. 1 Epiglottis. Secretory endpieces. Stain: AB/H/PAS. Magnification: X260.

3.20. 2 Similar area to above. Stain: AB/AY/H&E. Magnification: X260.

3.20.3 Epiglottis. Secretory endpieces. Stain: AB/H&E. The demilune cells are strongly acidophilic (bright pink). Clumps of plasma cells can be seen between endpieces. Magnification: X260.

3.20.4 Higher power photomicrograph of similar area to 3.20.3. Stain: PAS/H/Tartrazine. Note the abundant PAS positive granules in the demilune cells. Magnification: X650.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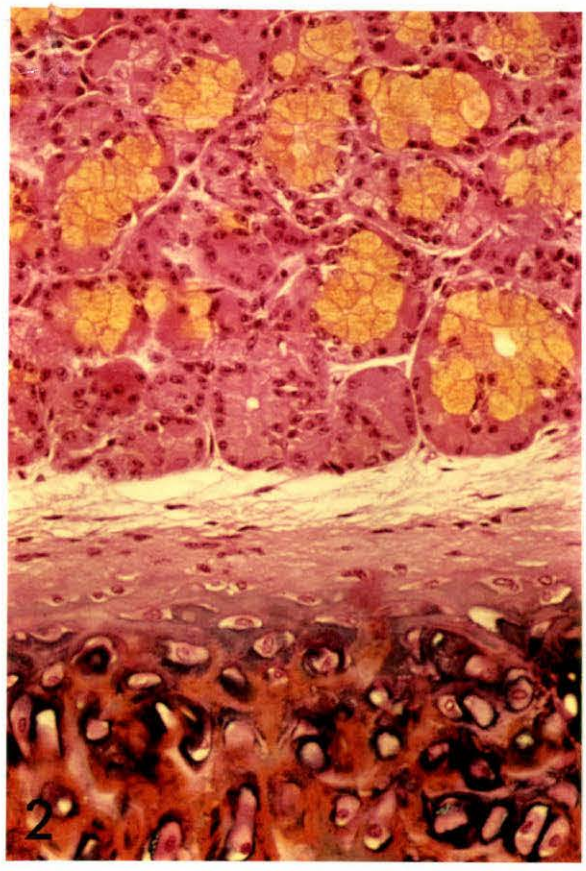
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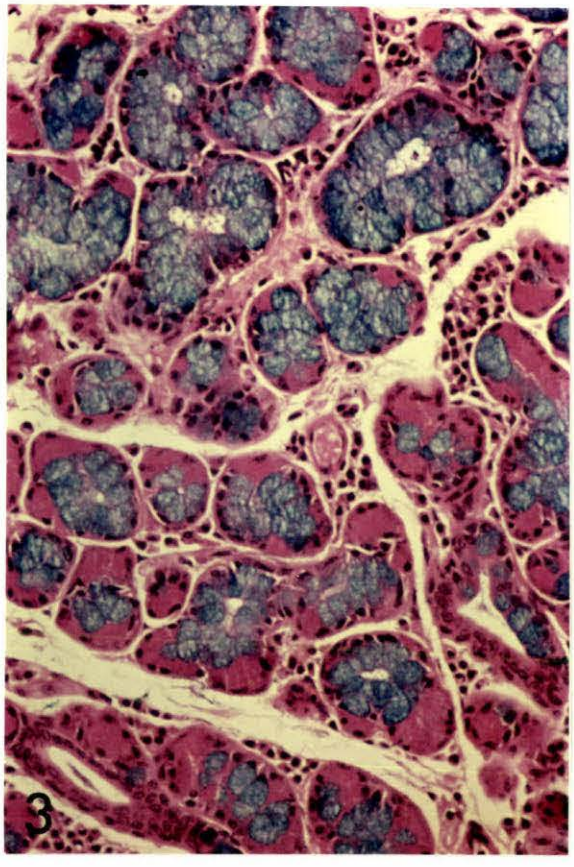
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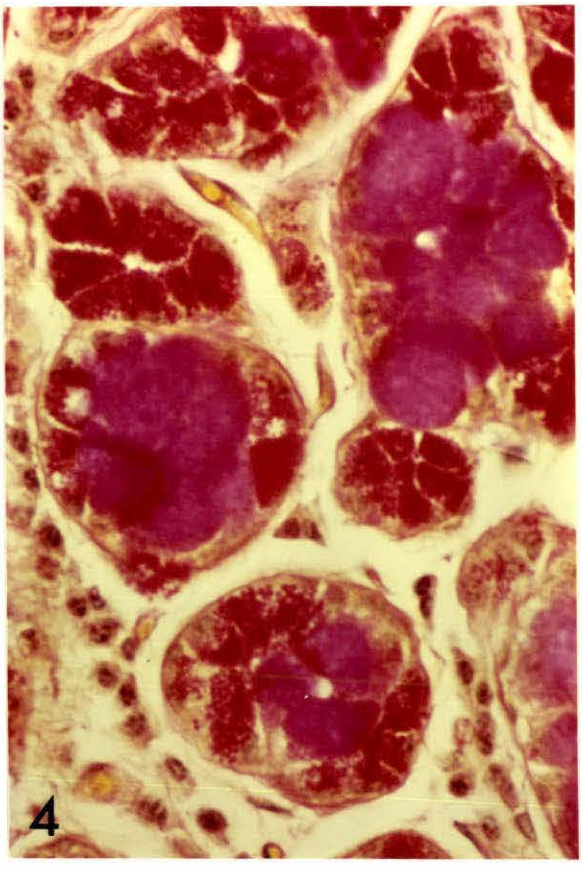
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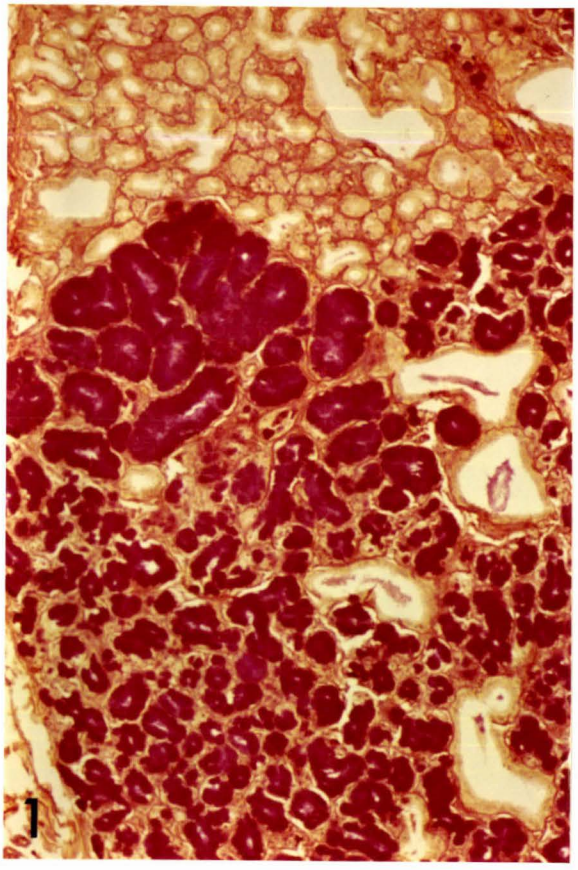
FIGURE 3.21

3.21.1 Dorsal buccal gland. Low power photomicrograph which illustrates three differing forms of secretory endpiece present within these and the intermediate buccal glands. Stain: PAS/H/Tartrazine. Magnification: X100.

3.21.2 Higher power view of upper part of Figure 3.21.1. Note the absence of PAS staining. Magnification: X650.

3.21.3 Higher power view of mid portion of Figure 3.21.1. Secretory endpieces are almost exclusively mucus producing. Magnification: X650.

3.21.4 Higher power view of lower part of Figure 3.21.1. Secretory endpieces are composed of both mucous and serous cells. Magnification: X650.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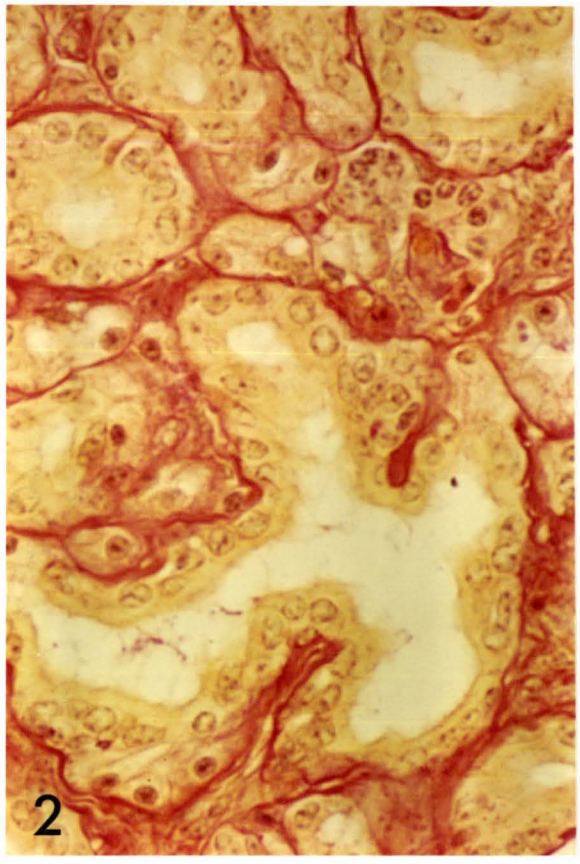
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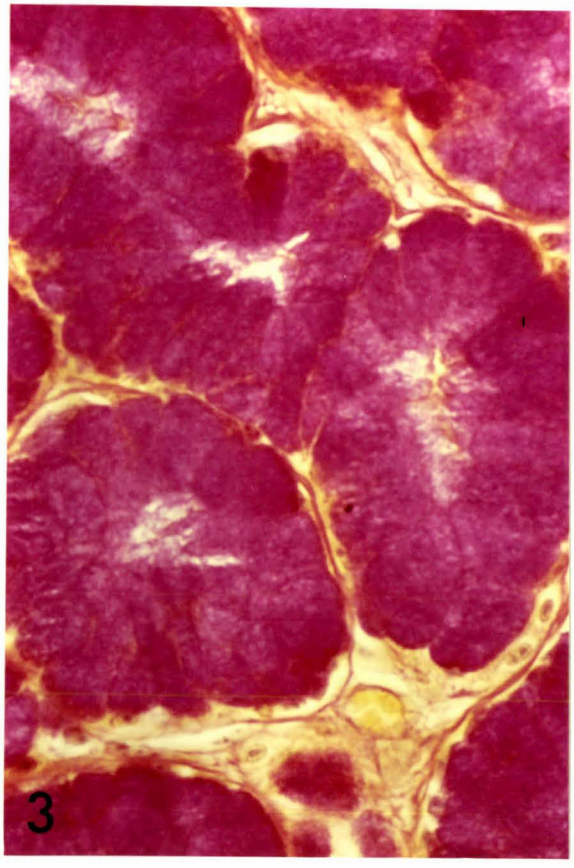
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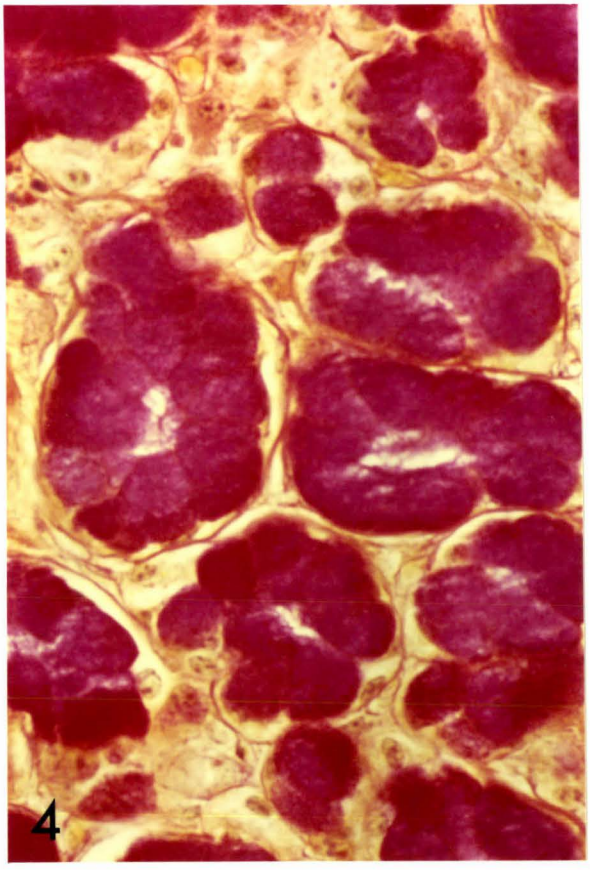
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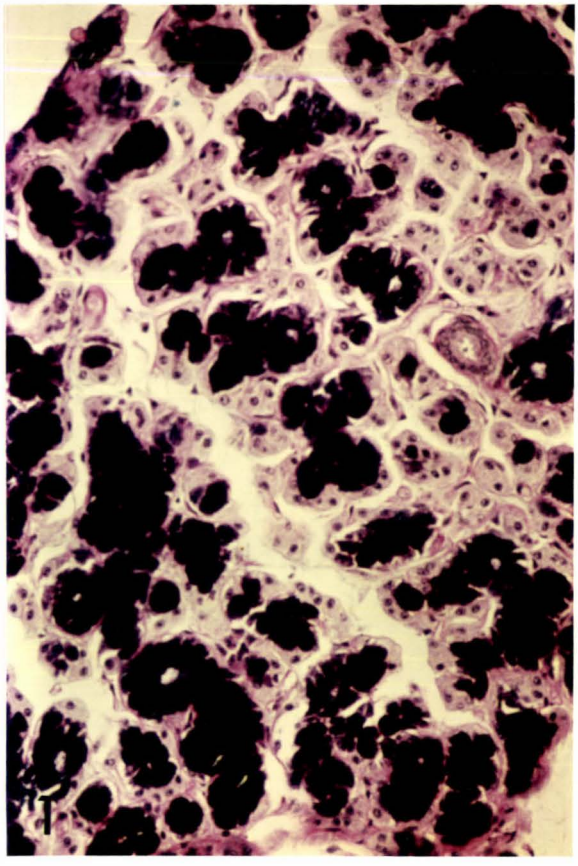
FIGURE 3.22

3.22.1 Intermediate buccal gland. Stain: AB/H/PAS. Magnification: X260.

3.22.2 Intermediate buccal gland. Note the pocket of entirely serous cells present. Stain: AB/H&E. Magnification: X260.

3.22.3 Higher power photomicrograph from Figure 20.2, showing mucous cells (blue) and demilune cells devoid of AB positive granules. Magnification: X650.

3.22.4 Similar area to Figure 20.3. Stain: PAS/H/Tartrazine. Note the absence of PAS positive granules within the demilune cells. Magnification: X650.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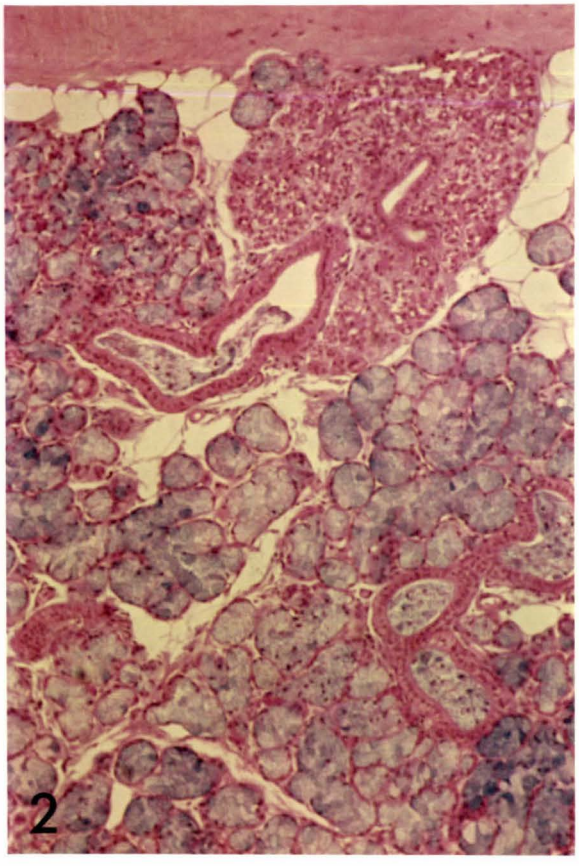
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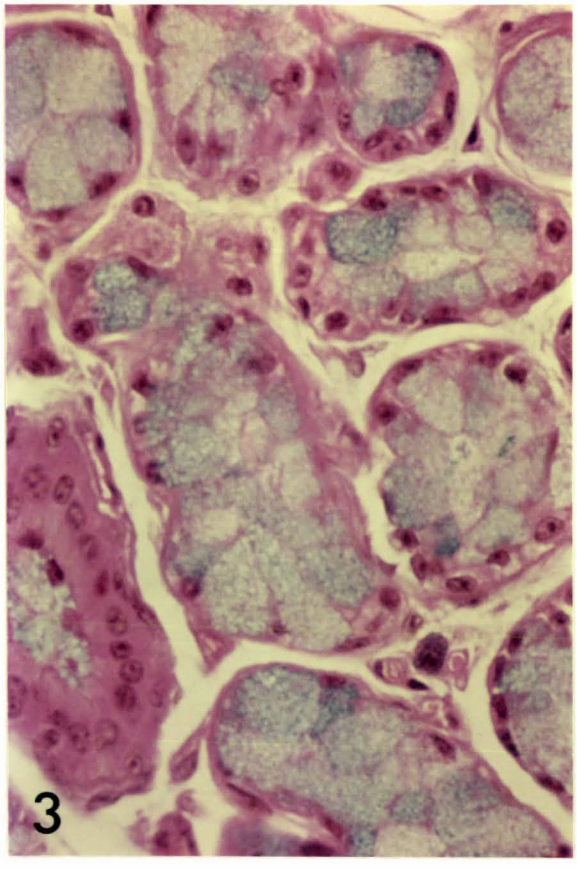
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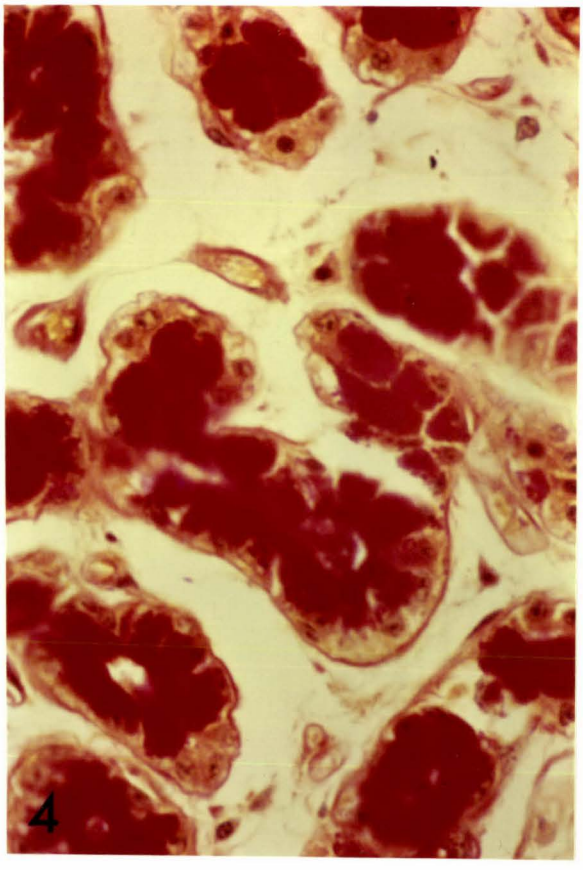
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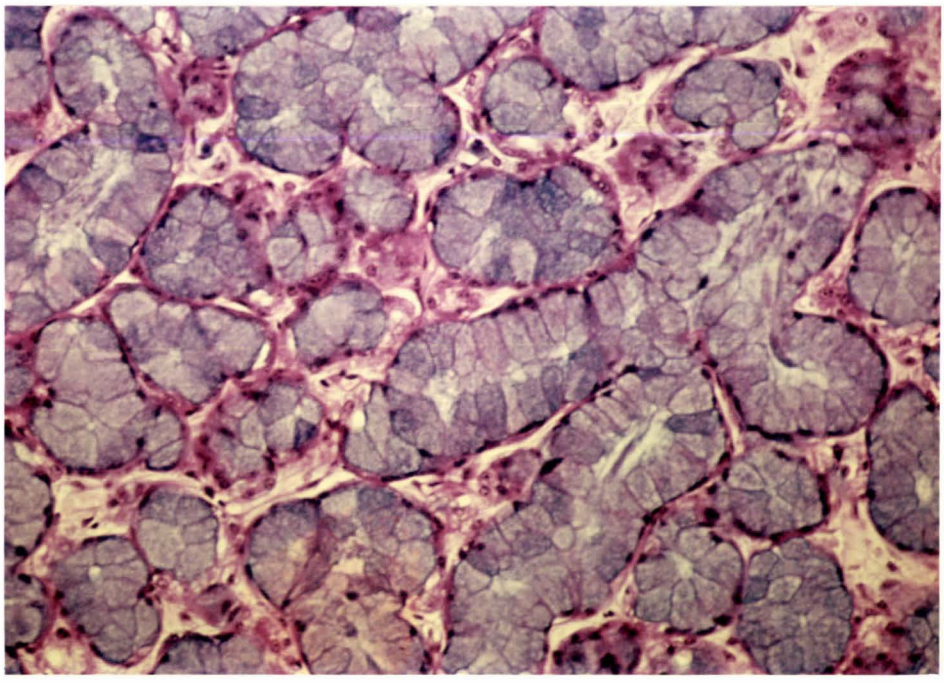
FIGURE 3.23

3.23.1 Dorsal buccal gland showing the more usual appearance of the secretory endpieces. Composed of mucous cells and mucoserous demilunes. Stain: AB/H&E. Magnification: X260.

3.23.2 Similar section to the above. Stain: AB/AY/H&E. Magnification: X260.



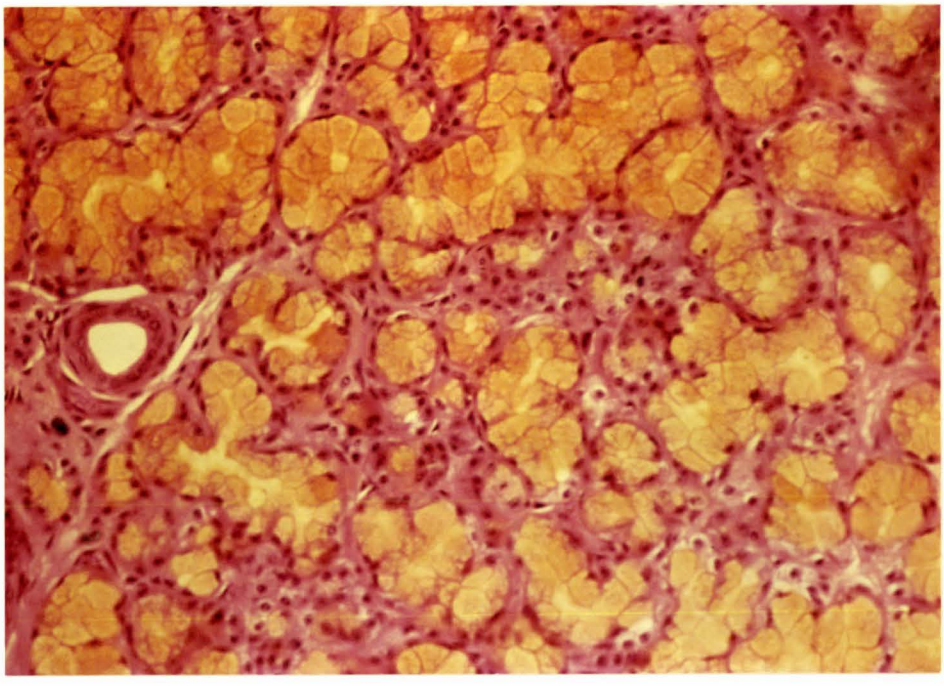


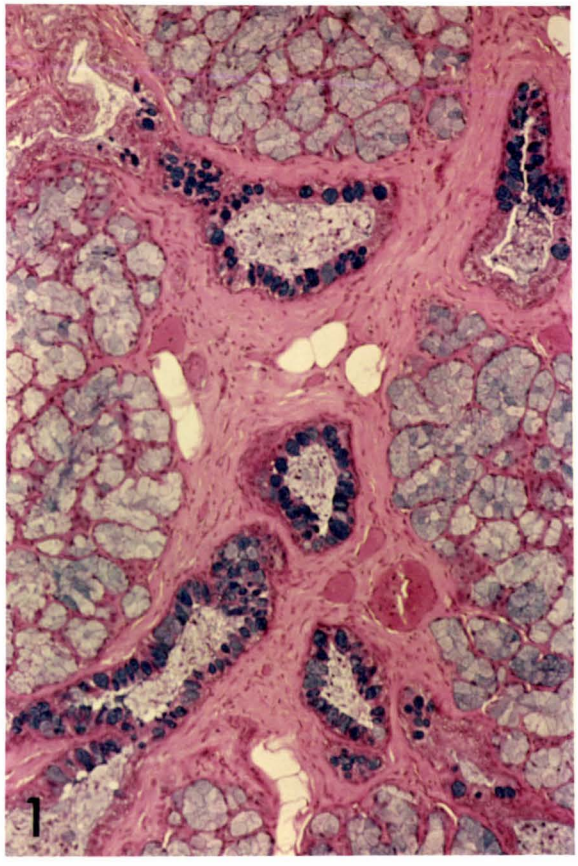
FIGURE 3.24

3.24.1 Dorsal buccal gland. Stain: AB/H&E. Dark blue staining. Goblet cells are present in the extra-lobular ducts. Magnification: X100.

3.24.2 Dorsal buccal gland. Showing secretory endpieces and an intralobular duct. Note duct lumen contents. Stain: AB/H&E. Magnification: X260.

3.24.3 Dorsal buccal gland. Secretory endpieces showing what resembles a holocrine secretory mechanism. Stain: AB/H&E. Magnification: X260.

3.24.4 Dorsal buccal gland. Cross sections through tubular secretory endpieces. Stain: PAS/H/Tartrazine. Magnification: X650.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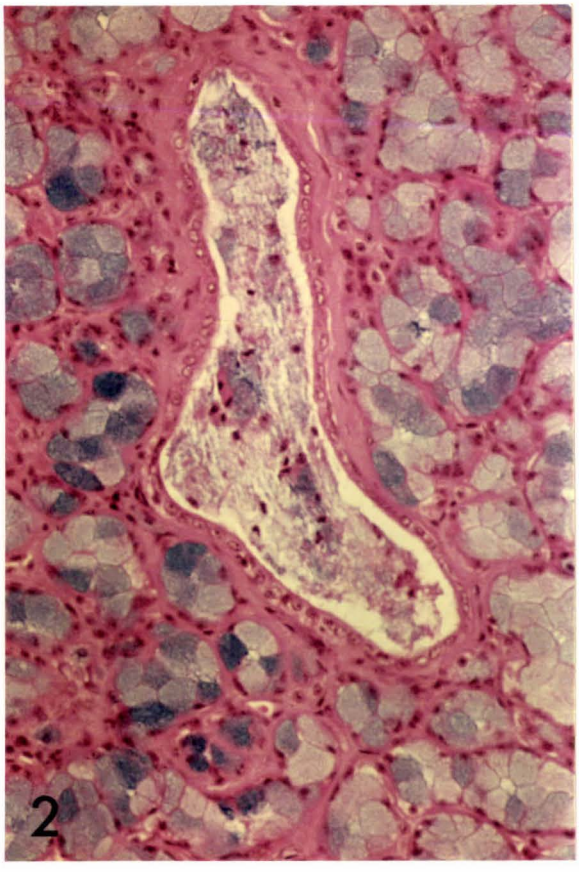
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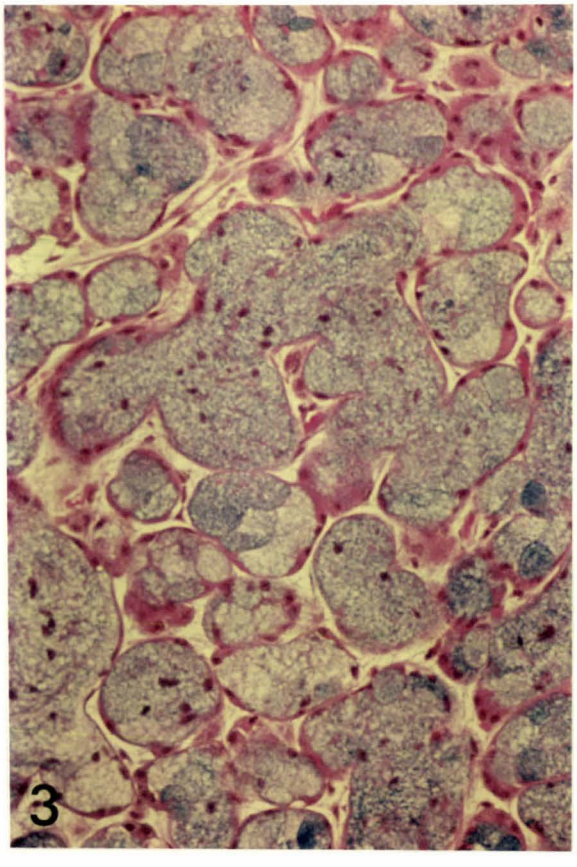
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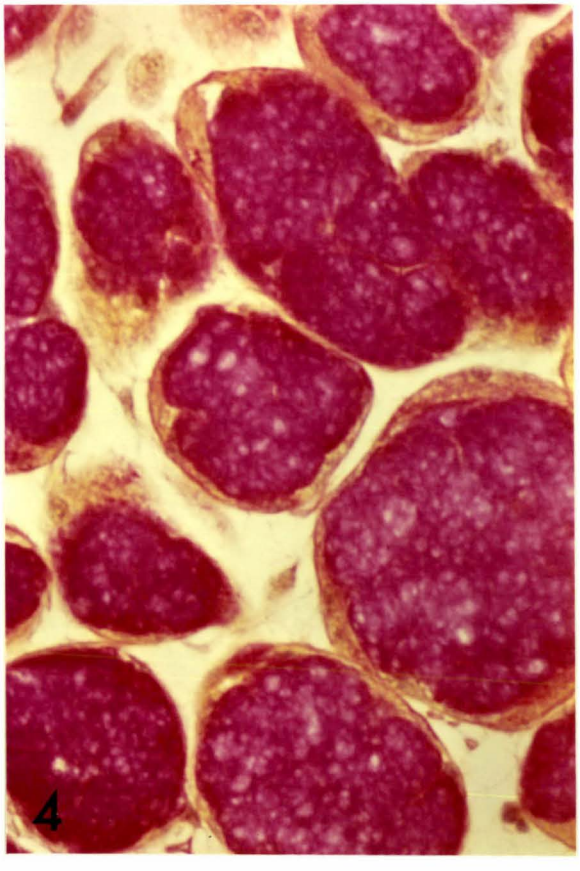
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3.3.7 VENTRAL BUCCAL GLANDS

The morphology and histochemistry of the ventral buccal glands were found to be identical with that of the parotid glands. See section 3.2.1 and Figures 3.8 - 3.10.

3.3.8 LABIAL GLANDS

Labial glands were composed of small lobules of glandular tissue which were scattered within loose connective tissue and fat. The secretory tissue consisted of small mucous endpieces capped with large demilune cells. The mucous cells were PAS, AY and weakly AB positive.

Unlike the dorsal and intermediate buccal glands the demilune cells of the labial glands contained eosinophilic granules which were also PAS positive. RER was sufficiently well developed within these cells to be identified with the light microscope using methyl green pyronin. The duct system of labial glands was short and the striated ducts poorly developed. Large numbers of plasma cells were seen residing in clusters within the connective tissue between secretory endpieces (Figure 3.25).

3.4 HIGH BLOAT SUSCEPTIBILITY COMPARED TO LOW BLOAT SUSCEPTIBILITY

Although the information was not available at the time their tissues were examined, animals 1 and 4 were known to have a high susceptibility to bloat. The parotid and ventral buccal glands from these animals contained abundant PAS positive cytoplasmic granules within secretory cells and the contents of duct lumens was strongly PAS positive. Another notable characteristic was the lack of plasma

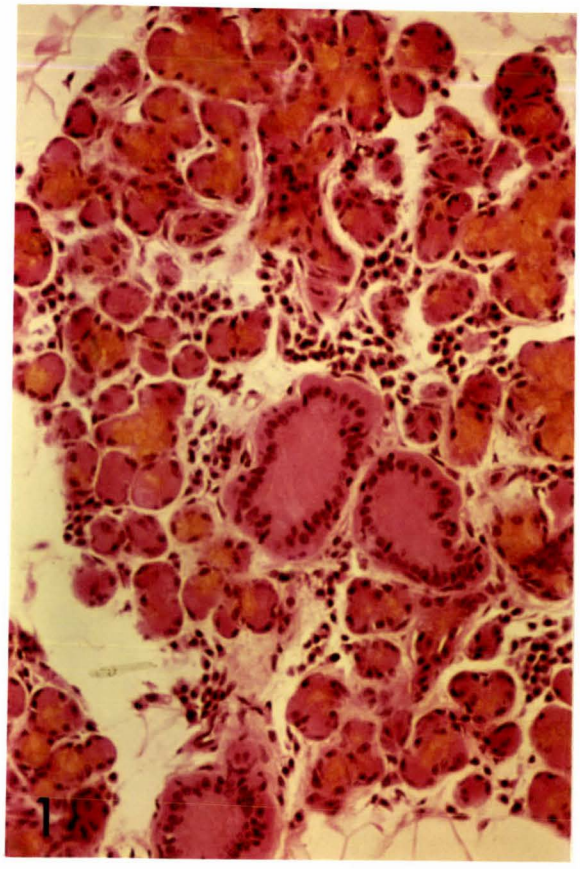
FIGURE 3.25

3.25.1 Labial gland. Lobule of secretory tissue surrounded by adipose tissue. Stain: AB/AY/H&E. Magnification: X260.

3.25.2 Higher magnification of Figure 3.25.1. Mucous cells are strongly AY positive, weakly AB positive. Demilune cells are strongly acidophilic. Clumps of plasma cells are found between the secretory endpieces. Magnification: X650.

3.25.3 Similar area to Figure 3.25.2. Demilune cells contain PAS positive granules. Stain: AB/H/PAS. Magnification: X650.

3.25.4 Similar section to Figure 3.25.2. Stain: Methyl green pyronin. This stain demonstrates the presence of cytoplasmic RNA. The basal regions of the demilune cells and the cytoplasm of plasma cells have reacted strongly. Magnification: X650.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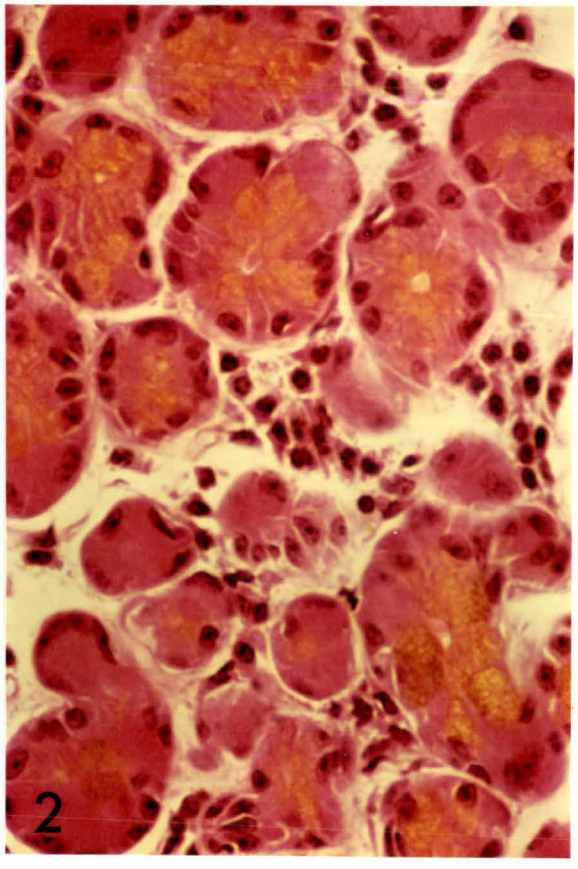
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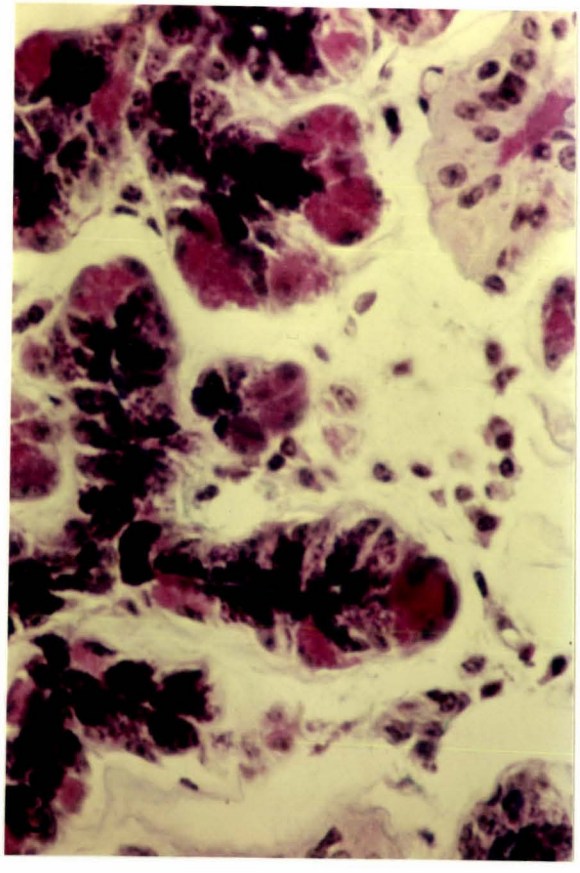

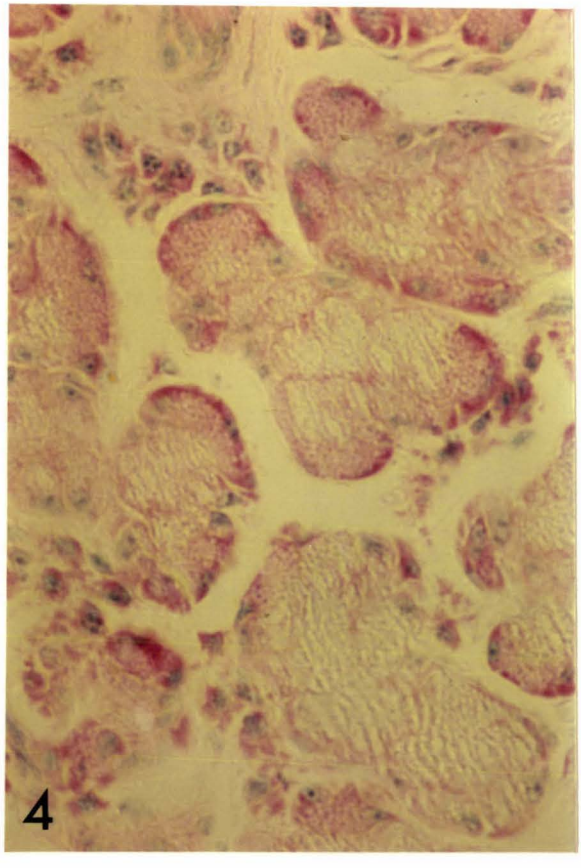
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cells or mast cells in the walls of intralobular ducts. In animal No. 1, the left mandibular glands contained areas where no mucous cells occurred, only serous cells were present while the right gland had small areas where only mucous cells were present, demilune cells were replaced by fibrous connective tissue. The demilune cells of the left mandibular gland from animal No. 4 showed a lack of granulation and the striated duct cells contained large perinuclear vacuoles not seen in other sections. The right gland appeared normal.

Animals 2 and 3 were known to have low susceptibility to bloat. Two differences in the parotid glands of these animals were observed :

(a) Secretory endpiece cells contained much less PAS positive granulation.

(b) Many "immigrant " cells were present within the walls of intralobular ducts (Table 3.2).

3.5 CANNULATED GLANDS COMPARED WITH NON-CANNULATED

3.5.1 ANIMAL NUMBER 7 CANNULATED LEFT PAROTID AND MANDIBULAR

At the time of death neither cannulae had functioned for 5 days. The cells of the secretory endpieces of the left parotid gland were small and flattened and the lumen of the endpiece was enlarged. Plasma cells, mast cells and PMN neutrophils were found in the connective tissue around the endpieces and ducts. The right gland appeared normal, with little PAS positive material in the duct lumens.

The left mandibular gland contained mucous endpiece cells which were smaller than those in the contralateral gland. Demilune cells were also smaller and lacked the usual degree of granulation.

Incresed numbers of plasma cells, mast cells and PMN neutrophils were present and the venules and lymphatic drainage vessels were dilated. The right mandibular gland appeared normal. Demilune cells of the right sublingual gland from this animal were also degranulated.

3.5.2 ANIMAL NUMBER 8 CANNULATED LEFT PAROTID AND MANDIBULAR

At the time of death the parotid cannula was functional but the mandibular cannula had ceased functioning 3 days previously. Lumens of the secretory endpieces from the left parotid gland were distended as were the intralobular ducts. The right gland had a normal appearance except for perinuclear vacuolation of the striated duct cells. The cannulated mandibular glands contained slightly dilated ducts and endpiece lumens along with mild cellular infiltration as in animal No. 7. The contralateral glands had wide lumen ducts and endpieces. Changes were noted in the dorsal buccal glands. The left dorsal contained normal mucous endpiece cells but the demilunes were granulated while in the right dorsal buccal gland the demilunes contained no stainable granules. The ventral buccal glands were normal.

3.5.3 ANIMAL NUMBER 9 CANNULATED LEFT PAROTID AND MANDIBULAR

The mandibular cannula ceased functioning 9 days prior to the time of death but the parotid cannula was still functional. Except for some mast cells present in the cannulated gland both parotids were normal. However, the left mandibular gland showed evidence of duct and endpiece damage. The mucous cells were small and reduced in number while the demilune cells were small and degranulated. Although the right gland was

normal it was notable that the striated ducts were highly developed. There were very few "immigrant" cells in either gland (Figure 3.26). As was noted in the previous animal both sublingual glands contained enlarged endpiece lumens, an increased number of demilune cells and intralobular ducts which were well striated. The same changes were noted between left and right upper buccal glands as recorded for Animal 8. The right ventral buccal gland had large endpieces with dilated lumens suggesting greater activity than the left gland.

3.5.4 ANIMAL NUMBER 10 LEFT PAROTID CANNULATION

The cannula was functional at the time of death. All the salivary glands studied in this animal appeared normal with the exception of the cannulated parotid gland which showed changes in intralobular duct and secretory endpiece lumen diameters and cellular infiltration consistent with changes observed in other cannulated glands.

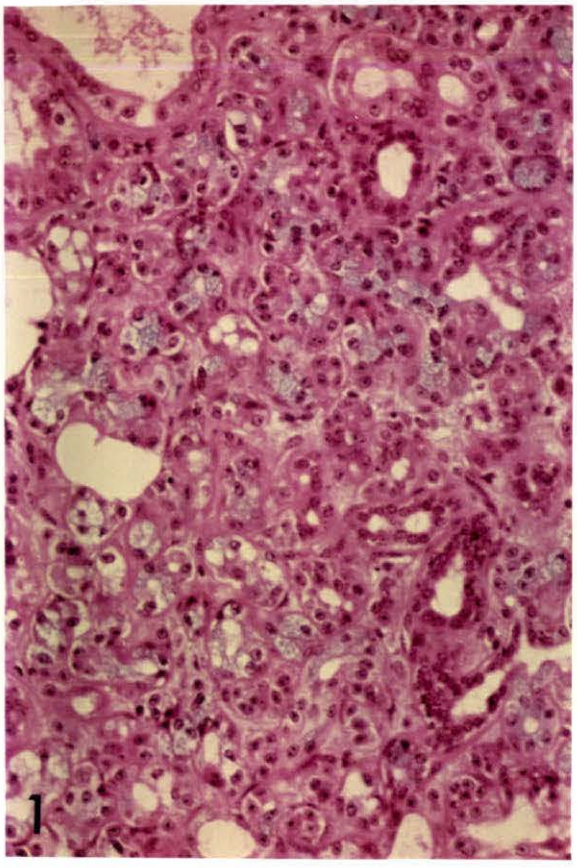
FIGURE 3.26

3.26.1 Left mandibular salivary gland from animal No.9. Duct and endpiece damage has resulted from an obstructed duct cannula. Stain: AB/H&E. Magnification: X260.

3.26.2 Higher magnification of Figure 3.26.1 showing mucous cells of smaller size and reduced numbers and degenerate demilune cells. Magnification: X650.

3.26.3 Right mandibular salivary gland from animal No.9. Note the abundance of demilune cells. The mucous cells appear normal. Magnification: X260.

3.26.4 Higher magnification of Figure 3.26.3. The intralobular ducts have well developed basal striations. Magnification: X650.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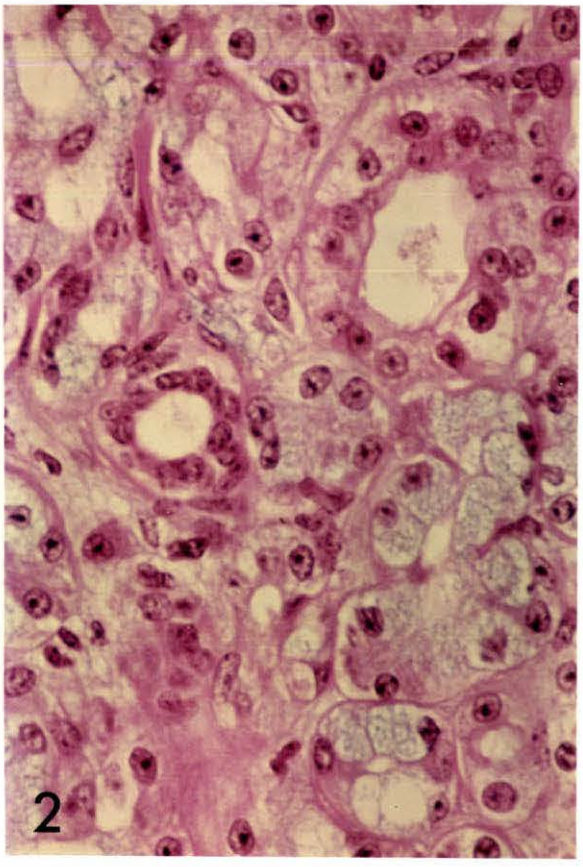
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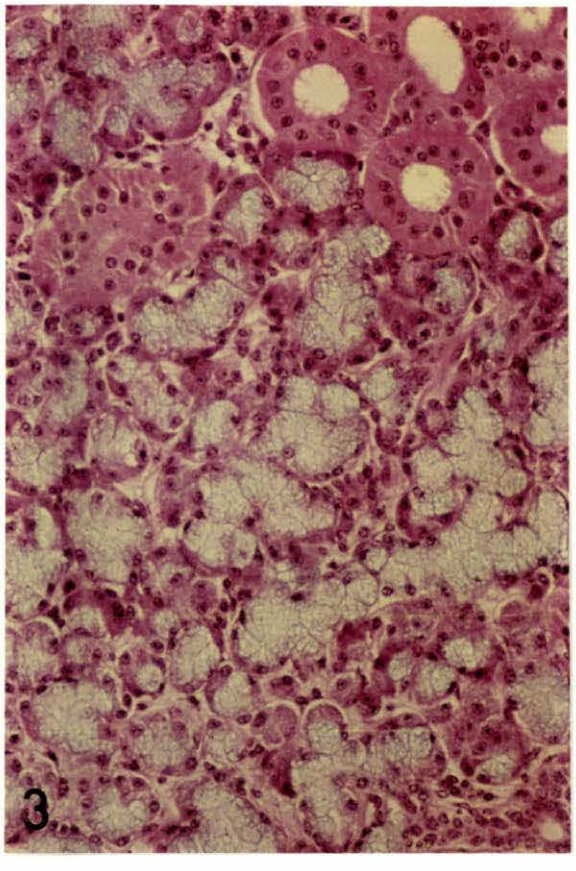
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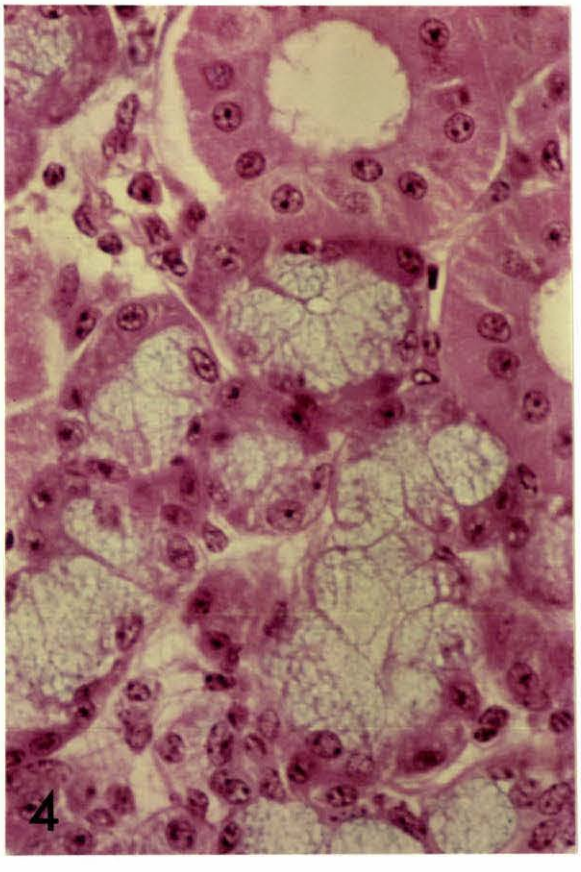
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4. DISCUSSION

4.1 SALIVARY GLAND WEIGHTS

A comparison between the ratio of the mean salivary gland weights and the bodyweights of the non-cannulated animals used in this study revealed no significant differences (Figure 3.1). This could be due to the relative maturity of the digestive tracts in these animals as no differences were noted between these results and those of Kay (1960) who studied salivary gland weights in calves 8 months old. Wilson and Tribe (1961) have shown that, in lambs, the parotid glands had reached maturity by about 3 months of age and Wilson (1963) correlated the development of the parotid salivary gland with the ingestion of solid material. However salivary gland weight variations were observed between left and right parotid and mandibular glands of individual animals (Figure 3.2 and 3.3). This cannot be accounted for from the evidence available but it is tempting to speculate that it reflects an animals preference to chew on one side relative to the other. In animals with cannulated glands a marked difference in weight between the cannulated and non-cannulated gland was noted (Figure 3.2). At least two factors may have contributed to this difference. Firstly, because the histology of the cannulated gland is altered (see section 3.5 and Figure 3.26) and its capacity for secretion consequently reduced the contralateral gland may be expected to undergo compensatory hypertrophy. Secondly, in the process of cannulation there may have been damage to the secreto-motor nerve which is closely associated with the excretory duct (Coats, Denton, Goding and Wright, 1956)

and a decrease in gland weight through partial dennervation. It is possible that the small (but variable) differences between left and right sublingual glands (Figure 3.4) are due to compensatory growth to offset reduced parotid output. No other reports involving differences in gland weights as a result of chronic cannulations appear to have been published.

In the present study the mean percentage contribution of each pair of glands to the total glands weighed is as follows :

Parotid	39.0%
Mandibular	47.8%
Sublingual	5.2%
Ventral buccal	8.0%

It can be seen that the percentage of secretory tissue of the parotid is less than that of the mandibular gland. Nevertheless, the rate and volume of saliva that the parotid glands contribute toward total salivary flow is much greater (Bailey, 1961).

In this study the intermediate and dorsal buccals, labial, palatine, lingual and pharyngeal glands were not dissected entirely or weighed because of their diffuse nature. However, Kay (1960) estimated that in sheep "residual saliva" (salivary secretion excluding parotid, mandibular and sublingual) flow was equal to parotid salivary flow. Because of the amount of secretory tissue present in the minor buccal glands and the soft palate in cattle (judged by dissection) it seems reasonable to extend Kay's (1960) findings to include cattle. Although the bovine mandibular is the heaviest salivary gland in that species its rate of secretion is approximately one fifth that of the parotid* and the predominantly

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mucous secreting glands which produce "residual saliva" secrete even more slowly. Therefore, it can be appreciated that, to produce a flow rate equal to that of the parotid, the weight of secretory tissue in the minor buccal glands and soft palate must be considerable.

4.2 HISTOLOGY AND HISTOCHEMISTRY

Salivary glands are usually described as compound, tubuloacinar glands, (Ham, 1974). Although this is a useful general definition it implies that all salivary glands are composed of secretory endpieces in the form of acini which drain into the first part of a branching duct system (Pinkstaff, 1980). As van Lennep et. al. (1977) noted, authors have tended to refer to all terminal secretory elements as acini, "a general term used in anatomical nomenclature to designate a small saclike dilation, particularly one found in various glands" (Dorland, 1965).

From the results of this study it can be seen that, at least in bovine parotid, sublingual and buccal salivary glands (Figure 3.15 and 3.23) this definition cannot be accurately applied. The arrangement of the secretory cells in these glands, rather than being in the form of a "saclike dilation", is in the form of a long, narrow, blind-ended tubule. A similar conclusion was reached by Shackleford and Wilborn (1968) who studied the histology of the sheep parotid gland.

In addition to the actual shape of the secretory endpieces there is controversy over the cell types which contribute to their formation. According to Pinkstaff's (1980) review most authors now agree that four cell types may participate in the formation of

secretory endpieces ; serous, mucous, seromucous and special serous cells.

A description of the histology and ultrastructure of serous cells is found in section 1.4.1. There are two possible implications in the designation "serous". The inference that serous cell secretions would contain secretory proteins might be drawn from the definition "of or resembling serum" (Dorland, 1965). On the other hand the adjective serous is commonly used to describe a watery fluid containing inorganic ions and possibly traces of serum derived proteins but being clearly distinct from mucus (Quintarelli, 1963). If Dorland's (1965) definition is accepted then secretory endpiece cells of the parotid and ventral buccal glands and the demilune cells of the sublingual, and labial glands are adequately described as serous cells despite the presence of protein granules in their cytoplasm (Figure 3.6, 3.16 and 3.25). In this case expressions such as "special serous", "proteoserous" or "atypical serous" need not be used. However, if the meaning expressed by Quintarelli (1963) is accepted then the abovementioned secretory cells could not be accurately described by the term serous and special serous or proteoserous would be more appropriate*. In the present study the use of the term serous is restricted to those cells, judged by their histochemical reactivity, or lack of reactivity, which produce a thin watery secretion containing inorganic ions and possibly small amounts of serum protein but no

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* In neither case could the term serozymogenic apply to the adult bovine species because the proteins in saliva do not appear to manifest enzymatic properties (Church, 1976).

detectable secretory protein. Cells which produce intrinsic secretory proteins or glycoproteins along with inorganic ions and water are described as proteoserous.

Mucous secreting cells are much more easily classified on the basis of cell morphology and histochemical reactivity than serous cells. Morphologically, mucous producing cells are irregularly cuboidal in shape and in the living state their cytoplasm contains numerous droplets of mucigen. In fixed and stained sections the droplets of mucigen are usually destroyed leaving only a loose network of cytoplasm and traces of precipitated mucigen which accounts for the characteristic "foamy" appearance of the cytoplasm of mucous cells (see Figure 3.12). Their nuclei are generally flattened, strongly basophilic and peripherally located (Bloom and Fawcett, 1975). Difficulties arise when information regarding the specific chemistry of the mucosubstance* is sought. A summary of the classification and histochemical methods for the demonstration of salivary carbohydrates is presented in appendices 6.1 and 6.2.

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*MUCOSUBSTANCE, MUCUS.
A viscous fluid characterised as carbohydrate rich glycoproteins and glycosamineglycans.

1. Glycoprotein, mucoprotein, mucin (Herp, Wu and Moschera, 1979).
Glycoproteins are molecules of high molecular weight (approximately 15,000 to 1,000,000) composed of two parts :

(a) protein moiety (25 - 50% by dry weight)
The protein core or backbone of the molecule is rich in serine and threonine, contains significant amounts of alanine and glycine and usually a large proportion of proline. It carries on average one O-glycosidically linked oligosaccharide side chain for every four amino acids.

(b) carbohydrate side chains
These are rich in sialic acid and hexosamines but often also

contain variable amounts of fucose and galactose. Among the sugars, sialic acid and fucose always occupy non-reducing terminal positions of the oligosaccharide side chain. The side chains are linked to the polypeptide core by an O-glycosidic bond through N-acetylgalactosamine to the hydroxyl group of serine and threonine.

2. Glycosamineglycans, acid mucopolysaccharide (Reid and Clamp, 1978)
These molecules also contain a polypeptide core but the carbohydrate component of glycosamineglycans differs from the glycoproteins in several respects.

(a) Side chains contain 50 or more monosaccharides whereas glycoproteins contain 25 or less.

(b) Linkage to the protein core is by xylose not N-acetyl-galactosamine.

(c) The side chains contain a repeating disaccharide structure. Glycoproteins have few or no repeating units.

(d) The shape of the carbohydrate component is linear and unbranched whereas in glycoproteins the side chains branch.

(e) Hexuronic acid is present in glycosamineglycans but absent in glycoproteins.

In general mucosubstance molecules may be conceived as comprising of long polypeptide backbones to which are linked numerous carbohydrate side chains of glycoprotein, glycosamineglycans, or sometimes a mixture of both.

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There exist several schools of thought concerning the criteria for classifying seromucous secretory cells. Shackleford and Klapper (1962) and Shackleford and Wilborn (1968) proposed a method of classification of secretory cell types in salivary glands based upon the histochemical staining of mucosubstances. They suggested that cells having the usual morphological characteristics of protein secreting cells and which lack acidic mucosubstances (AB and AY negative) be called serous cells (these cells may contain neutral mucosubstances and thus be PAS positive). Shackleford and Wilborn (1968) believed that the term seromucous should only be applied to those cells that contained appreciable amounts of acidic mucosubstances. These cells

would therefore stain positively with AB or AY and probably with PAS also. Munger (1964) proposed a classification system in which cells that contained appreciable amounts of mucosubstances should be termed seromucous irrespective of the type of mucosubstance. This latter system of classification has never gained wide acceptance.

The fourth cell type which may be present in a secretory endpiece is the special serous cell. It is defined as a cell that is poor in mucosubstance; the mucosubstance may be neutral, but it cannot be acidic (Pinkstaff, 1980). At the ultrastructural level, special serous cells are seen to have very low concentrations of RER. Their cytoplasmic granules have an ultrastructural appearance much like that of mucous granules but they do not stain like mucous granules when histochemical stains are used. This type of cell has been found to occur in the secretory endpieces of bovine and ovine parotid glands (Shackleford and Wilborn, 1968). van Lennep et. al. (1977) have verified the special serous nature of the sheep parotid gland secretory endpiece cells although they do not use the term "special serous".

Although it has long been considered to be absent (McDougall, 1948, Kay, 1960) the presence of protein in parotid saliva of ruminants has recently been established by physiological and biochemical investigations by Patterson and Titchen (1976) and Gurnsey, Jones and Reid (1980). Immunocytochemistry performed as part of the present study provides evidence that at least some of this protein is produced by the secretory endpiece cells (see Figure 3.6). The high concentration of goblet cells in the interlobular, interlobar and excretory ducts (Figure 3.10) would also add small quantities of glycoprotein to parotid saliva. It is not possible, from these results, to infer

a function for this goblet cell secretion except perhaps to provide a protective glycoprotein layer on the surface of the duct cells to prevent bacterial and viral adherence.

The validity of using the PAS technique for demonstrating the presence of certain conjugated proteins, glycoproteins in secretory cells may be questioned on the grounds that PAS reactivity is a characteristic of carbohydrates not proteins. However, Leblond, Glegg and Eidinger (1957), in a paper related to the specificity of PAS as a sensitive test for carbohydrate-protein complexes, produced conclusive evidence that the PAS positive materials investigated included hexosamine, galactose, fucose and mannose. These investigators were among the first to show that sialic acid could also give a positive PAS reaction. On the basis of their results, they proposed that PAS reactive substances, with the exception of glycogen, represent carbohydrate-protein complexes.

Quintarelli, Tsiuka, Hashimoto and Pigman (1960) reported that bovine mandibular gland mucous endpiece cells were not metachromatically stained with toluidine blue but were selectively stained by AB. The present work confirms this finding and in addition has shown the same cells to be AY and PAS positive (see Figure 3.12 and 3.13). On the basis of these three staining reactions it may be suggested that bovine mandibular gland secretory cells synthesize three different mucosubstances which co-exist, i.e. sulphated mucopolysaccharide (AB positive) carboxylated mucopolysaccharide (AY positive) and a neutral glycoprotein (PAS positive). However, before such a suggestion can be proven further factors must be given consideration. Nisizawa

and Pigman (1959) found that the bovine mandibular gland contained only traces of neutral sugars. Quintarelli et. al. (1960), using influenza virus neuraminidase noticed an unmistakable reduction in the intensity of PAS staining following enzymatic hydrolysis. Taking these two results together, it seems that the PAS reaction could be accounted for only by unsubstituted glycols of N-acetylgalactosamine, sialic acid or both. If, therefore, a PAS reduction is noticed after neuraminidase treatment (Quintarelli et. al., 1960) or after mild acid hydrolysis or acetylation (Shackleford and Klapper, 1962), such reduction should be interpreted as due to the removal of sialic acid thus proving that the acid, in itself, if PAS reactive. In the present study it was found that in addition to the mandibular, mucous cells of other glands; dorsal buccal, intermediate buccal, labial and the glands of tongue and soft palate, reacted in a similar way to AB and PAS (see Figures 3.17, 3.18, 3.22 and 3.25). Thus it can be argued that these glands produce a mixed mucosubstance containing neutral glycoprotein along with sulphated and carboxylated mucopolysaccharides. The PAS positive nature of the parotid gland secretory cells (Figure 3.6) is probably due to neutral glycoproteins.

The granular cells observed within the walls of intralobular ducts of the parotid glands (Figure 3.9) have also been noted by other workers (Shackleford and Klapper, 1962). They described these cells as resembling mast cells and found that they were metachromatic (before and after sulphation) but did not stain well with PAS or AB. The results from the present study show that the "intra-striated duct cells" are also present in the ventral buccal glands. The cells were

AB positive, weakly reactive to PAS and strongly pyroninophilic. Sometimes the cells had the appearance of mast cells but more often they closely resembled plasma cells (Figure 3.9). Because of their sialic acid content (Tomasi and Grey, 1972) immunoglobulin may cause positive reactions with AB (Figure 3.9). This study also revealed that these granular cells were not a consistent feature of all bovine parotid or ventral buccal glands studied. It is tempting to correlate the abundance or lack of these cells to the animals bloat susceptibility (see table 3.2). Perhaps the presence of these granular cells within the walls of the intralobular ducts of the parotid and ventral buccal glands in some ways modifies the composition of the saliva formed by altering the physiological status of the surrounding duct cells by releasing a pharmacologically active secretory product. Alternatively, the granular cells may produce some additional substance which is active, or becomes active, in the reticulo-rumen to control microbial activity or prevent the formation of a stable foam. One possibility is that the granular intra-striated duct cells of the parotid gland are plasma cells producing IgA. If this were the case it would certainly serve to emphasize the protective function of salivary secretion in the ruminant.

In the mandibular and other glands the plasma cells are found in the connective tissue around intralobular ducts and secretory endpieces (Figure 3.13 and 3.25). Plasma cells synthesize and release IgA, having a molecular weight of 170,000 daltons and consisting of four polypeptide chains, into the interstitial fluid. By transudation these molecules enter the cytoplasm of the intralobular duct epithelial cell where two molecules of IgA become united to each other by an additional polypeptide chain called the J chain. The resulting complex

is bound to a glycoprotein with a high carbohydrate content, called the secretory piece, which is produced by the epithelial cells (Bloom and Fawcett, 1975). The resultant molecule of secretory IgA has a molecular weight of 390,000 daltons and if assembled in the cell would be released into the duct lumen by exocytosis. Lamm (1976) has shown that secretory component can be demonstrated in the supranuclear Golgi zone of the cell. The cells of the intralobular ducts of the parotid gland and to a lesser extent in the mandibular gland show variability in their reactivity to PAS. The PAS positive staining of these duct cells is not due to stored glycogen because staining persists following diastase digestion (Figure 3.8) and the possibility thus exists that the PAS positive granulation is due to the presence of secretory component. Since these cytoplasmic granules are located throughout the upper part of the cell and included within "apical blebs" a suggestion made by Lamm (1976) that the possible location of assembly of the secretory IgA molecule is within the interior of the cell, seems appropriate (see Figure 3.8).

Just over a decade ago, Watson and Lascelles (1970) speculated that the high levels of secretory IgA in non-parotid saliva could be explained by the tissues producing this saliva being constantly subjected to antigenic stimulation due to the low flow rates and the comparitive proximity of the glandular masses to the buccal epithelium. Conversely they argued that the reason why parotid saliva contains very low levels of secretory IgA was due to the continuous flow and large volume of secretion from the gland which prevented significant local antigenic stimulation. More recent

work suggests that this explanation is an over-simplification of the processes involved in stimulating IgA producing plasma cells. Tomasi (1976) and Fudenberg, Stites, Caldwell and Wells (1976), have shown that antigenic stimulation of small lymphoctes which transforms them into the immunoblast line of cell differentiation requires the help of T lymphocytes and the site of antigenic stimulation of B lymphocytes may not necessarily be at the same location where IgA synthesizing plasma cells are found. The exact origin of the precursors of plasma cells in mammals is still unsettled.

It thus seems, in the light of more recent work, that direct antigenic stimulation of salivary gland tissues (if, in fact it does occur) is probably not responsible for the establishment of populations of IgA producing plasma cells. Why concentrations of plasma cells vary considerably from one salivary gland to another is a question still to be satisfactorily answered.

4.3 CANNULATED GLANDS COMPARED WITH NON-CANNULATED

Apart from the weight variations already discussed the general effect of cannulation on the parotid and mandibular glands was to distend the intralobular duct system and cause degenerative changes in the secretory endpiece cells. These changes are assumed to be pressure related and may have arisen due to back pressure on the gland caused by ;

(a) long cannulae

(b) transmitted pressure waves within the cannulae generated by a mechanical pump used to both measure the volume of saliva secreted and return it to the animal

(c) blockage of the cannulae by cellular debris or inflammation of the duct-cannulae junction.

Cells associated with the inflammatory process, namely small lymphocytes, PMN neutrophils, plasma cells and mast cells were not a consistant feature of histological changes in chronically cannulated glands. The contralateral mandibular, sublingual and minor buccal glands in cannulated animals showed evidence of increased output (possibly as a compensatory mechanism) as a reduction of secretory granules or degranulation of their demilune cells. These results emphasize the dangers involved in relating salivary flow rates and compositional studies from cannulated glands to be normal physiological state in non-cannulated animals.

5. CONCLUSION

The contribution of salivary secretion to the fermentative digestion of ruminants is well established (McDougall, 1948, Turner and Hodgetts, 1955, Kay 1960). In addition to this, total salivary secretion or components of that secretion exhibit important protective functions. The mucus component of saliva, because of its viscous nature, adhere to epithelial surfaces thus resisting their dissication. The lubricating qualities of mucus aid in deglutition and protect the epithelial linings of the buccal cavity, oesophagus and reticulo-rumen against mechanical damage or abrasion from the often coarse diet. In addition, mucus is a nutrient and source of nitrogen for ruminal microbes. In the bovine species this mucus is produced from a wide variety of glands associated with the mouth and pharynx - of the glands studied in the present investigation only the parotid and ventral buccal glands were found not to contain mucous secretory endpiece cells. Total saliva contains large volumes of water and electrolytes which act as a taste vehicle, soften the food as an aid to mastication, promote oral hygiene and maintains the correct level. consistancy and pH of fluid in the rumen to create optimum conditions for fermentative digestion. The secretory endpiece cells of the parotid, ventral buccal and demilune cells of the dorsal buccal glands, histologically display the characteristics of cells capable of producing water and electrolytes as well as various proteins. Salivary proteins affect microbial activity and replication so that a paucity of certain proteins (band 4 salivary protein and probably others) may

influence an individual animals susceptibility to bloat. Lysozyme is a cationic, low molecular weight protein (enzyme) found in salivary secretion which reduces the local concentration of bacteria by attacking the micropeptides of their cell walls. Saliva thus contains an antibacterial substance in addition to immunoglobulins. The principal immunoglobulin present in salivary secretions is IgA which has antiviral and antibacterial properties as well as the ability to inhibit absorption of nonviable antigens across epithelial cells of mucous membranes. In monogastric species salivary IgA acts in a protective capacity in the mouth and oesophagus and it seems likely in ruminants this function is extended to include the epithelial linings of the rumen, reticulum and omasum since no plasma cells are normally found within the walls of these organs. The distribution of plasma cells throughout the salivary glands varies from one gland to another. The parotid ventral buccal, intermediate buccal, dorsal buccal, posterior tongue, soft palate contain very few plasma cells whereas the mandibular, sublingual, labial and the glands of the epiglottis are bountifully populated.

5.1 BOVINE SALIVARY GLAND CLASSIFICATION

Based on the results of this study the parotid and ventral buccal glands are proteoserous while the mandibular, sublingual, intermediate buccal, labial and the glands of the lingual surface of the epiglottis are mixed; mucous endpiece cells with proteoserous demilunes. Only one gland was found to be truely mucoserous: the upper buccal. The glandular masses associated with the posterior

tongue and soft palate are predominantly mucus producing with a small proteoserous component.

The mucous cells of bovine salivary glands react uniformly with AY and PAS indicating the production of carboxylated mucopolysaccharide and neutral mucopolysaccharide respectively. AB staining was variable indicative of differing levels of sulphated mucopolysaccharide production.

Based on their negative reactivity to mucosubstance and glycoprotein histochemistry the demilune cells of the dorsal buccal glands were assessed to be purely serous. All other demilune cells contained cytoplasmic granules which reacted positively with AB or PAS or both.

Arrangements of secretory cells within the salivary glands have traditionally been called acini or alveoli but, despite the persistance of this term in the literature, it conveys a false idea of the shape or form of the secretory cell arrangement. The 'secretory endpiece' is a preferred term, since, at least in bovine salivary glands, the secretory cells are arranged in a blind-ended tubular fashion of variable diameter and length.

While the objective of this study was to broaden understanding of the histochemical nature of the cells which produce salivary secretions in the bovine, deficiencies in current knowledge have also been identified in certain areas. In particular this study raises questions over :

1. The possible relationship between intra-striated duct cells of the parotid gland and bloat.

2. The specific location of cells responsible for the synthesis of salivary proteins other than band 4.

3. The specific influence of organic components of saliva (glycoproteins, glycosamineglycans and secretory IgA) on fermentative digestion.

4. The stimulus for the differential populations of plasma cells resident within salivary glands.

It is the authors hypothesis that all biological structures and cellular secretions possess a physiological role. At present the functions of many cellular structures and components of exocrine secretion are unknown. Many relationships between histochemical findings and function have been well established while other histologically demonstratable properties of salivary glands have a proposed function and yet others provide a challenge for further investigation.

6. APPENDICES

6.1 APPENDIX

SUMMARY OF THE NATURALLY OCCURRING POLYSACCHARIDES

Group I Neutral Polysaccharides - Non ionic heteroglycans

1. Glycogen
2. N-acetyl-glucosamine containing

Group II Acid mucopolysaccharides - Anionic heteroglycans (glycosamineglycans)

This entire group are thought to be attached to protein (but not by co ordinate bonds).

1. Carboxylated
2. Sulphated

Group III Glycoproteins - Mucins, Mucoproteins

1. Neutral
2. Carboxylated - sialoglycoprotein. Contains sialic acid but no sulphate.
3. Sulphated - sulphated sialoglycoprotein. Contains both sialic acid and sulphate.

Group IV Cerebrosides and Phosphatides

These have been inserted only for completeness.

Cerebrosides have a fatty residue bound to a carbohydrate. The only connection they have with the above is that they are periodic acid Schiff positive (PAS+ve).

(Culling, 1974)

6.2 APPENDIX

6.2.1 HISTOCHEMISTRY OF SALIVARY GLAND CARBOHYDRATES

Most of the carbohydrates present in saliva are polymers of carbohydrate linked covalently to protein and in which the protein moiety may constitute a majority or minority of the residues present. The ratio of protein to carbohydrate is one form of classification but a useless one in a histochemical study since the demonstration of carbohydrate and protein at the same site is no proof that they are part of the same molecule. The only information that can be derived histochemically is based on the reactive groups that are demonstratable in carbohydrates ;

(i) 1:2 glycols

(ii) Carboxyl and Ester Sulphate

Additional information can be gained from enzyme hydrolsis using

(i) diastase

(ii) hyaluronidase

(iii) sialidase

(iv) various chemical procedures such as blocking techniques, methylation, and saponification.

6.2.2 HISTOCHEMICAL METHODS FOR THE DEMONSTRATION OF GROUP III POLYSACCHARIDES

1. PERIODIC ACID SCHIFF (PAS)

Periodic acid oxidises tissue and cleaves C-C bonds where they have adjacent OH groups (1;2 glycol) and one of the reaction products is an aldehyde which then reacts with Schiffs reagent. Any substance that has a :

(i) 1:2 glycol

(ii) 1:2 amino hydroxyl

(iii) Oxidation product CHOH.CO will give a PAS+ve reaction providing :

(i) the substance will not diffuse during the course of fixation;

(ii) the oxidation product of the substance will not diffuse;

(iii) the final concentration is sufficient to give a detectable colour.

2. ALCIAN BLUE (AB)

Is a water soluble copper phthalocyanin, thought to stain by salt linkage to acidic groups when used at a low pH. By the use of AB at specific pH, and in combination with other stains and reactions, it is possible to identify the acidic groups present as either sulphate (OSO_3H) or carboxyl (COOH).

A. Alcian Blue at pH 1.0

OSO_3H groups	AB+ve
COOH groups	AB-ve

B. Alcian Blue at pH 2.5

OSO_3H groups	AB-ve
COOH groups	AB+ve

C. AB/PAS

This combination can be used to differentiate between acid mucopolysaccharides and neutral mucopolysaccharides but provides no information regarding sulphate or carboxyl groups present. It should be pointed out that sialic acid, which is present in relatively large amounts in salivary mucoproteins, will react positively to both AB and PAS because the molecule contains a carboxyl group in

addition to a 1:2 glycol.

OSO_3H and COOH groups - blue

Neutral groups - pink

Mixed groups - purple

D. AB/Gomoris Aldehyde Fuchsin

Gomoris Aldehyde Fuchsin is a combination of basic fuchsin and aldehyde in the presence of a strong mineral acid. It positively identifies sulphated mucopolysaccharides and in combination with AB stains :

OSO_3H groups - purple

COOH groups - blue

Mixed groups - violet purple

E. AB/Alcian Yellow

Alcian yellow is a related dye to alcian blue that has been used in combination with alcian blue to confirm the presence of carboxyl or sulphate groups.

OSO_3H groups - blue

COOH groups - yellow

Mixed - green

F. AB/Safranin

Safrainin is a red coloured dye which possesses metachromatic properties (orange). It has an affinity for carboxyl groups and thus can be used in combination with alcian blue to distinguish between carboxyl and sulphate groups.

Strongly OSO_3H groups - blue

Strongly COOH groups - red-orange

3. HALES COLLOIDAL IRON

The reaction is based on the affinity of acid groups for colloidal iron at low pH. Iron forms a chelate with OSO_3H. The bound Fe^{++} can then be demonstrated by Perls Prussian Blue reaction.

4. TOLUIDINE BLUE

The metachromatic properties of this dye are ultilised to demonstrate high molecular weight substances with free anionic (acid) groups e.g. acidic glycoproteins. Metachromasia may be defined as the staining of a tissue component so that the absorption spectrum of the resulting dye tissues complex differs sufficiently from that of the original dye and from its ordinary tissue complexes, to give a marked contrast in colour. The change in colour is thought to be due to polymerisation. The presence of high molecular weight substances with free anionic groups is essential for metachromasia. Sulphonate groups (SO_3H) are stronger chromotropes than are carboxyl (COOH) or phosphate (PO_4) groups. Metachromasia of polysaccharides can be induced by sulphation using sulphuric acid.

6.2.3 METHODS FOR SIALIC ACID IDENTIFICATION

Large amounts of sialic acid have been reported to be present in the salivary secretions of sheep and cattle (Quintarelli, 1963). Because sialic acid contains both 1:2 glycol grouping and COOH groups it will react with both AB and PAS. However, AB+ve staining and PAS+ve reaction does not necessarily indicate the presence of sialic acid.

Positive identification of sialic acid is mainly carried out by staining the tissue with AB and PAS before and after blocking

techniques. (Blocking techniques are those which, although failing to give a colour reaction with a certain tissue element, will combine with that tissue element in such a way as to prevent it from giving a colour reaction with other agents). These techniques can be used to identify the reactive groups responsible for AB+ve staining or PAS+ve reaction. The more commonly applied blocking techniques are :

(i) Aldehyde Blocking Technique

By blocking aldehyde groups in a section oxidised by periodic acid, it can be shown that a positive Schiff reaction may be given to substances other than aldehydes. The blocking is done by treating the section with an aniline/acetic acid mixture.

(ii) Acetylation

Will block the reactivity of 1:2 glycol grouping. A PAS+ve substance, after treatment with acetic anhydride will give a PAS+ve reaction. This would indicate that the original reaction was due to a 1:2 glycol; grouping. Acetylation can be reversed by potassium hydoxide treatment call deacetylation or :

(iii) Saponification (KOH treatment)

Will restore basophilia or AB+ve staining of COOH groups which have been esterified by methylation or restore PAS+ve reactivity of 1:2 glycol groups following acetylation. Saponification may also increase PAS reactivity of certain Group III polysaccharides.

(iv) Methylation

Entails the treatment of tissue sections with a mixture of methyl alcohol and hydrochloric acid. Methylation blocks or eliminates AB staining of COOH groups, including sialic acid and OSO_3H attached to

glycoproteins.

Thus the demonstration of sialic acid utilising blocking techniques in association with AB and PAS techniques is essentially a negative one. Sialic acid can be removed by :

1. Methylation
2. Mild acid hydrolysis
3. Sialiadase digestion

The removal of sialic acid is shown by loss of AB+ve staining.

Chemical studies of different epithelial mucosubstances have greatly clarified their chemical composition. In human and animal tissues it has been found that the predominent glycoprotein carbohydrate is made up of hexosamine glactose and fucose (Quintarelli, 1963). It is also reported that all mucous secretions studies contain varying amounts of sialic acid. Thus two main types of glycoproteins are recognised : Fucomucins and sialomucins. A third group of carbohydrates, the acid mucopolysaccharides or glycosamineglycans have also been found to be present in mucous secreting cells.

6.3 APPENDIX

6.3.1 HAEMATOXYLIN AND EOSIN H&E

METHOD

1. Dewax and bring sections to water.
2. Stain in Mayers Haemalum for 10 minutes.
3. Rinse in tapwater.
4. Blue in Scotts tapwater for 2 minutes.
5. Rinse in tapwater.
6. Stain in 1% aqueous Eosin Y for 2 minutes.
7. Rinse rapidly in tapwater.
8. Dehydrate, clear and mount.

6.3.2 ALCIAN BLUE AB

METHOD

1. Dewax and bring sections to water.
2. Stain in 0.3% Alcian blue 8GX in acetic acid pH 2.5 for 10 minutes.
3. Rinse in tapwater.
4. Dehydrate, clear and mount or counterstain as desired.

6.3.3 TOLUIDINE BLUE

METHOD

1. Dewax and bring sections to water.
2. Stain in 0.5% toluidine blue in acetate buffer pH 4.5 for 2 minutes.
3. Rinse quickly in tapwater.
4. Rapidly dehydrate, clear and mount.

6.3.4 ALCIAN YELLOW AY

METHOD

1. Dewax and bring sections to water.
2. Stain in 0.5% alcian yellow in 3% acetic acid for 30 minutes.
3. Wash in tapwater.
4. Counterstain as desired or dehydrate, clear and mount.

6.3.5 PERIODIC ACID SCHIFF PAS

METHOD

1. Dewax and bring sections to water.
2. Oxidise in 1% periodic acid for 8 minutes.
3. Wash in running tapwater for 5 minutes.
4. Wash in at least 3 changes of distilled water.
5. React with Schiffs reagent for 10 minutes.
6. Wash in running tapwater for 10 minutes.
7. Stain in Mayers Haemalum for 5 minutes.
8. Rinse in tapwater.
9. Blue in Scotts tapwater for 2 minutes.
10. Rinse in tapwater.
11. To counterstain, rinse in 70% and 95% alcohol.
12. Stain in a saturated solution of tartrazine in cellosolve (ethylene glycol monoethyl ether) for 2 minutes.
13. Differentiate and dehydrate in 2 changes of absolute alcohol.
14. Clear and mount.

TO PREPARE SCHIFFS REAGENT

To 400 ml of distilled water warmed to 37°C add 2 grams of parafuchsin and agitate until dissolved. Add 7.6 grams of sodium metabisulphite and 2 millilitres of concentrated hydrochloric acid. Shake well for 10 minutes. Store in a dark cupboard at room temperature overnight. The solution should then be straw coloured. Add 2 grams of activated charcoal, shake well and filter. The solution is ready for use. Store in refrigerator.

6.3.6 ALCIAN BLUE/HAEMATOXYLIN AND EOSIN AB/A&E

METHOD

1. Follow AB method steps 1-3.
2. Continue with H&E method steps 2-8.

6.3.7 ALCIAN BLUE/HAEMATOXYLIN/PERIODIC ACID SCHIFF AB/H/PAS

METHOD

1. Follow AB methods steps 1-3.
2. Continue with PAS method steps 2-10.
3. Dehydrate clear and mount.

6.3.8 ALCIAN BLUE/ALCIAN YELLOW/HAEMATOXYLIN AND EOSIN AB/AY/H&E

METHOD

1. Follow AB method steps 1-3.
2. Continue with AY method steps 2-3.
3. Then H&E method steps 2-8.

6.3.9 ALCIAN BLUE/ALCIAN YELLOW/HAEMATOXYLIN/PERIODIC ACID SCHIFF AB/AY/H/PAS

METHOD

1. Follow AB method steps 1-3.

2. Continue with AY method steps 2-3.

3. Continue with PAS method steps 2-10.

4. Dehydrate, clear and mount.

6.3.10 ALCIAN YELLOW/HAEMATOXYLIN/PERIODIC ACID SCHIFF AY/H/PAS

METHOD

1. Follow AY method steps 1-3.

2. Continue with PAS method steps 2-10.

3. Dehydrate, clear and mount.

6.3.11 METHYL GREEN PYRONIN (unna Pappenheim)

METHOD

1. Dewax sections and bring to water.

2. Stain in Unna Pappenheim solution for 30 minutes.

3. Blot sections dry with filter paper.

4. Dehydrate using absolute acetone.

5. Transfer to a solution of 50% acetone, 50% xylene.

6. Clear and mount.

TO PREPARE METHYL GREEN PYRONIN SOLUTION

0.5% aqueous pyronin Y	37 ml
0.5% aqueous methyl green	13 ml
acetate buffer pH 4.8	50 ml

Methyl green should be extracted using chloroform before use.

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