A STUDY OF MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN THE MANDIBULAR GLAND OF THE SHEEP ASSOCIATED WITH EATING AND DIRECT STIMULATION

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ABSTRACT

A STUDY OF MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN THE MANDIBULAR GLAND OF THE SHEEP ASSOCIATED WITH EATING AND DIRECT STIMULATION

by Punnipa Ariyakulkaln

This study was undertaken to investigate relationships between the structure of the mandibular gland of the sheep and its secretory activity in response to feeding, direct stimulation of autonomic nerves, or pharmacological agents that mimic the action of autonomic transmitters. Forty-five crossbred Romney ewes and wethers were used in acute experiments and twenty-two in chronic experiments.

Histochemical and electron microscopical examinations of the structure of mandibular glands confirmed that their secretory endpieces are composed of mucous tubulo-acinar cells and seromucous demilunes. The mucous acini contained a single type of electron lucent granules, whereas the granules of demilunes typically exhibited a tripartite structure. The intercalated ducts were relatively short and lined by non-secretory, simple cuboidal cells and occasional basal cells. Striated ducts were numerous and lined by four cell types, the most common of which (type-I) were tall, columnar, electron lucent cells with well developed membrane infoldings basally with associated mitochondria and small, dense, apical bodies. Myoepithelial cells were distributed densely around the secretory endpieces and within the basement membranes. Myoepithelial cells were also found embracing the intercalated duct cells.

Both AChE-positive and biogenic-amine fluorescent nerve fibres were present around the secretory endpieces and the walls of blood vessels. Fewer biogenic-amine fluorescent fibres were seen in relation to duct cells. Electron microscopy showed unmyelinated fibres in both epilemmal and

hypolemmal sites. The epilemmal axons were frequently found close to a variety of effector cells, while hypolemmal axons were observed occasionally in the intercellular space between adjacent striated duct cells and between intercalated duct and mucous cells. Axons containing large granular vesicles were also found within interstitial nerve bundles.

Mandibular secretion was studied after cannulation of the mandibular duct in both acute and chronic experiments. In anaesthetized animals, stimulation of either the chorda lingual nerve (3-8V, 5-10Hz, 0.2 msec) or injection of carbachol (40 $\mu g\ kg^{-1}$ body weight, iv) within 10-25 sec caused a copious secretion (0.33-0.74 $g\ min^{-1}$) of low protein content (0.44-1.56 $mg\ ml^{-1}$). This response was completely blocked by atropine (0.1 $mg\ kg^{-1}$ body weight). In contrast, stimulation of cervical sympathetic trunk (3-8V, 5-10 Hz, 0.2 msec) after a latency of 35-102 sec caused a meagre secretion (0.01-0.06 $g\ min^{-1}$) of high protein concentration (4.02-25.68 $mg\ ml^{-1}$). Isoprenaline had similar effects. Secretory responses to sympathetic stimulation were blocked by propranolol (1.0 $mg\ kg^{-1}$ body weight). Studies involving gel electrophoresis demonstrated major protein bands exclusively in the sympathetic nerve or isoprenaline stimulated saliva. These major protein components (both soluble and insoluble) were found by immunocytochemical studies to be localized in the demilunes and some striated duct cells of the resting gland.

It was found that in sheep fed lucerne chaff (ca. 1,000 g daily) a rapid and sustained mandibular flow only occurred during eating, although, short term increases were seen, for example, during drinking. Flow was absent during rumination and slight ($0.95 \pm 0.09\ g\ h^{-1}$) or absent at rest. Saliva produced during eating had its highest protein concentration almost immediately as eating commenced ($1.65 \pm 0.06\ mg\ ml^{-1}$) and remained at a high level during the first hour of eating ($1.55 \pm 0.06\ mg\ ml^{-1}$). Propranolol (1.0 $mg\ kg^{-1}$ body weight, iv) caused significant reductions

in protein secretion during eating ($p<.001$) without associated changes in flow. Gel electrophoretic studies confirmed the presence of a major protein band similar to soluble protein band X found in sympathetically evoked saliva. The intensity of this major protein band in saliva collected during eating was also reduced after propranolol treatment. Saliva collected during teasing had a high protein concentration (2.73 ± 0.20 mg ml^{-1}). It is concluded that sympathetic activation was involved mainly early in the eating period and that parasympathetic nerves were active throughout. The latter was confirmed by a great reduction in flow after injection of atropine (0.1 mg kg^{-1}, iv).

Morphological studies of the glands of sheep whose food had been withheld for 20 hours revealed that both the mucous acini and seromucous demilunes were filled with secretory granules. Stimulation of the chorda lingual nerve for 2-4 hours caused acini to discharge their contents of secretory granules, but no appreciable changes in the demilunes. On the other hand, stimulation of the cervical sympathetic trunk produced varying degrees of degranulation in the demilunes, with, in some cells, vacuolation. Infusion of isoprenaline (2h; 0.3 µg kg^{-1} min^{-1}) produced similar changes in demilunes. Striated duct cells showed reduced PAS-staining, and disruption of their basal regions, particularly after stimulation of sympathetic nerves. Concurrent stimulation of both sympathetic and parasympathetic nerves resulted in a combination of the above separate effects.

Eating led to extensive degranulation and greater evidence of synthesis in the mucous acini than parasympathetic nerve stimulation, the changes increasing with the duration of eating, and a depletion of secretory granules in demilunes that could be prevented by propranolol. (1.2 mg kg^{-1} body weight, iv). The morphological changes in demilunes were not proportional to the duration of eating but were greatest in its early phases. Evidence of small dense bodies which were apparently discharged *via* the apical membrane of striated duct cells and a loss of PAS-staining in these cells suggest that they secrete during eating. However, neither damage

to striated duct cells nor secretory endpieces was evident.

The results suggest that the sheep mandibular gland is naturally stimulated by both divisions of the autonomic nervous system, with acinar cells predominantly under the parasympathetic and demilunes under the sympathetic control. The sympathetic stimulation of salivary protein secretion appears to be mainly mediated *via* a β-adrenergic mechanism whereas the secretion of fluid and probably also mucus glycoproteins is an atropine-sensitive parasympathetic effect.

On both morphological and physiological grounds it is suggested that in sheep mandibular glands, myoepithelial cell contraction is important in assisting the secretion of viscous saliva.

Further studies on the following areas would seem appropriate: (i) systematic morphological studies using stereological analysis of changes in the acinar cells, demilunes, striated ducts and their cytoplasmic components; (ii) ultrastructural examinations of the innervation pattern in this gland under normal conditions, after specific denervation and reinnervation; (iii) studies of the nature and origin of the salivary proteins secreted during eating and nerve stimulation and (iv) the use of chronically cannulated animals for studies of the influence of different conditions of feeding.

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CHAPTER I

INTRODUCTION & LITERATURE REVIEW

A. INTRODUCTION

This thesis describes an experimental study of the mandibular[1] salivary gland in sheep (*Ovis aries* Linnaeus, 1758) undertaken to examine the relationships between the morphology of the gland, its neural activation and the nature of its secretion with a view to explaining the changes that occur during eating. The basic features of the gland are presented diagrammatically in Figure 1.1, which illustrates its mixed population of secretory cells and their relationships with each other and with cells of the duct system.

As a prelude to describing the experimental work and to give it perspective, this chapter first briefly reviews the development of studies on salivary secretion. A detailed description of the anatomical and histochemical characteristics of the mandibular gland is then followed by a discussion of the literature on the composition of mandibular saliva and the regulation of its secretion in ruminants.

B. HISTORICAL BACKGROUND

The importance of saliva for moistening food and lubricating its passage to the stomach has long been recognized. Three centuries ago Regner de Graaf (1677) observed, from a chronic fistula of the mandibular duct in a dog, an increase of salivary flow during mastication and swallowing, and also noted that salivation occurred in

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[1] The terms mandibular, maxillary, submaxillary and submandibular have all been used for this gland. The term "mandibular gland" is prescribed by the Nomina Anatomica Veterinaria (1973).

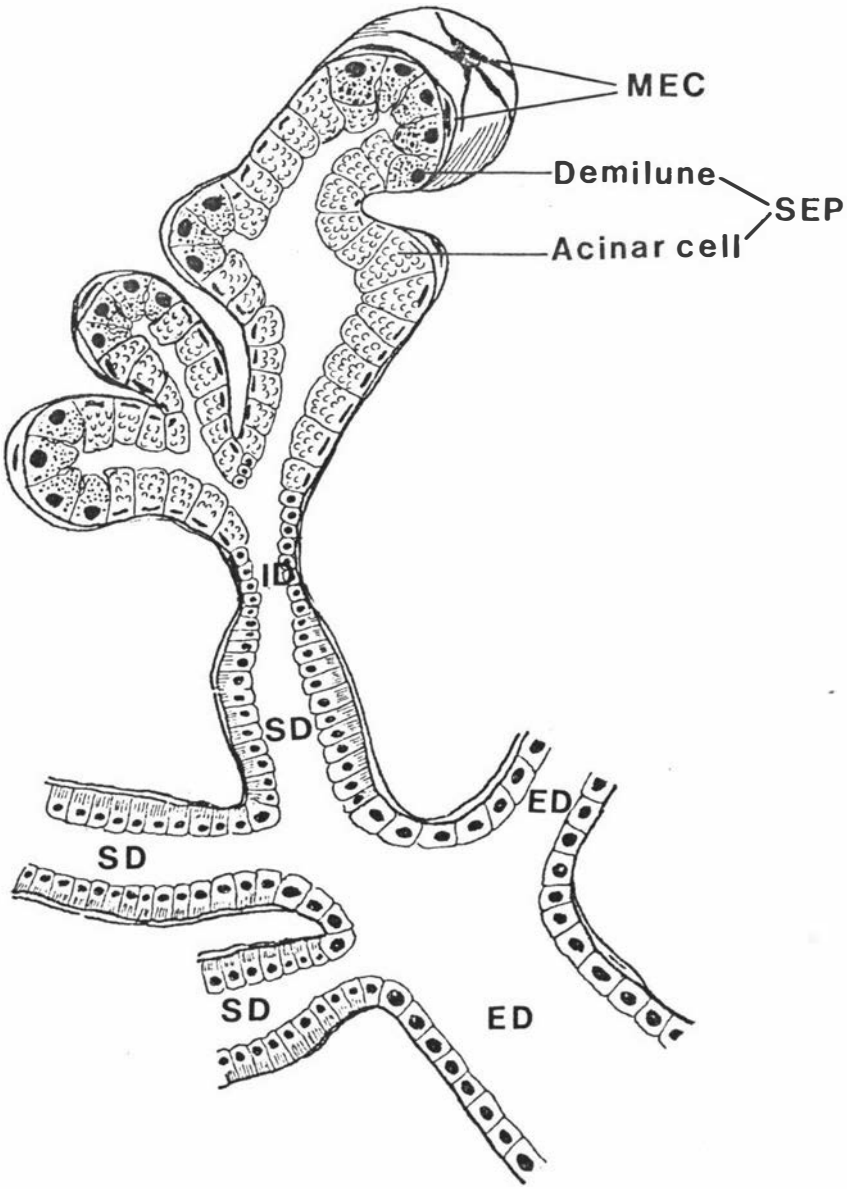

Figure 1.1: Diagrammatic representation of the structure of a mandibular salivary gland.
The gland is composed of secretory endpieces (SEP) and a system of intralobular ducts comprising intercalated ducts (ID) and intralobular striated ducts (SD) which lead to the extralobular duct system, excretory ducts (ED). All excretory ducts empty into the main excretory duct which opens in turn into the oral cavity. Myoepithelial cells (MEC) embrace the secretory endpieces and intercalated ducts.

response to the smell and taste of food. It was not until 19th century, however, that major advances were made toward understanding how secretion is regulated: the role of nerve fibres in secretion was established experimentally by Ludwig (1851) who demonstrated that saliva flowed from the dog's mandibular gland when the distal end of a cut lingual nerve was stimulated. Bernard (1856, 1858) furthered our understanding of salivary secretion with key observations on its reflex nature, and introduced the concept of salivary centres in the central nervous system. He also noted an increased glandular blood flow during stimulation of the chorda tympani nerve and argued that vasodilator nerve fibres were responsible (Bernard, 1858).

At about that time, as shown mainly through the work of Heidenhain (1868, 1883) and Langley (1879, 1889) there developed an interest in the cellular mechanisms associated with secretion. Langley (1879) studied the relationship between the histological structure of salivary gland cells and their secretory activity during stimulation of the autonomic nervous system. Using rabbits, cats and rats, he demonstrated that the acini of parotid glands of hungry animals are loaded with granules, and become depleted of these granules after eating or after electrical stimulation of autonomic nerves. Langley (1879) also commented that such degranulation was especially pronounced after dual sympathetic and parasympathetic stimulation, when the remaining granules tended to form a thin layer in the luminal regions. Langley's understanding of neural regulation was highly developed and he hinted (Langley, 1889) that sympathetic and parasympathetic nerves might supply different gland cells. This idea has been sustained by numerous workers in the intervening years (Stormont, 1926; Rawlinson, 1933, 1935; Babkin, 1950), and persists to the present day (Garrett, Harrop, Kidd and Thulin, 1977). The fact that the salivary glands appear to be almost entirely under the influence of nerves has meant they have received much attention from those interested in secretory processes.

The development of a universal understanding of salivary secretion, however, has been hindered by the variations in response to nerve stimulation that occur both between species and between different glands of the same species (Heidenhain, 1868; Langley, 1879; Emmelin, 1967). Thus Langley (1878-79) found in the cat's mandibular gland that sympathetically evoked saliva is poorer in organic material than the saliva evoked by chorda tympani nerve stimulation, whereas Heidenhain (1886) showed that the reverse applied in the dog. In ruminants, Colin (1868) had earlier reported that secretion from the mandibular gland of cattle occurred only during eating whereas that from the parotid gland was continuous. This variability has contributed to an enduring controversy regarding the roles of autonomic nerves - a controversy that has come to involve not only the secretomotor nerves but also those innervating myoepithelial cells and blood vessels (Emmelin, 1972; Emmelin and Gjörstrup, 1974; Schneyer and Emmelin, 1974; Garrett and Emmelin, 1979).

Advances in electron microscope and histochemical techniques have disclosed substantial variability in the pattern of organization of nerves within salivary glands (Garrett, 1972, 1974, 1975*b*, 1976), and have proved valuable in interpreting the diverse physiological responses observed. In a recent review, Garrett (1976) pointed out that this diversity precludes the development of unified concepts and simple classifications. It is thus necessary for detailed studies to be made of each gland system in animals of biological and economic importance if the understanding of their secretory activity in health and disease is to be adequately understood.

In ruminants, there has been extensive work on the control of secretion in the parotid gland (Coats, Denton, Goding and Wright, 1956; Kay, 1958*a*,*b*; Ash and Kay, 1959; Carr, 1974; Brightling, Patterson and Titchen, 1977; Patterson and Titchen, 1975, 1979, 1980). This gland has also been the subject of several ultrastructural studies (Shackleford and Wilborn, 1969*a*; Patterson, Petersen and Titchen, 1976; van Lennep, Kennerson and Compton, 1977).

The other salivary glands of ruminants, however, have received much less attention, and little information exists on the cellular processes concerned with the production of saliva in these animals generally, despite the obvious importance of lubricating the fibrous diet of man's most important source of protein (Ruckebusch and Thivend, 1980).

C. THE ANATOMY OF RUMINANT MANDIBULAR SALIVARY GLANDS

1. Gross Anatomy

The mandibular gland is typically located caudal to the mandible although its size and precise location varies between the different species (Shackleford and Wilborn, 1968). In the sheep, it is located partly caudal and medial to the angle of the mandible and it appears more accurately described as having an irregularly quadrilateral shape with rounded angles (May, 1970) rather than as a roughly triangular organ (Habel, 1975). It is related medially to the larynx and pharynx, dorsally to the common carotid artery and hypoglossal nerve, laterally to the maxillary and external jugular veins and the parotid gland, rostrally to the mandibular lymph nodes and ventrally to the skin (May, 1970). The mandibular gland in adult sheep is lobulated, creamy pink in colour and weighs about 9 g (Kay, 1960) while in the ox it is also lobulated, but pale yellow, and weighs about 140 g (Habel, 1975).

The mandibular duct, formed by the union of a dorsal and a ventral radicle in the middle of the gland, leaves the middle of the concave rostral border of the gland and passes medially to the digastricus muscle, and then between the mylohyoid and hyoglossal muscles, and alongside the sublingual glands to end at the sublingual caruncle (Habel, 1975). The arterial blood supply is *via* the cranial laryngeal and lingual arteries: the veins drain into the linguofacial vein.

Nerves supplying the mandibular gland arise from the autonomic nervous system. The parasympathetic secretomotor fibres are carried in the chorda tympani nerve from the

facial to the lingual nerves. They then leave the lingual nerve as it crosses the rostral border of the medial pterygoid muscle, and pass caudally alongside the mandibular duct to the gland (Habel, 1975). In sheep, both the mandibular and sublingual glands receive several twigs from the mandibular ganglion: these course along the mandibular duct to penetrate the glands (Habel, 1975). The sympathetic fibres run in the ipsilateral cervical sympathetic trunk, relay in the cranial cervical ganglion and then reach the mandibular gland after running alongside the external carotid artery and its branches (Langley, 1898; Habel, 1975).

2. Microanatomy and Histochemistry

The general organization of the mandibular gland is such that its parenchyma is divided into lobules by connective tissue septa. The interlobular stroma contains excretory (extralobular) ducts, large blood vessels, bundles of nerve fibres and small ganglia. The lobular parenchyma in turn is composed of secretory endpieces (Figure 1.1) associated with an intralobular duct system and myoepithelial cells, as well as fine nerve fibres and capillaries.

Structurally, the mandibular gland of ruminants has been considered to be a mixed gland ever since the studies of Ziegler (1927). Many subsequent histochemical investigations of the mandibular glands in sheep, cattle, and goats have been reported (Quintarelli, Tsuiki, Hashimoto and Pigman, 1961; Shackleford and Klapper, 1962; Quintarelli, 1963; Leppi and Spicer, 1967; Shackleford and Wilborn, 1968). These investigations, by utilizing a series of methods which stain carbohydrate-rich substances (mucosubstances), have indicated that in both sheep and cattle the mandibular glands consist of mucous acini and seromucous demilunes (Shackleford and Klapper, 1962; Shackleford, 1963). The former have large amounts of acidic carbohydrates, while the latter possess an appreciable amount of acidic and neutral carbohydrates.

a. Secretory Endpieces

As stated above, the secretory endpieces of bovine mandibular glands are composed of mucous acinar cells which are capped with the cells of seromucous demilunes (Quintarelli *et al.*, 1961; Shackleford and Klapper, 1962; Quintarelli, 1963; Shackleford, 1963; Junqueira Fava-de-Moraes and Paulo, 1965; Shackleford and Wilborn, 1968), but those of the goat are mainly mucous tubules with serous demilunes and a few serous acini (Junqueira, *et al.*, 1965). This report by Junqueira *et al.* (1965) appears to be the only one which describes serous demilunes in the mandibular glands of ruminants.

Leppi and Spicer (1967) found that mucous acini of both bovine and ovine mandibular glands produced sialylated-glycoprotein (sialomucin), but they did not detect any sulphated-glycoprotein (sulphomucin). The demilunes, by contrast, mainly contain neutral glycoproteins, together with a minimal quantity of sulphated-glycoprotein: demilunes in bovine mandibular glands also contain sialic acid (Leppi and Spicer, 1967). Varying forms of sialylated-glycoprotein have been distinguished histochemically in the salivary glands of both ruminants and non-ruminants, but the mucous acini of ovine mandibular glands appear to produce only a single form of sialylated glycoprotein, which is fully susceptible to neuraminidase (Leppi and Spicer, 1967). On the other hand, two kinds of sialylated glycoproteins, neuraminidase sensitive and neuraminidase resistant, are present in bovine glands (Quintarelli *et al.*, 1961; Leppi and Spicer, 1967). In addition, Ravetto, Galluzzo and Siervo (1964) described the presence of a ganglioside in the demilunes of bovine mandibular glands that is neuraminidase resistant.

These general conclusions, based on histological and histochemical findings, are supported by biochemical studies which suggest that mucus glycoproteins in ovine mandibular glands contain only N-acetylneuraminic acid (see Herp, Wu and Moschera, 1979), whereas a mixture of neuraminic acid

and N-glycolyl-neuraminic acid can be isolated from bovine mandibular glands (see Herp, *et al.*, 1979). About 25% of the sialic acids in bovine glands are neuraminidase resistant (Gottschalk, 1957).

Although the ultrastructure of secretory endpieces has only been briefly described in sheep (Young and van Lennep, 1978), more detailed studies have been reported in other ruminants. Thus, Shackleford and Wilborn (1970*a*) examined the mandibular glands of the calf, Bloom and Carlsöö (1974) those of the cow and Kayanja (1973) those of several species of East African ungulates. The detailed description which now follows is, therefore, largely of the bovine glands. Shackleford and Wilborn (1970*a*) and Bloom and Carlsöö (1974) demonstrated distinct ultrastructural differences between the acini and demilunes of mandibular glands.

(i) Acinar cells

The mucous acini contain electron lucent granules, the encapsulating membranes of which are often fused with one another, and within some granules electron dense foci are seen. Based on a comparison of the dimensions of the fine structure of contents of these granules (as calculated from electron micrographs) with the dimensions of glycoproteins isolated from whole mandibular glands as determined by physical methods, e.g. for the sheep, its viscosity and sedimentary velocity - reported by Gottschalk and McKenzie [1961]), Gallagher Marsden and Robards (1969) suggested that in the sheep and cattle the secretory granules in acinar cells contain glycoprotein molecules. Nuclei in mucous acini are often irregular in shape and flattened towards the cell base, and few mitochondria are evident.

Shackleford and Wilborn (1969*a*,*b*) described differences in the morphological appearance of cellular organelles in different functional states of the cells: in the synthetic phase, acinar cells contained a high concentration of rough endoplasmic reticulum (RER) with dilated cisternae throughout the cytoplasm and particularly at the cell base. Golgi complexes were conspicuous specially in the proximity of

immature droplets. As secretory products accumulated within the cells, the RER became limited to small angular areas between mucous droplets or at the base of the cells.

(ii) Demilunes

By contrast with the cytology of the acini, the cytoplasm of demilunes contains numerous secretory granules with a bipartite pattern that resembles the granules described in human salivary glands by Tandler and Erlandson (1972). The significance of this bipartite structure is obscure. The nuclei of demilunes are located at the base of the cells, with one or more prominent nucleoli often apparent. This basal region is also the site where other cellular organelles are usually seen. A well developed RER is present as uniform parallel arrays of densely packed ribosome-studded cisternal profiles which Bloom and Carlsöö (1974) observed to be in association with a labyrinth of interconnecting tubules: mitochondria are numerous.

Secretion from demilunes evidently reaches the central acinar lumen either *via* intercellular canaliculi located between adjacent demilunar cells, or through narrow apical extensions of demilunar cells which border the lumen in common with acinar cells (Shackleford and Wilborn, 1970*a*). The intercellular canaliculi are provided with short microvilli and limited by junctional complexes between the cell membranes of adjacent cells: only a narrow intercellular space is present at the junctions of the demilunar cells, acinar cells and intercalated duct cells.

b. The Duct System

The intralobular duct system in the mandibular gland (Figure 1.1) consists of relatively short intercalated ducts which connect the mucous acini with the intralobular striated ducts and then drain into a series of excretory ducts including interlobular ducts, interlobar ducts and the main excretory duct.

(i) Intercalated ducts are lined by simple cuboidal epithelial cells whose nuclei are extremely heterogenous in both shape and size. The cytoplasm of these cells contains scattered mitochondria, ribosomes, a few segments of RER and Golgi complexes which are small and tend to be located just apical to the nucleus. Also present are large numbers of intercellular fibrils and filaments that are postulated by Wilborn and Shackleford (1969*a*) to afford rigidity to the cytoplasm and to assist in anchoring apposed cell surfaces. The adjacent cells are joined by tight junctions at their apical surfaces and by desmosomes along their lateral surfaces. A few granules of moderate electron density have been observed in the cytoplasm basal to the nucleus in bovine intercalated duct cells (Bloom and Carlsöö, 1974).

(ii) Striated duct cells are of the simple columnar type with large spherical or oval nuclei located at approximately the centre of the cells. This part of the duct system seems to be devoid of goblet cells (Kay, 1960). Striated ducts are relatively long and numerous - Pérez Clavier (1976) showed the volume of striated duct in the bovine (*Bos indicus*) mandibular gland to be about 8% of the gland volume, compared with 9% in the parotid gland.

Ultrastructurally, the striated duct cells show characteristic basal infoldings occupying their basal third, with mitochondria among these folded membranes. (The two together form the 'basal striation' observed by light microscopists - see Young and van Lennep, 1978). Shackleford and Wilborn (1970*a*) have pointed out that the striated ducts of the mandibular gland have relatively more numerous and more prominent basal infoldings than those of the parotid gland (Shackleford and Wilborn, 1969*a*). Such infoldings are, however, a prominent feature in the parotid gland of other members of the same zoological family (Family Bovidae, Order Artiodactyla), viz. sheep (van Lennep *et al.*, 1977) and wild East African ungulates (Kayanja and Scholz, 1974). Kayanja (1973) stated that as the ducts became larger, basal striation became less apparent.

Like that of intercalated duct cells, the cytoplasm of striated duct cells usually contains abundant fine fibrils and filaments, a sparse RER and Golgi complexes. Small granulated vesicles (70-220 mμ diameter) are present in the apical cytoplasm of the principal cells and some striated duct cells possess apical blebs.

It is also characteristic of striated duct cells that they exhibit varying degrees of electron density and this has led various investigators to distinguish them as 'dark', 'light', or 'intermediate' cells. Other cells are also distinguished because of their appearance, e.g. 'basal cells'. Shackleford and Wilborn (1970*a*) observed that dark cells reach the basement membrane of striated duct epithelium by means of narrow processes which pass between the more common light cells and occasional basal cells. Basal cells are distinguished by their lack of basal infoldings and in their typical location adjacent to the basement membrane have no cytoplasmic extensions reaching the lumen. Bloom and Carlsöö (1974), on the other hand, make no mention of basal cells but draw attention to dark cells, light cells and intermediate cells. They point out that the intermediate cells correspond to the light cells described by Shackleford and Wilborn (1970*a*).

(iii) Excretory (Extralobular) Ducts Relatively little information on the cellular structure of the extralobular duct system has been provided from ruminant mandibular glands. In general, the epithelium forming the ducts varies with location, ranging through simple columnar, stratified cuboidal or columnar and pseudostratified epithelium (Pinkstaff, 1980). In bovine mandibular glands, the interlobular ducts are lined by stratified columnar epithelium and do not contain goblet cells (Birtles, 1981), but in the ovine mandibular gland, Quintarelli (1963) reported the presence of large goblet cells in the main excretory duct.

The only information about the fine structure of the excretory duct of the ruminants appears to be from the studies of Hayes, McCombs and Faherty (1970) on the calf's parotid

gland. These authors found that the main excretory duct in the calf has a stratified epithelium six to eight cells deep and they divided these cells into 3 zones: a basal zone, a zone of differentiating cells, and a zone of differentiated cells.

c. Myoepithelial Cells

Myoepithelial cells are present in salivary glands and many other mammalian exocrine glands, e.g. sweat glands (Ellis, 1965), mammary glands (Linzell, 1955), lacrimal glands (Leeson, 1960), and even in bronchial glands (Sorokin, 1965), but not in the pancreas (Garrett, Lenninger and Ohlin, 1970).

The structure of salivary myoepithelial cells has been well described for many species and reviewed by many investigators (Tandler, 1965; Tamarin, 1966; Tandler, Denning, Mandel and Kitscher, 1970; Young and van Lennep, 1977, 1978; Garrett and Emmelin, 1979). Typically, salivary myoepithelial cells have 2 basic shapes depending on whether they are in association with a secretory endpiece or an intercalated duct. Those cells that are associated with secretory endpieces usually are of stellate shape and typically there is only one myoepithelial cell per endpiece although as many as 3 cells may be present. Each consists of a central cell body with 4-8 processes radiating from it. These processes give rise to 2 or more generations of branches. The processes of cells associated with endpieces neither fuse nor extend on to the intercalated duct, but the reverse does not hold and the myoepithelial cells of intercalated ducts commonly run up to the base of endpieces. Myoepithelial cells associated with intercalated ducts have a spindle shape and may be without processes (as reviewed by Young and van Lennep, 1977) or with few processes (Garrett and Emmelin, 1979), and lie longitudinally along the ducts. Usually there is no conspicuous association of myoepithelial cells with striated ducts, although the sheep parotid gland appears to be an exception (van Lennep *et al.*, 1977).

The following account of the ultrastructure of the myoepithelial cell is largely based on recent reviews by Young and van Lennep (1977) and Garrett and Emmelin (1979). A characteristic of myoepithelial cells is the presence of cytofilaments which resemble myofilaments. These filaments frequently aggregate to form electron-dense structures similar to the dark bodies observed in smooth muscle cells (Rhodin, 1962) and are secured to the plasma membrane by attachment devices. The majority of the cytofilaments are thin (approximately 4 nm diameter) and usually occupy the space nearer to the basal lamina with their orientation longitudinally along the processes. Thick filaments (10 nm diameter) are scattered among the thinner filaments. Another conspicuous feature of the cells is the presence of minute vesicles and numerous caveolar invaginations of plasma membrane on the stromal aspect facing the interstitium. The visceral aspect of the cells, which faces the underlying secretory cells or the epithelial cells of intercalated ducts, however, is relatively smooth with fewer caveolae. Tamarin (1966) has pointed out that peripheral caveolae and vesicles similar to those at the stromal side of myoepithelial cells, are characteristic of the superficial cytoplasm (surface membrane) invaginations in smooth muscle cells as well. The ultrastructure of myoepithelial cells supports the notion that they are contractile in function.

The non-filamentous part of myoepithelial cells contains the nucleus and other organelles. The nucleus is ellipsoidal and distributed near it are free ribosomes, a few RER cisternae, Golgi complexes, mitochondria and lysosome-like bodies. Most of the organelles are confined to juxtanuclear zones. The cells lie within the periparenchymal basement membrane and are attached to acinar or ductal epithelium by a small number of desmosomes.

In bovine mandibular glands myoepithelial cells are abundant and present in relation to both demilunes and acini: they are described inside the basement membrane of secretory endpieces and also at the basal aspect of the intercalated

duct epithelium. (Shackleford and Wilborn, 1970*a*; Alm, Bloom and Carlsöö, 1973; Bloom and Carlsöö, 1974).

d. Innervation

The morphological evidence of the innervation of ruminant mandibular glands seems to have been studied systematically only in the bovine: Alm *et al*. (1973) demonstrated that both adrenergic nerve fibres (revealed by a formaldehyde-induced fluorescence method) and cholinergic nerve fibres (demonstrated by acetylocholinesterase [AChE] activity) were present around the secretory endpieces and blood vessels.

Electron microscopy has revealed only unmyelinated axons in the location which Garrett (1975*b*; 1976) has described as 'epilemmal' (i.e. axons that lie outside the basement membrane of an effector cell at a distance of 100 nm or more), in association with mucous acini, demilunes and myoepithelial cells (Bloom and Carlsöö, 1974). They stated that two groups of axons with characteristics, respectively, of adrenergic and cholinergic nerve terminals, seemed to be present within the same bundle (Bloom and Carlsöö, 1974). The adrenergic nerve terminals were distinguished through the occurrence of small dense cored vesicles (40-50 nm diameter) after permanganate fixation. Abundant evidence is available to support the contention that the electron dense core in these vesicles represents the neurotransmitter noradrenaline (Wolfe, Potter, Richardson and Axelrod, 1962; Bloom and Barnett, 1966; Tranzer, Thœnen, Snipes and Richards, 1969). The criterion for identifying cholinergic nerve terminals is the presence of a homogeneous population of small agranular electron-lucent vesicles, about 40-50 nm diameter, which are probably the storage site of acetylcholine (Whittaker, Michaelson and Kirkland, 1964; Tranzer *et al*., 1969).

No 'hypolemmal' nerves (i.e. axons that penetrate beneath the basement membrane to be within 20 nm of the adjacent effector cells - Garrett, 1975*b*, 1976) have been reported in bovine mandibular glands. However, Kayanja (1973) did observe a hypolemmal nerve terminal at the base

of a large duct of the mandibular gland of an impula. He was unable to distinguish the nerve type involved.

D. MANDIBULAR SALIVARY FLOW

Ruminant mandibular glands secrete a weakly buffered hypotonic saliva of variable mucous content which is considered to be important in moistening and lubricating masticated food (Colin, 1868; Kay, 1960). The total volume of mandibular secretion is about 5% of the estimated total volume of saliva secreted by the two parotid glands (Kay, 1960). Most of the mandibular saliva is secreted in response to feeding: an observation reported by Colin (1868) in experiments on cattle and by Scheunert and Trautmann (1921) on sheep, and confirmed recently by Kay (1960) and Carr, Reid and Verkerk (1977). The reasons why remastication during rumination is not an effective secretory stimulus to the mandibular gland remain to be elucidated; this feature and the absence of a spontaneous secretion are in marked contrast to the activity of the parotid gland.

E. COMPOSITION OF MANDIBULAR SALIVA

Mandibular saliva has a high water content and a variety of inorganic and organic components. The overall composition of the mandibular saliva in the cow, for example, in per cent is water, 99.11; inorganic matter, 0.53; and organic matter, 0.35; respectively (Dukes, 1955). Hence the specific gravity of saliva is only slightly greater than 1.0; as in the sheep it is about 1.01 (Kay, 1960).

1. Electrolyte Content

Phillipson and Mangan (1959) and Kay (1960) determined the chemical composition of mandibular saliva produced by both conscious and anaesthetized animals. Their findings (summarized in Table 1.1) show that, compared with plasma, the secretion has a low sodium and a relatively high potassium ion content. Mandibular saliva usually contained much lower concentrations of Na^+, HCO_3^- and HPO_4^{--} than

Table 1.1: The composition of mandibular and parotid saliva collected from sheep and cattle under various conditions.

Origin of Saliva	Nature of Stimulation	Nitrogenous Constituents (mg 100 ml^{-1})			Electrolytes (mmole l^{-1})						References
		Total N	Urea N	Protein N	Na^+	K^+	Ca^{++}	HCO_3^-	HPO_4^{--}	Cl^-	
SHEEP											
Mandibular	Anaesthetized	-	-	-	3-66	15-51	-	1-9	7-88	2-9	a
	Conscious	-	-	-	4-16	10-25	-	5-14	1-5	9-16	a
Parotid	Anaesthetized										
	Unstimulated or reflex	-	-	-	163-189	5-14	-	88-99	8.5-50	9-34	a
	Unstimulated	-	-	-	162	7	-	98	17	26	b
	Conscious	-	-	-	147-185	5-31	0.1	100-140	13-32	9-17	a,c,d
CATTLE											
Mandibular	Anaesthetized										
	Sympathetic n.	127.6	31.4	90.4	-	-	-	-	-	-	e
	Rumen inflation	116.8-165.2	13.1-13.2	90.3-96.3	16-70	14-16	4	17	0.2-20	49	a,e
	Carbachol	26.4-29.2	9.0-15.9	15.0-19.5	13-14	13-15	3-5	17-20	0-0.6	13-58	e
	Mixed stim.	44.0-53.0	7.3-10.6	27.4-36.7	13-42	14-16	3-4	18-19	0-0.3	17-29	e
Parotid	Anaesthetized										
	Unstimulated	15.4-16.2	12.1-13.3	1.0-2.0	134-143	11-14	1.4	104-107	9-11	6-18	e
	Rumen inflation	14.8-20.2	12.5-16.4	1.0-3.5	122-123	14-16	1-2	105-116	9-14	9-12	e
	Carbachol	14.6-19.0	11.5-16.1	1.3-2.5	115-164	12-17	1-2	100-117	7-14	10-31	e

[a] From Kay (1960); [b] Compton, Nelson, Wright and Young (1960); [c] McDougall (1948); [d] Denton (1957a); [e] Phillipson and Mangan (1959).

parotid saliva, while the concentrations of potassium and chloride ions were approximately the same in the two secretions. The electrolyte content of mandibular saliva is influenced by the rate of its secretion, and by the sodium status of the animal (Kay, 1960). When the mandibular secretory rate is increased (by electrical stimulation of the submaxillary nerve) to 1g h^{-1} g^{-1} gland, the concentrations of Na^+, K^+, HPO_4^{--} and Cl^- fall, while at higher rates of secretion the concentrations of Na^+, HCO_3^- and Cl^- increase, the concentration of K^+ remains constant and the concentration of HPO_4^{--} continues to fall (Kay, 1960). Under conditions of sodium depletion the sodium ion content in the mandibular saliva is reduced, the deficit being replaced by potassium ions in a manner similar to that reported by Denton (1956) for the parotid gland. Kay (1960) observed a reduction in the Na^+:K^+ ratio from 0.71 in the normal animal to 0.25 in the sodium depleted animal.

2. Protein/Glycoprotein Content

Surprizingly little attention has been paid to the non-electrolyte components of mandibular saliva. One of the few detailed analyses of protein levels for ruminant saliva is that given by Phillipson and Mangan (1959; Table 1.1). This shows that although saliva from the mandibular gland has a higher protein nitrogen or mucoprotein content than that from the parotid gland, the urea concentrations do not differ significantly.

a. Mucoprotein (Mucus Glycoprotein[2])

Phillipson and Mangan (1959) suggested that the relatively high protein content of mandibular saliva in ruminants is due mainly to the presence of mucoproteins. Because many investigations have been interested in biochemical rather than physiological events, there has been a tendency to use

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[2] Reid and Clamp (1978) recommended the term "mucus glycoprotein" in place of the terms "mucoprotein" and "mucin" in order to produce terms common to histochemistry and biochemistry, and so avoid the considerable confusion of terminology in the literature.

the materials extracted from the salivary glands rather than to obtain pure samples of secretion for analysis. It is now well established from such biochemical studies that salivary mucins are high molecular weight glycoproteins which, it appears, are the major contributors to the viscosity of saliva (Herp *et al.*, 1979).

Based on Herp *et al.* (1979)'s review, the mucus glycoproteins in mammalian salivary glands comprise a protein core carrying, usually, an O-glycosidically linked oligosaccharide side chain for every 4 amino acids. The carbohydrate side chains in bovine and ovine mandibular gland mucins are made up by sialic acid and N-acetylhexosamine (N-acetylgalactosamine and N-acetylglucosamine) - together these account for more than 50% of the total mucoproteins. There are also traces of fucose and galactose. The protein moiety contains nearly all of the common amino acids, with a preponderance of serine, glycine, threonine, alanine and proline.

b. Immunoglobulins

Various classes of immunoglobulins, including IgG_1, IgG_2, IgM and IgA, have been found in the saliva of ruminants. IgA is the major immunoglobulin present in mixed saliva of sheep, cattle and goats (Pahud and Mach, 1970; Mach and Pahud, 1971) and in the non-parotid saliva of sheep and cattle (Watson and Lascelles, 1971; Cripps and Lascelles, 1976). Cripps and Lascelles (1976) also reported that although the flow from the mandibular gland was considerably less than from the parotid gland there was an extremely high concentration and output of IgA in the mandibular saliva of sheep compared with the parotid.

The high IgA content of mandibular saliva indicates that the mandibular glands possess a local IgA synthesizing system (the products being termed secretory IgA). Tissue culture studies on the incorporation of labelled amino acids into IgA have provided evidence of local synthesis of secretory IgA by the bovine salivary glands (Mach and Pahud, 1971), as well as by human salivary glands and other exocrine

glandular tissues (see Lamm, 1976). Histological studies in sheep show a relative absence of lympho-plasma cells from the parotid tissues when compared with the mandibular gland, in which they are numerous in the intra-alveolar areas (Watson and Lascelles, 1971). This is consistent with the relative levels of IgA in sheep mandibular and parotid saliva.

The secretory IgA is composed of a dimer of 7S serum IgA (which possesses no secretory antigenic determinant) and an additional glycoprotein segment called secretory component (Mach and Pahud, 1969; Bienenstock, Tourville and Tomasi, 1969; Pahud and Mach 1970). The secretory component (SC) is present in a free form and also in association with dimers of serum IgA (Pahud and Mach, 1970).

It has been suggested that the IgA and SC are synthesized separately by different salivary gland cells: the interstitial plasma cells being the source of the IgA monomers, while the salivary gland parenchyma cells synthesize SC (Mach, Pahud and Isliker, 1969; Brandtzaeg, 1974). Immunohistochemical studies by Brandtzaeg (1974) in the human submandibular glands demonstrated that free SC was found intracellularly in the Golgi region whereas both free and IgA bound SC were detected on the cell membranes and in the apical cytoplasm.

Cripps and Lascelles (1976) showed that all of the IgG_2 and most of the IgG_1 in the mandibular saliva is derived from plasma. A significant amount of IgG_1 (about 16% of the total IgG_1) is derived locally and the selective transfer of IgG_1 is of a similar magnitude in the mandibular and the parotid glands, but there is no relationship between the selective transfer of IgG_1 and the secretion of IgA.

3. Enzymes

The mandibular saliva of ruminants is devoid of amylase, as is the saliva of the dog, cat and horse (Scheunert and Trautman, 1921; Burgen and Emmelin, 1961). Chauncey and Quintarelli (1961) and Chauncey, Herrinques and Tanzer (1963) reported the presence in sheep of the enzymes acid phosphatases

and esterases both in the mandibular saliva and in the demilunes and ducts of the mandibular gland itself, as well as the presence of peptidases in the demilunes and acini (Arvy, 1963). In addition, galactosidase activity has been detected in the demilunes and duct cells of mandibular glands of sheep and cattle but not in the secretions from the gland (Chauncey and Quintarelli, 1961; Chauncey *et al.*, 1963).

Bovine mandibular glands also contain lactoperoxidases (Morrison and Allen, 1965; Carlsöö, 1971). Histochemical techniques, at both electron microscopic and ligh microscopic levels, show the endogenous lactoperoxidase activity to be present only in the demilunes and the secretory products of the gland (Carlsöö, 1971; Bloom and Carlsöö, 1974). The enzyme is present within the secretory granules, in both electron lucid and electron dense portions, and in RER (including perinuclear cisternae as well as the labyrinth connecting the RER). This suggests that lactoperoxidases in mandibular saliva are synthesized, stored and secreted from seromucous demilunes. One role that has been postulated for these proteins is in a defence system for the oral cavity (Carlsöö, 1971). Strum and Karnovsky (1970) suggest that peroxidase has an important anti-bacterial action in saliva and may be involved in iodination reactions.

Peroxidase activity has also been demonstrated in many other salivary glands, e.g. in the endpiece cells of the parotid gland of the cat, (Garrett and Kidd, 1976) and sheep (van Lennep *et al.*, 1977) and in the endpiece cells of mandibular glands of the rat (Strum and Karnovsky, 1970), guinea pig and man (Carlsöö, Kumlien and Bloom, 1971). Garrett and Kidd (1976) suggest that enzymes such as peroxidase and acid phosphatase are non-obligatory secretory proteins, i.e. the enzymes may by-pass the Golgi cisternae during incorporation into secretory granules - a pathway different from the "segregation hypothesis" proposed by Palade (see Palade, 1975).

F. CONTROL OF MANDIBULAR SALIVARY SECRETION OF RUMINANTS

Under most circumstances variations in salivary flow are due to alterations in the activity of nerve fibres to the secretory cells (Burgen and Emmelin, 1961). These fibres are excited centrally. If parallels can be drawn with the other species, afferent fibres located chiefly in the trigeminal and glossopharyngeal nerves carry taste and general sensation to the salivary centres located in the reticular formation of the medulla oblongata (Emmelin, 1967).

Parasympathetic fibres originating in the superior salivary nucleus leave the brain stem in the facial nerve and pass to the gland, as stated earlier (Section C.1) *via* the chorda tympani and lingual nerve. Descending reticulo-spinal fibres connecting with the sympathetic outflow have similarly been suggested to arise in medullary nuclei (Velo and Hoff, 1961). The sympathetic fibres leave the spinal cord in the ventral roots of the first, second or third thoracic spinal nerves and pass up the sympathetic chain to relay in the cranial cervical ganglion and thence reach the gland (as stated in Section C.1).

However, the precise routes taken by the parasympathetic and sympathetic nerves to the sheep mandibular gland have not been studied. Although Garrett and Kemplay (1977) found the cranial cervical ganglion to be the sole source of adrenergic nerves to the mandibular gland in the cat, there is evidence suggesting that secretory nerves may arrive at some glands through unknown channels (Burgen and Emmelin, 1961; Garrett and Holmberg, 1972; Ekström and Emmelin, 1974). For example, in the dog's parotid gland, Garrett and Holmberg (1972) found that after cranial cervical ganglionectomy, some adrenergic nerves of unknown origin remained intact in the gland. In addition, the parotid gland has also been found to receive parasympathetic secretory nerves *via* sources other than the classical (auriculo-temporal) route, e.g. *via* nerves accompanying the internal maxillary artery and the facial nerve (Garrett and Holmberg, 1972).

1. Psychic Stimulation

Psychic influences on salivary secretion popularized by Pavlov's extensive studies on dogs early this century (Pavlov, 1910) have also been reported in ruminants. Scheunert and Trautmann (1921) observed that stimulation, by showing a sheep its food, caused a slight psychic secretion of mandibular saliva. Blokh (1939) observed in the bull that the sight of food, or the arrival of usual attendant evoked mandibular secretion and a large decrease in the continuous parotid secretion, and this type of reflex secretion has also been reported in the goat (Tikhomirov and Fomin, 1950). Denton (1957*b*) in his behavioural study on conditioned reflexes in sheep suggested that the observed increases or decreases in salivary secretion reflect cortical influences.

2. Reflex Stimulation

In ruminants, secretion by the mandibular gland is stimulated by feeding but not by rumination (Colin, 1868; Scheunert and Trautmann, 1921; Kay 1960; Carr *et al.*, 1977). This involves excitation of afferent fibres from the mouth - chiefly the trigeminal and glossopharyngeal nerves, salivary centres in the brain stem and efferent nerve fibres to effector cells in the mandibular gland. There is little secretion when the animal is not eating.

The effect of mechanical stimulation of the gut is variable. Stimulation of the oesophagus or rumino-reticulum by inflation to pressures of less than 20 mmHg causes little or no mandibular secretion (Kay and Phillipson, 1959; Kay, 1960), although in the hands of Phillipson and Mangan (1959) these stimuli evoked the secretion of a viscid saliva rich in mucoprotein (see Table 1.1). Ash and Kay (1959) studied the reflex secretion of mandibular saliva in 2 conscious sheep and noted that distension of the cardia or the reticulo-omasal orifice, as well as tactile stimulation of the anterior wall of reticulum caused a moderate secretory response. The afferent fibres concerned this reflex secretion

probably course in the vagus nerves as with parotid secretion (Kay, 1958*a*). Extensive studies on the reflex control of parotid secretion in sheep reveal that mechanical stimulation of the oesophagus (particularly the lower thoracic and abdominal portions), as well as distension of the cardia, reticulo-omasal orifice and reticulo-ruminal fold (Titchen, 1958; Kay, 1958*a*; Ash and Kay, 1959) excite parotid secretion provided a vagus nerve is intact.

3. Parasympathetic Stimulation

Beilenson, Smaje and Schachter (1968) reported that stimulation of the chorda tympani nerve induced secretion from the mandibular gland of sheep and an accompanying vasodilation. Phillipson and Mangan (1959) had earlier reported from the calf that intravenous injection of carbachol (5-10 mg kg^{-1} body weight) induced a greatly enhanced mandibular salivary secretion of a low protein content, (see Table 1.1) and markedly different appearance from that produced by sympathetic nerve stimulation or by inflation of the rumen.

The phenomenon of parasympathetic vasodilation, which has been known since the work of Bernard (1858), is still controversial. Intravenous injection of atropine blocks the secretion induced by parasympathetic stimulation but dilation of blood vessels to the sheep mandibular gland is not affected (Beilenson *et al.*, 1968). Similar responses have been obtained in the mandibular glands of the cat and the dog, although in the sheep parotid and the rabbit mandibular glands the parasympathetic vasodilation is atropine sensitive (Kay, 1958*b*; Schachter, 1970). The explanation of this atropine-resistant vasodilation in salivary glands has been a point of contention for many years. Opposing concepts to explain the phenomenon are release of vasodilator agents, e.g. kallikrein (Hilton and Lewis, 1957), or the presence of vasodilator nerves (Schachter, 1970).

Recently, additional neurotransmitters that are polypeptides have been shown to have a vasoactive action. Vasoactive intestinal peptide-like (VIP) immunoactivity has been described within nerve terminals of sublingual glands of the rat and the mandibular glands of the piglet and cat (Bloom, Bryan, Polak, van Noorden and Wharton, 1979; Bloom and Edwards, 1979). It is proposed that the VIP may modify salivary electrolyte composition (Denniss and Young, 1978) and mediate the atropine resistant vasodilation (Bloom and Edwards, 1980; Lundberg, Aggard, Fahrenkrug, Hökfelt and Mutt, 1980; Uddman, Fahrenkrug, Malm, Alumets, Hakanson and Sundler, 1980). The latter role for VIP is supported by the observation of marked rises of VIP levels assayed in the venous effluent from the cat mandibular gland during stimulation of the chorda lingual nerve both in the presence and absence of atropine. The marked vasodilation induced by intra-arterial infusion of VIP is also atropine resistant (Lundberg *et al.*, 1980). Kidd and Garrett (1979) have speculated that the large dense-cored (ca. 100 nm diameter) vesicles observed in some nerve fibres in the cat's mandibular gland may be sites of peptide storage. Recently, morphological analysis using a combination of indirect immunofluorescence and AChE staining techniques have suggested the presence of VIP in postganglionic cholinergic neurones (Lundberg *et al.*, 1980).

4. Sympathetic Stimulation

Under most circumstances sympathetic nerve stimulation elicits vasoconstriction in the sheep, as in the dog and the cat (Kay, 1958*b*; Burgen and Emmelin, 1961; Emmelin, 1967). Stimulation of the sympathetic nerve alone is reported to cause a small increase in the volume of secretion from the sheep mandibular gland - this secretion being viscous and cloudy in appearance, with high mucoprotein content (Phillipson and Mangan, 1959).

Patterson and Titchen (1977), in an examination of the effects of sympathetic stimulation on background flows of

mandibular saliva evoked by intravenous infusion of pilocarpine in anaesthetized or totally pithed or decerebrated animals, found that stimulation either *via* the cervical sympathetic trunk or by injection of isoprenaline (1-2 μg kg^{-1}, iv) produced increases in flow and also the protein and glycoprotein content (measured from the free and bound neuraminic acid content). Following sympathetic nerve stimulation, the saliva changed from a clear viscid secretion to a stringy opalescent one. All of these effects were blocked by propranolol (300 μg kg^{-1}, iv). Patterson and Titchen (1977) proposed that the sheep mandibular gland has a β-adrenergic innervation contributing to its secretion of protein or glycoprotein.

G. THE PRESENT STUDY

Previously only limited studies on the fine structure of the sheep mandibular gland (Young and van Lennep, 1978), and its secretion during nerve stimulation (Phillipson and Mangan, 1959) and feeding (Kay, 1960) have been undertaken. These are incomplete and rather fragmentary.

Several special attributes of the sheep and its mandibular gland formed the basis for the present study of secretory processes. First, under natural conditions the gland secretes relatively large volumes of a saliva that is important in lubricating the animals' fibrous diet and because this diet is bulky and of low nutritional quality the gland is normally active over a feeding period of several hours. This relatively long time-span provides the opportunity to observe either gross or graded changes in response to the natural stimuli involved in eating. Secondly, it can be argued that the sheep mandibular gland is a particularly suitable salivary gland for an investigation of relationships between physiological events and morphological changes because its mixed population of secretory cells offers the prospect of observing, in the one gland, changes in both mucous and seromucous cell types. Since sheep are easily

trained for laboratory life the results of such chronic studies can be referred back to those produced under acute conditions where greater analysis of relationships between the secretory response, nerve stimulation, and morphological changes is possible.

CHAPTER II

MATERIALS & METHODS

The experiments to be described examined the structure of mandibular glands from animals whose food had been withheld for 20-24 hours (the glands are hereafter referred to as resting glands) and from animals whose mandibular glands were stimulated to secrete either naturally or artificially. In addition, a detailed examination was undertaken of the flow of mandibular saliva and its protein content. Observations were therefore made under three general conditions:

(i) On tissues from sheep that had not been fed for 20-24 hours. This included observations on the contralateral resting glands from sheep used in nerve stimulation experiments in (ii) below.

(ii) In acute experiments on anaesthetized sheep when secretion was stimulated by either direct stimulation of autonomic nerves or pharmacological agents that mimic the action of autonomic transmitters.

(iii) In chronic experiments in which the stimulus for secretion was the natural one afforded by eating. In this circumstance different groups of animals were used for the physiological and morphological studies.

A. ACUTE EXPERIMENTS ON ANAESTHETIZED SHEEP

1. Animals, Diet and Housing

Forty-five crossbred Romney ewes and wethers weighing 16-49 kg were used. They were housed indoors and fed an unrestricted diet of lucerne chaff until 20-24 hours before experiments. Water was available *ad libitum*.

2. Anaesthesia and Dissection

a. Anaesthesia

Sodium pentobarbitone solution ('Anathal', V.R. Laboratories Ltd., N.S.W.) was injected into a jugular vein in a dose of 25-30 mg kg^{-1} body weight for induction of anaesthesia. In some experiments anaesthesia was maintained with supplementary injections of 60-180 mg sodium pentobarbitone *via* a venous cannula, while in others a solution of 1% chloralose (F. Merck C.G., Germany) in normal saline at 70°C, was given instead to a total dose of 70 mg kg^{-1} body weight.

In all anaesthetized animals a clear airway was maintained by insertion of a glass tracheal cannula. This cannula was also used to facilitate artificial respiration (with a Palmer respiratory pump) when regular breathing ceased.

b. Cannulation of Both Mandibular Ducts

A midventral skin incision, about 8-10 cm long, was made beneath the jaw and deepened between the mylohyoid muscles to expose the mandibular salivary ducts situated between the mylohyoid and styloglossal muscles in close association with the lingual nerve. On each side the duct was dissected free of surrounding tissues rostral to the lingual nerve for 10-20 mm, tied with a ligature orally and a V-shaped incision made into its wall close to the ligature. A cannula of vinyl tubing (either ID 0.58 mm, OD 0.96 mm; or ID 0.97 mm, OD 1.27 mm; Dural Plastics & Engineering Pty., Ltd., Dural, N.S.W.) was inserted about 10 mm into the duct, towards the gland. The free end of the cannula was connected to a photo-electric drop counter. Care was taken not to damage the lingual nerve.

c. Exposure of Autonomic Nerves

(i) Chorda Lingual Nerve

At the time of cannulation of the mandibular duct, the lingual nerve was exposed between the mylohyoid and stylohyoid muscles. The mandibular duct usually crossed under the lingual nerve at the position where the nerve divided into superficial and deep branches. The chorda lingual nerve appeared as a fine branch running from the lingual nerve to innervate the mandibular gland: its identity was confirmed when electrical stimulation rapidly produced a flow of clear saliva from the mandibular cannula.

(ii) Cervical Sympathetic Trunk

Dissection uncovered the ipsilateral vagus nerve, cervical sympathetic trunk and common carotid artery. The cervical sympathetic trunk could usually be identified as a trunk clearly separated from the vagus nerve when exposed close to the thoracic inlet. It was isolated over about 20 mm and cut and its identity was confirmed by observing the ipsilateral palpebral margin during electrical stimulation of its peripheral end. Effective sympathetic stimulation caused widening of the palpebral margin. In some experiments the compound action potential in the cervical sympathetic trunk was recorded peripherally to the stimulating electrodes. In three acute experiments, the cervical sympathetic trunk was left intact and the cranial cervical ganglion extirpated instead.

3. Recording Techniques, Apparatus and Estimations

a. Arterial Blood Pressure

Arterial blood pressure recording was *via* a polyethylene cannula (ca. OD 2.0 mm, ID 1.8 mm; Dural Plastics and Engineering Pty., Ltd.) inserted into a femoral artery and connected to a pressure transducer (Statham Laboratories Inc., U.S.A.) in conjunction with a heat stylus chart recorder (Devices Ltd., Welwyn Garden City, England).

b. Salivary Flow Recording and Collection of Saliva

In acute experiments mandibular salivary flow was recorded as the number of drops in each 30 second interval, using a photo electric drop counter, an impulse integrator (Neurolog, Digitimer, Welwyn Garden City, England) and the heat stylus chart recorder, except for a few early experiments when salivary flow was recorded on the smoked drum of a kymograph (C.F. Palmer Ltd., England). The saliva was collected into graduated tubes held in a reservoir of iced water, and stored at -20°C.

c. Nerve Stimulation

Stimulation of the cervical sympathetic trunk and the intact chorda lingual nerve was performed in a pool of paraffin oil (National Dairy Association N.Z. Ltd., Palmerston North) using bipolar electrodes (chlorided silver) and a stimulator (Type 5112, Model 104A, American Electronic Laboratories Inc., U.S.A.) supplied with stimulus isolation unit. The applied voltage was monitored on an oscilloscope (Tektronix Inc., U.S.A.) in circuit with the electrodes. The stimulation frequency was usually within the range of 5-10 Hz, the pulse duration 0.2 msec, and the voltage supramaximal (3-6V).

d. Estimation of Salivary Protein

The concentration of total protein in mandibular saliva was determined by using a copper-containing reagent in the modified Biuret method of Koch and Putnam (1971). This method is based on the absorption of the copper-peptide complex at a wave length 330 nm. It can be applied over a range of 0.25-7.00 mg ml^{-1} protein. The absorbance of copper-peptide complex was read in 4 ml silica cells (UV grade, BS type 1A) at 330 nm and 392 nm with either a Gilford spectrophotometer (Beckman) or a SP500-2 Spectrophotometer (Pye, Unicam Ltd., Cambridge). A reagent blank was prepared from 1.0 ml distilled water and 4.0 ml of the copper-containing reagent. Interference from light scattering due to turbidity was corrected for as recommended by Koch and Putnam (1971). A

standard curve (or a regression line) was plotted between the concentrations of standard protein (mg ml^{-1} bovine serum albumin) and the corrected values of its corresponding absorbance. Salivary protein concentrations were expressed in terms of equivalent concentrations of bovine serum albumin (Fraction V, Sigma Chemicals Co.). When high concentrations of protein were encountered, such as in sympathetically evoked saliva, the samples of saliva were diluted with distilled water. Interference due to precipitation in such saliva did not occur since the saliva cleared in the presence of strongly alkaline copper-containing reagent. To reduce the errors, the estimations on all the samples from a given experiment were always done at the end of the experiment but not in sequential order. Samples were estimated in duplicate.

e. Acrylamide Gel Electrophoresis

A preliminary study of the protein components of mandibular saliva secreted during stimulation of the autonomic nerve supply to the gland and during eating was performed using the vertical flat sheet polyacrylamide gel electrophoresis system of Reid and Bieleski (1968). The apparatus provided direct electrical contact with the top and bottom edges of the gel through the buffer in the buffer troughs. Two polyacrylamide gels were employed: (i) 7.5% polyacrylamide gel for soluble salivary proteins, and (ii) 15% polyacrylamide gel containing 1.0% sodium dodecyl sulfate (SDS) for insoluble salivary proteins.

Gel preparation and electrophoresis procedures:

(i) Soluble Proteins (7.5% polyacrylamide gel)

To prepare the gel, 15 ml of a 15% acrylamide (Eastman 5521, Kodak Co., New York) solution containing 0.3% Bis (N, N'-methylene bis-acrylamide, Eastman 8383) (w:v) was mixed with 12 ml of 1.0 M Tris/glycine (Sigma Chemicals Co.) buffer and 3 ml distilled water. The solution was degassed for about 5 minutes and then 0.3 ml of a 10% TEMED (N,N, N',N'-tetramethylene diamine, Eastman 8178) solution

and 0.3 ml of 10% ammonium persulfate (analytical grade reagent, BDH Chemicals Ltd.) were added. Immediately after the addition of these catalysts the gel solution was poured into a 'Plexiglas' slab assembly designed to produce a 140x100x2 mm gel slab. Care was taken to avoid the formation or entrapment of air bubbles within the gel chamber. A mould of 10 or 20 slots was quickly inserted into the top edge of the gel solution. This was kept in an upright position and at least 30 min allowed for polymerization to occur.

Samples for analysis were prepared by adding a small amount of dextrose (5%) and bromophenol (as a tracking dye) to the saliva. Five to ten microlitres of sample was layered with a microsyringe directly into the bottom of each buffer-filled well at the top edge of the gel. Electrophoresis was carried out in 0.1 M Tris/glycine buffer, pH 8.3, for about 2 hours. Current was applied according to the following scheme:

(a) pre-run at 12.5 mA for 30 minutes before applying samples,

(b) run at 12.5 mA for 20 minutes after replacing the buffer in the upper trough and loading the samples,

(c) run at 24 mA for up to 1.5 hours.

After electrophoresis, the gel was stained with a 0.25% (w:v) solution of Coomassie Brilliant Blue R250 (Sigma Chemicals Co.) in a methanol:acetic acid: water mixture, 4:1:1 (v:v:v), for about one hour at room temperature on a shaking bath to demonstrate the protein components. Destaining (elimination of the background stain) was carried out by electrophoresis for 20-30 minutes in a solution containing methanol: acetic acid: water, 25:7:68 (v:v:v). Any remaining excess stain was removed by shaking the gel in the same solution overnight.

(ii) Insoluble Proteins (15% polyacrylamide gel containing 1.0% SDS).

The gel slab was prepared by adding 15 ml of a 30% acrylamide/0.17% Bis (w:v) solution to 3 ml of 1.0 M Tris/

glycine buffer, and 11 ml distilled water. After deaerating for 10 minutes, 0.3 ml of 10% SDS was added, followed by 0.3 ml of 10% TEMED and 0.3 ml of 10% ammonium persulfate.

Insoluble components of saliva were obtained for analysis by centrifugation. To eliminate the residual soluble proteins the precipitate was washed in two changes of phosphate buffer. It was then prepared in 2% SDS and 1% glycerol and allowed to dissolve by boiling at 100°C for two minutes. Bromophenol red was added as a tracking dye.

Electrophoresis, using 0.1 M Tris/glycine buffer containing 0.1% SDS, pH 8.9, was carried out at 50 V for 30 minutes and then at 150 V for about 3.5 hours. The SDS gel was prerun at 150 V for 30 min prior to applying samples to obtain a uniform condition, as the current fell from 30 mA to 24 mA before becoming constant.

Staining of proteins in SDS gel was performed using 1.0% Coomassie Brilliant Blue R250 in methanol: acetic acid: water, 5:1:5 (v:v:v), for 0.5-1.0 hour at room temperature. A destaining solution containing a higher concentration of methanol (methanol: acetic acid: water, 4:1:4; v:v:v) was used in order to wash out the excess SDS. Gels were stored in a holding solution of methanol: acetic acid: water (8:2:30, v:v:v).

4. Drug Administration

A cannula of polyethylene tubing was inserted into either a lateral saphenous or a femoral vein for administration of drugs. These drugs were given either as a bolus or by steady slow administration *via* the venous cannula using a constant infusion pump (Harvard Apparatus Co., Mass.).

The following drugs were used: Atropine sulphate solution (National Dairy Association of N.Z. Ltd., Wellington), 0.1 mg kg^{-1} body weight; carbachol solution (May and Baker Ltd., Dagenham, England), 40 µg kg^{-1}; isoprenaline sulphate (Burroughs Wellcome and Co., Ltd), either 2 µg kg^{-1} or 0.3 µg kg^{-1} min^{-1}; phentolamine mesylate BP solution ('Rogitine',

CIBA Laboratories, Sussex), 1.0 mg kg^{-1}; pilocarpine nitrate (Macfarlan Smith Ltd., Edinburgh), 5.8-7.4 µg kg^{-1} min^{-1}; and propranolol hydrochloride solution ('Inderal', Imperial Chemical Industries Ltd., England), 1.0 mg kg^{-1}.

5. Investigation of the Cellular Origins of Salivary Proteins Secreted in Mandibular Saliva During Sympathetic Stimulation

a. Preparation of Antigens

Two types of antisera were raised in rabbits by multiple injection of antigens I and II which were prepared as follows:

(i) Antigen I

Antigen I contained the major soluble salivary proteins found in sympathetically evoked saliva. The proteins were isolated by using 7.5% polyacrylamide gel electrophoresis. Two hundred microlitres of saliva containing soluble proteins were applied to the top edge of a gel slab and electrophoresis performed as previously described. Following this, two narrow gel strips, each about 10-15 mm wide, were cut from each lateral edge of the gel slab and stained with 0.06% Coomassie Brilliant Blue G250 (Sigma Chemicals Co.) in 3.0% perchloric acid solution at room temperature (Reisner, Nemes and Bucholtz, 1975). This step allowed demonstration, within 10 minutes, of the zone containing the two major protein bands and required no destaining. The corresponding zone on the remaining gel slab was then cut out and the antigen prepared by grinding this with 3.0 ml sterile normal saline solution and subsequently mixing with Freund's adjuvant. This provided sufficient preparation to inject into 2 rabbits.

(ii) Antigen II

Antigen II contained the insoluble precipitates of mandibular saliva. Salivary precipitates obtained from centrifugation of turbid saliva collected during stimulation of the cervical sympathetic nerve (either alone or on a background of pilocarpine infusion) were pooled and washed

in 0.1M phosphate buffer (pH 7.2) and then freeze dried. Twenty milligrams of precipitate was redissolved in 3.0 ml of sterile normal saline solution and mixed vigorously with an equal volume of Freund's adjuvant. The antigen preparation was used for subcutaneous injection into 2 rabbits.

b. Production of Antisera and Isolation of Immunoglobulins

Antisera were produced in rabbits according to a conventional schedule (Harboe and Ingild, 1975). The first injection given was antigen in complete Freund's adjuvant and this was followed by additional injections with incomplete Freund's adjuvant on days 14, 28 and 42. Bleeding was usually performed from an ear vein and was first done on day 50. Every sixth week thereafter further bleeding was performed (the animals had received an antigen injection 8-10 days before each bleeding). About 35-40 ml of blood was obtained on each occasion. Finally, blood was drawn by cardiac puncture with the animal under (terminal) ether anaesthesia.

Serum was obtained from the clotted whole blood by centrifugation at 2,000 g for about 10 minutes and stored at -20^{o}C. Such serum can be frozen and thawed several times without damage to the antibodies (Work and Work, 1970). The immunoglobulins from rabbit antisera were isolated following the procedures described by Harboe and Ingild (1975) of salting out with ammonium sulfate and ion exchange chromatography using DEAE Sephadex A 50 equilibrated with acetate buffer, pH 5.0. This procedure recovers the IgG and IgA quantitatively but IgM is lost.

The antibody titre was determined by single radial immunodiffusion on an agarose gel plate (Mancini, Carbonara and Heremans, 1965). The specificity of antibody was tested using double immunodiffusion in agar.

c. Immunocytochemical Localization of Antigens in Gland Sections

The rabbit immunoglobulins raised against insoluble and soluble salivary proteins secreted in sympathetically evoked saliva were applied to paraffin embedded sections of mandibular glands which had been fixed in Bouin's fixative. Sites of immunoreactive precipitates were assessed at light microscopic level using an unlabelled peroxidase anti/peroxidase (PAP) method (after Taylor, 1978; Sternberger, 1979). Non-immune swine serum was applied prior to the specific antiserum and as diluent to reduce background staining (Burns, 1975). (Step by step details of this method are presented in Appendix 1).

Control sections were included in every study. In these either rabbit non-immune immunoglobulin or Tris/phosphate buffer was substituted for the primary antiserum.

6. Tissue Specimens

In the acute experiments specimens of mandibular glands were taken for morphological studies, as follows:

a. Nerve Stimulation

		Duration of Stimulation	No. of Sheep
(i)	Chorda lingual nerve	2h	4
	" " "	4h	1
(ii)	Cervical sympathetic trunk	2h	4
(iii)	Cervical sympathetic trunk after injection of phentolamine (0.1 mg kg^{-1} body weight, iv)	2h	1
(iv)	Chorda lingual nerve plus ipsilateral cervical sympathetic trunk	2h	1
	" " "	3h	1

Specimens from all 12 sheep were prepared for both light and electron microscopy. The contralateral unstimulated gland was always used as control.

b. Pharmacological Stimulation

Sheep No.

SP1 & SP2 Stimulation of left chorda lingual nerve for 3h, during the last 2h (SP1) or 2.5h (SP2) isoprenaline (0.3 μg kg^{-1} body weight min^{-1}, iv) was infused.

SP3 & SP4 Infusion of pilocarpine (5.8-7.4 μg kg^{-1} body weight min^{-1}, iv) for 2h (SP3) and 5h (SP4). In each case, the left cervical sympathetic trunk was stimulated for the last 2h.

Specimens from all 4 sheep were prepared for light microscopic examination and specimens from SP1 and SP3 were also prepared for electron microscopy.

B. CHRONIC EXPERIMENTS ON CONSCIOUS SHEEP

1. Animals, Diet and Housing

Twenty-two crossbred Romney ewes and wethers, weighing 26-48 kg were used. They were housed in individual metabolism crates and loosely restrained with head stocks. Lucerne chaff (about 1000 g) was given at 9:30a.m. daily and the residue removed 4 hours later. The amounts of food eaten after 1h, 2h, and 4h of feeding were estimated. Water and a block of salt lick were usually provided *ad libitum*. Surgical cannulation of the left mandibular duct in seven of these sheep was performed after they had been housed for at least two weeks so that they were well accustomed to a regular feeding regime and animal house procedures before experimental observations began. To minimize unnecessary environmental influences, access to the animal room was restricted at all times. Experimental observations were begun at 10-14 days after the surgery.

2. Experimental Procedures

The two aspects of the feeding experiments were studied in separate groups of sheep.

a. Morphological Procedures

Specimens of mandibular glands were taken from 15 sheep under the following conditions:

		No of Sheep
(i)	Before feeding (after the food had been withheld for 20h). This was taken as the resting condition.	2
(ii)	After eating for 1h.	3
(iii)	After eating for 4h.	3
(iv)	One hour postprandially	2
(v)	Two hours postprandially	2
(vi)	After eating for 1h with a prior injection of propranolol (1.2 mg kg^{-1} body weight) *via* a jugular vein (2 sheep); a third sheep, used as control, was given sterile normal saline solution (1.2 ml kg^{-1} body weight) instead.	3

b. Physiological Studies

Observations on mandibular salivary flow and salivary protein concentrations during feeding and between feeds were made in seven conscious sheep. Each animal had the left mandibular duct chronically cannulated and the cannula exteriorized on the dorsal aspect of the neck. The ambient temperature in the animal room was 8-15^{o}C.

In 18 experiments on four animals the effect of teasing with food was studied. The teasing procedure involved feeding two sheep in the animal room at the usual feeding time while the other two sheep were denied both food and water and, instead, teased with food for some minutes.

3. Anaesthesia and Surgery

a. Preparation for Anaesthesia and Anaesthesia

Twenty-four hours before surgical procedures, food, but not water, was withdrawn. All surgical procedures were performed with aseptic precautions. Anaesthesia was induced with a short acting steroid anaesthesia ('Saffan CT 341', Glaxo Laboratories Ltd., England) injected into a jugular vein in a dosage of 12-18 mg kg^{-1}. A cuffed endotracheal tube was inserted while the animal was in the sitting position, using a laryngoscope to depress the tongue and expose and illuminate the aditus laryngeus. With the animal supine the endotracheal tube was connected to an anaesthetic apparatus ('Halox' evaporizer, British Oxygen Co., Ltd., London) which delivered a halothane(B.P.)/ oxygen mixture. The concentration of vaporized halothane was adjusted to maintain surgical anaesthesia, and was usually 1.0-2.5%.

b. Surgery: Cannulation of the Mandibular Duct

The left mandibular duct was cannulated at the same site as described for acute experiments, and the cannula was anchored in position with 00-silk (Ethicon Ltd., Sydney). From the point of cannulation a loop of tubing was secured subcutaneously close to the mandibular symphysis with particular care being taken to avoid forming a bend that would occlude the lumen. The cannula was anchored at several points (using silk ties) as it ran caudally toward the angle of the jaw. It was then exteriorized *via* a skin incision on the dorsal aspect of the neck 4-5 cm caudal to the ears (Figure 2.1). Care was taken to avoid the external jugular vein when establishing this subcutaneous route. The incision into mylohyoid muscle, and the dead space, was closed with 00-chromic gut (Ethicon Ltd., Sydney) and that in the skin with Michelle clips. To minimize resistance to salivary flow the cannula inserted into the duct was kept as short as possible (less than 2 cm) and its free end tied into

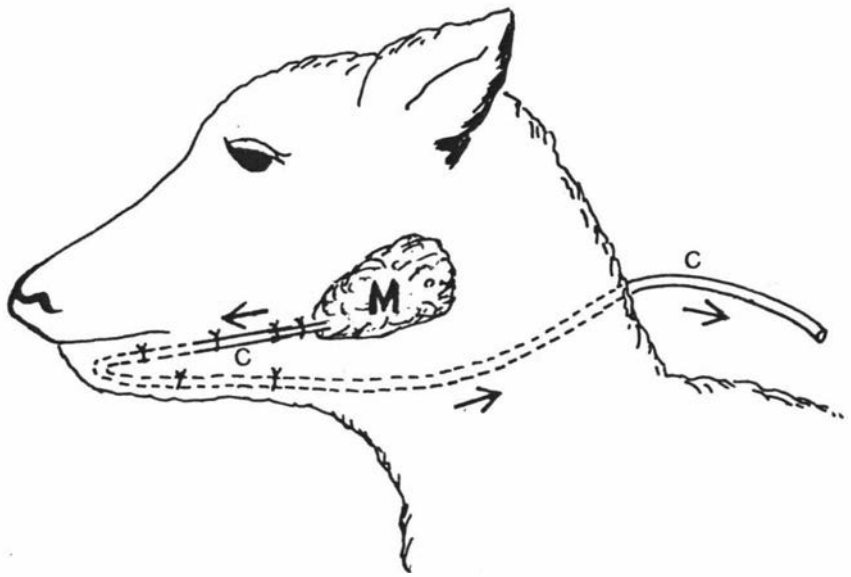

Figure 2.1: Diagram of a chronically placed cannula (c) in the left mandibular duct. Arrows indicate the direction of salivary flow from the left mandibular salivary gland (M). Dotted lines represent the tube that is situated subcutaneously

another tube (silicone rubber; Silastic, Dow Corning Ltd., Michigan, U.S.A.) of larger diameter. Particular care was taken not to constrict the tubes at the connecting points.

At the completion of the surgery the animal was disconnected from the anaesthetic apparatus, but the endotracheal tube was not removed until a brisk swallowing reflex had returned. This procedure was adopted to limit the possibility of inhalation of regurgitated ruminal contents.

Postoperatively the animal was given a long acting penicillin combination ('Prolopen', Glaxo Laboratories Ltd.) by subcutaneous injection, as a routine precaution. None of the sheep suffered complications during the recovery period, and all continued to eat well.

4. Recording Techniques, Apparatus and Estimations

a. Salivary Flow Recording and Collection of Saliva

The exteriorized cannula was connected to a polyethylene tube (OD 3 mm, ID 2 mm) which was run from the neck along the back of the sheep and into a collection bottle. This tube was formed into a coil as it passed along the neck region to ensure that extension of the neck to feed or drink did not place undue tension on the collection system. The tube was anchored to the animal by adhesive nylon strips (Velcro (N.Z.) Ltd., Wellington) which were glued (with Ados-F_2, Ados Chemical Co., Ltd., N.Z.) to the closely clipped skin. When detailed recordings were not being made the saliva was collected into a reservoir bottle which was weighed daily. During intensive collections mandibular salivary flow was recorded in the same manner as described in acute experiments.

Collections of sequential salivary samples from conscious sheep, however, utilized an LKB Fraction Collector (Type 3401, LKB Ltd., Sweden). When the daily supply of fresh food was given the saliva in the dead space of the cannula was collected into a graduated test tube. Following elimination of the dead space saliva, serial saliva samples

of 2-minute duration were collected into weighed test tubes for the first 20-22 minutes. Samples were then taken at 10 minute intervals throughout the remainder of the eating period. Flow rates were expressed as grams of saliva per minute (g min^{-1}). Samples to be analysed on the day of the experiment were kept in chilled tubes; others were stored at -20°C.

In some experiments the animals were teased with food. Immediately before the teasing commenced, a smaller diameter polyethylene tube was inserted into the cannula and the known volume of saliva in the dead space of cannula was withdrawn. When teasing began, another polyethylene tube was inserted along the cannula as far as possible toward the mandibular duct and the secretion was sucked into a 2 ml syringe. Saliva secreted during teasing was collected in a graduated test tube held for convenience at the back of the neck. Teasing was continued until about 1 ml of saliva was collected. The polyethylene tube used for aspiration was removed as soon as teasing stopped and the secretion pooled and stored at -20°C. In some experiments, samples of saliva were also collected into the test tubes held at the back of the neck for the first 20 minutes of feeding after the teasing collection.

b. Estimation of Salivary Protein

The concentrations of salivary proteins in the mandibular saliva collected during teasing and feeding were estimated by the modified Biuret method described in the acute experiments (Section A.3d).

c. Gel Electrophoresis

Samples of total mandibular saliva collected during feeding (from 2 sheep) were also studied electrophoretically on a 7.5% polyacrylamide gel.

5. Venous Cannulation and Administration of Drugs

A temporary intravenous cannula (Portex Ltd., Hythe, Kent, England) was introduced into the jugular vein of the conscious sheep *via* a needle inserted through the skin under local anaesthesia (2% 'Xylocaine'; Astra Chemical Pty. Ltd., Ryde, N.S.W.). This cannula was anchored to the neck with adhesive tape. Clotting in the venous cannula was prevented by the use of heparinized ('Pularin', Evans Medical Ltd., England) saline (1,000 U ml^{-1}) which was withdrawn and replaced afresh at least once daily.

Either propranolol hydrochloride ('Inderal'; 1.0-1.2 mg kg^{-1} body weight), or atropine sulphate (0.1 mg kg^{-1}) solution was injected *via* the temporary venous cannula using sterile disposable plastic syringes. Sterile Ringer's solution (McGraw Ethicals Ltd., Auckland) was administered in the same manner.

Normal saline solution used in chronically prepared animals was made from sterile pyrogen-free water (Water for Injection Sterile Pyrogen Free, May and Baker Ltd., Dagenham, England) and analytical grade sodium chloride.

C. PREPARATION OF TISSUES FOR MORPHOLOGICAL STUDIES

Thin wedges of tissues (4-5 mm cubes) were excised transversely from several lobes of each mandibular gland in anaesthetized animals. In an initial study, paraffin sections of 8 pieces of tissues taken from a variety of sites within a resting gland were examined and found to exhibit a similar histological appearance under light microscopy. Tissues for morphological studies were therefore subsequently taken from random sites in the glands, which were either unfixed or previously perfused *in situ*. They were then immediately placed in fixative and processed for light and electron microscopy following the procedures described in (1) and (2) below. The methods outlined were used on tissues from animals in both acute (Section A.6) and chronic (Section B.2) experiments, as well as on tissues

of resting glands from animals that had not been fed for 20 hours. In the latter case thin wedges of tissue were also removed from unfixed glands for examination of cryostat sections in enzyme histochemical studies (see Section C.3). For comparative purposes, the paired glands were always processed, stained and examined under identical conditions.

1. Light Microscopy

a. Preparation of Paraffin Sections

In the earlier studies, two pieces of tissues (4-5 mm cube) from each gland were fixed in 10% formal saline and Bouin's fluid (Culling, 1974) for at least 24 hours. Bouin's fluid fixation was complete within 24 hours (Culling, 1974) and specimens were transferred into 70% alcohol. Later, pieces of glands that had been fixed by arterial perfusion with half-strength Karnovsky's fixative[3] (Karnovsky, 1965) were immersed in the same fixative for a few days and then washed overnight in 0.1M cacodylate buffer before processing.

Following fixation, the specimens were processed for paraffin embedding following the routine ethanol-chloroform procedure (see Appendix 2), using a Shandon, Elliott automatic tissue processor (Watson, Victor, Ltd.).

Paraffin sections of 5-6 μm were cut using a microtome (C. Reichert, Germany), floated on warm water and mounted onto lightly albuminized 3" x 1" glass slides then air dried at 60°C.

b. Staining Methods for Paraffin Sections

For histological and histochemical studies of the resting glands, the following staining methods were applied:

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[3] In the present study, the half-strength Karnovsky's fixative contained 2.5% glutaraldehyde (EM grade, Agar Aids, Essex) and 2.0% paraformaldehyde (BDH Chemicals Ltd., England) in 0.1M cacodylate buffer, pH 7.2-7.4.

(i) Haematoxylin and eosin (H&E).

(ii) Periodic acid Schiff and haematoxylin (PAS/H) with or without pretreatment with 1.0% malt diastase (BDH Chemicals Ltd.) solution for 30 minutes.

(iii) Alcian Blue 8GX (Gurr, High Wycombe, Bucks, England), pH 2.5, haematoxylin and eosin (AB/H&E).

(iv) Alcian Blue, pH 1.0, Alcian Yellow, pH 2.5 (Carlo, 1964) and haematoxylin (AB, 1.0/AY/H).

(v) Alcian blue, pH 2.5, periodic acid-Schiff and haematoxylin (AB/PAS/H).

A step by step description of the staining methods may be found in Appendix 3.

Based on the results obtained with the above stains, it was decided to stain paraffin sections for the study of the morphological changes in the glands with AB/PAS/H, PAS/H, and AB/H&E.

In one study, serial sections of Karnovsky's fixed tissues were not only stained with PAS/H and AB/PAS/H, but also by omitting the step of periodic acid oxidation (i.e. Schiff's reagent/H, and AB (2.5)/Schiff's reagent/H). These latter sections were used as the controls to assess whether fixation with glutaraldehyde in Karnovsky's fixative interfered with the PAS-staining of mandibular tissues (Hopwood, 1967; 1969).

c. Semi-thin Sections

Semi-thin sections (0.5-1 μm thick) were also cut for light microscopy from resin-embedded tissues prepared for electron microscopy. These sections were stained for 1 minute with 1% toluidine blue (BDH Chemicals Ltd.) in 0.1 M phosphate buffer (pH 7.2) on a hot plate (C. Reichert, HK120, Germany) at 80°C and mounted with DPX mountant (BDH Chemicals Ltd.).

2. Electron Microscopy

a. Conventional Ultrathin Sections

Tissues for electron microscopy were usually fixed *in situ* by dripping the cold fixative onto the exposed glands during arterial perfusion. Artificial respiration (with a Palmer respiratory pump) was maintained *via* the tracheal cannula of the anaesthetized animal during arterial perfusion. Perfusion with phosphate buffered saline (Culling, 1974) preceded that of the half-strength Karnovsky's fixative (Karnovsky, 1965) used for the primary fixation. Perfusion was always *via* the common carotid arteries for about 5-10 minutes using a perfusion apparatus which consisted of 2 bottles, one containing fixative and the other phosphate buffered saline. The outlet tubes from these were joined with a Y-connector which in turn connected to the arterial cannula(e). Cannulation of the carotid arteries was performed conventionally using polyethylene tubes through which flow was controlled by three-way stopcocks. Perfusion pressure was monitored by an aneroid type of pressure gauge connected at the top of each bottle. Perfusion pressures of 100-120 mmHg were used and the temperature of solutions was either 37°C or room temperature. Perfusion was continued until the glands blanched and became firm.

After perfusion, wedges of tissues were quickly taken from the glands, placed in petri-dishes containing fixative and cut into about 1 mm cubes which were then immersed in the fresh fixative and left at room temperature for 2-4 hours. In some early experiments tissues were fixed by immersion overnight at 4°C either in half-strength Karnovsky's fixative or in 6.25% glutaraldehyde in sucrose phosphate buffer, pH 7.2-7.4. In all cases the tissues were washed overnight in 0.1 M buffer containing 7.0% sucrose before postfixing in 1.0% buffered OsO_4 at 4°C for 1.5 hours, and dehydrated in graded ethanol either with or without *en bloc* staining, and then embedded in the resin.

Two different techniques were employed to enhance the contrast of electron micrographs: (i) *En bloc* staining with 1.0% uranyl acetate during dehydration in graded ethanol. These tissue blocks were then embedded in Araldite (Fluka, Switzerland). (ii) Using an Epon 812 (Agar Aids, Essex) - Araldite (Fluka) mixture as embedding medium (without *en bloc* staining) following the method described by Mollenhauer (1964).

Ultra-thin sections of approximately 70 nm thickness were cut on an LKB Ultrotome III using glass knives, and mounted on unsupported copper grids. The sections were stained with saturated uranyl acetate in 50% ethanol for 6 minutes and in lead citrate (Venable and Coggeshall, 1965) for 8-10 minutes and then examined at 60 KV in a Philips 200 transmission electron microscope.

In two experiments, insoluble precipitates of sympathetically evoked saliva were prepared for electron microscopic assessment, following the fixation methods described by Glauert (1974).

(i) Fixation in a Pellet

The saliva was centrifuged in pellet (at 2,000 r.p.m. for 5 minutes) before being fixed with half-strength Karnovsky's fixative for 15-30 minutes, postfixed in 1.0% OsO_4, then dehydrated and embedded in Araldite.

(ii) Fixation in Suspension in Medium

Saliva was fixed directly in the cold fixative after being collected *via* a mandibular cannula during stimulation of the cervical sympathetic trunk. The saliva was allowed to fix for 15-30 minutes, centrifuged to form a pellet, then processed in the same way as in (i) above.

b. Freeze-Etching Study

A comparison of the structure of secretory granules in the secretory endpieces of resting mandibular glands was made between electron micrographs obtained from thin sections and freeze-etching replicas. Tissues used for this purpose

were prepared initially in the same manner as for other examinations with the electron microscope but after 30 minutes of immersion fixation were placed in 30% glycerol (in the same buffer) and stored at 4°C. The freeze-etching study used the simple cold-block freeze-fracture technique described by Bullivant and Ames (1966) and Bullivant (1973; 1980) and was kindly undertaken by Dr S. Bullivant (Cell Biology Department, University of Auckland, Auckland). Replicas were produced, either (i) by a normal freeze-fracturing method, using tissues with or without postfixation in 1.0% OsO_4, or (ii) by a method involving deep-etching of the complementary replicas.

3. Preparation for Enzyme Histochemical Studies under Light Microscopy

Cryostat sections (12-30 µm thick) of tissues from resting mandibular glands were prepared for enzyme histochemical studies. They were cut either after being rapidly quenched in isopentane cooled by liquid nitrogen or after being frozen directly in a Lipshaw Electric Cryotome (Lipshaw Manufacturing Co., Mich., U.S.A.) at -30°C. Sections were then mounted on clean slides without adhesive and allowed to dry in air for 1-3 hours to ensure adherence. With each enzyme, tissues from the parotid glands were used for comparative study.

The following enzyme activities were undertaken:

a. Alkaline Phosphatase Activity (to demonstrate the myoepithelial cells) using a calcium cobalt sulphide method suggested by E.W. van Lennep (personal communication). Tissues from kidney were used as a standard.

b. Acetylcholinesterase Activity (to demonstrate cholinergic nerves) following the method of Gerbtzoff (1959). Sections were incubated in a medium containing different substrates with or without inhibitor as follows:

(i) Acetyl-thiocholine iodide (Koch-Light Laboratories, Ltd., England) plus 3 x 10^{-5} M eserine (Physostigmine salicylate, Macfarlan Smith Ltd., Edinburgh) for control sections.

(ii) Acetyl-thiocholine iodide plus 3 x 10^{-6} M iso-OMPA (tetrapropylpyro-phosphoramide, Koch-Light Laboratories Ltd.) for AChE staining (after Garrett, 1966a).

(iii) Butyryl-thiocholine iodide (BDH Chemicals Ltd.) for nonspecific cholinesterase (BuChE) staining.

c. Biogenic Amine Activity (to demonstrate adrenergic nerves). The sucrose-potassium phosphate glyoxylic acid (SPG) method, as modified by de la Torre and Surgeon (1976) to give high specific sensitivity for monoamine histofluorescence, was used.

More detailed descriptions of these enzyme histochemical methods used may be found in Appendix 4.

CHAPTER III

OBSERVATIONS ON THE STRUCTURE OF THE RESTING MANDIBULAR GLAND IN SHEEP

A. RESULTS

In every animal examined the mandibular glands were irregularly quadrilateral with rounded angles. The mean wet weight of the adult resting gland was 10.55 ± 0.63g($\bar{X}$ ± SE, n = 46) with differences of 0.81 ± 0.13 g (n = 23) present between glands of individual animals. The parenchyma was organized into lobules, separated by interlobular connective tissue septa and neurovascular structures.

1. Histology

The present results confirmed that histologically, the gland may be described as a compound tubulo-acinar organ, composed of secretory endpieces and a ductal system which conveys secretory products to the oral cavity through a the intercalated, intralobular striated and excretory ducts.

As shown in Figure 3.1, the secretory endpieces consisted of 2 distinct cell types: the tubulo-acinar cells and demilune cells. The acinar cells, whose shape varied from pyramidal to rectangular, were arranged around a central lumen so that in a single plane the acinus consisted of 5-6 cells. These cells stained poorly with haematoxylin-eosin (H&E) in paraffin sections, but displayed metachromasia with toluidine blue on 0.5 µm resin-embedded sections. Their nuclei were characteristically located in the basal region and were usually seen to be indented by secretory granules. About 1-3 nucleoli were present within each nucleus. In the resting gland the central acinar lumen was very small but nevertheless always readily seen on 0.5 µm sections.

Demilunar cells contained granules which were acidophilic in paraffin sections (Figure 3.1A) and, in resin embedded sections, stained darkly with toluidine blue (Figure 3.1B-D). In an Epon-Araldite mixture, the cells showed metachromasia with toluidine blue (Figure 3.1C). The nuclei of demilunar cells were rounded and located basally. At the apical end cellular extensions frequently passed between two mucous cells to reach the acinar lumen.

The intercalated ducts formed short segments between acinar cells and the striated ducts and were lined with low cuboidal epithelial cells with large centrally located nuclei. (Basal cells were occasionally observed in this order of duct). Striated ducts were branched and widely dispersed throughout the lobules. Paraffin sections stained with H&E revealed no staining differences between individual duct cells. This contrasted with resin-embedded sections treated with toluidine blue in which cells of the striated ducts showed varying intensities of staining, ranging from pale to dark. The typical cells in such sections were columnar, pale, possessed basal striations and large, round or oval, centrally placed nuclei. Dark cells were present in small numbers.

Myoepithelial cells, distinguished by their elongated nuclei and cell profile, lay at the base of demilunes or acinar cells (Figure 3.1C). They were also commonly located at the base of the intercalated ducts (Figure 3.1B,D).

Large blood vessels were found in interlobular connective tissue spaces and many capillaries were indented with the striated duct cells. Interstitial plasma cells were present in juxtaposition to the endpiece secretory cells and striated ducts in relatively large numbers.

2. Histochemistry

As shown in Figure 3.3, acinar cells from the resting gland stained strongly with Alcian Blue solution (AB) and Alcian Yellow solution (AY) at pH 2.5, but not with AB at pH 1.0, which suggests they have a rich content of acidic nonsulphated glycoproteins (Ravetto, 1964). Intense purple-

blue staining was obtained with the AB (2.5)/PAS sequences.

Judged from the PAS-positive reactions after digestion with malt diastase the acinar cells also contained both glycogen and neutral glycoproteins (Figure 3.2). Variations of PAS-reactions occurred among individual glands. The acini in most mandibular glands displayed a uniformly faint PAS-staining following diastase, as illustrated in Figure 3.2, and this treatment also diminished the AB (2.5) staining in acini.

Somewhat different staining reactions were encountered in demilunes where, in individual cells, granules displayed varying degrees of positive-staining for carbohydrate (Figures 3.2; 3.3). Staining was (i) mildly positive with AY and AB at pH 2.5, but not with AB at pH 1.0, (ii) PAS-positive, diastase-resistant, or (iii) both AB (2.5) and PAS positive. Unlike the acinar cells, diastase treatment had no effect on the weakly alcianophilic granules in demilunes and PAS-reactivity remained strongly evident.

No positive-staining with AB or PAS was demonstrated in intercalated ducts, but in striated and excretory duct cells AB-negative and diastase-resistant, PAS-positive reactions were usually present. Large goblet cells (diastase-resistant, PAS-positive and AB negative) were always found in the excretory duct.

Hopwood (1967; 1969) reported that when tissues are fixed with glutaraldehyde, they become Schiff-positive because the glutaraldehyde-protein complexes contain free carbonyl groups introduced by the fixation. However, in controls carried out in the present investigation by omitting the periodic acid-oxidation, sections of Karnovsky's fixed tissues showed only a mildly non-specific positive staining (Figure 3.3D,E) which did not complicate the observation of PAS-stained tissues.

In Karnovsky's fixed paraffin sections, cells of the striated ducts displayed abundant cytoplasmic granules which were strongly PAS-positive and diastase-resistant. These

granules were present particularly around the nuclei and in the apical regions (Figure 3.2C,D). On the other hand, with tissues fixed in Bouin's fluid (Figure 3.2A,B) or formal saline solution, constant features of striated duct cells were large empty areas surrounding the nuclei and the PAS-positive reaction products were present only in the region of the apical membrane.

3. Enzyme Histochemistry

a. Alkaline Phosphatase Activity

The distribution of myoepithelial cells was assumed to coincide with that of alkaline phosphatase activity. In the mandibular glands of sheep strong reaction products were present in the basal surface of endpiece secretory cells and also at basal surface of excretory ducts (Figure 3.4A). Reaction products were seldom detected in the basal regions of striated ducts but were more frequent at the luminal surface (Figure 3.4B) - a finding similar to that reported for the parotid glands of sheep by van Lennep *et al.* (1977). The distribution of alkaline phosphatase activity in the parotid glands is shown in Figure 3.4D. The characteristic processes of these 'basket-like' cells were readily observed in both mandibular and parotid glands (Figure 3.4C,D).

b. Biogenic-Amine Activity

A study of the biogenic-amine activity in the sheep mandibular gland indicated that the gland has a rich adrenergic innervation (Figure 3.5B). Fluorescent fibres traversed the interlobular connective tissue spaces before giving smaller branches to secretory endpieces but no fluorescence could be localized within individual acini. Occasionally fibres located at the basal regions of striated ducts were observed but associations with individual cells were not discernable. Strong fluorescence was present within the muscle coat of arterioles (Figure 3.5C), and small blood vessels.

Light microscopic observations on the innervation pattern of parotid glands were also carried out to allow a comparison between the mandibular and parotid glands which elaborate different types of secretion. Figure 3.5A shows fluorescent products, as very fine strands and dots, distributed throughout the parenchyma in the same general way as in the mandibular gland, though in a lesser density. The striated and interlobular ducts of the parotid were apparently devoid of an adrenergic innervation, whereas rich fluorescence was present in the blood vessel walls.

c. Acetylcholinesterase (AChE) Activity

Judged by the occurrence of AChE activity the distribution pattern of cholinergic nerve fibres to the mandibular gland was essentially similar to that of the adrenergic nerves (Figure 3.6B,C). Positive reactions for AChE activity were confined to the basal aspects of secretory endpieces where they gave the appearance of a very fine network. Major nerve trunks coursed in interstitial connective tissue spaces adjacent to blood vessels and main ducts while relatively large numbers of smaller nerve bundles were evident in the interlobular connective tissues in close relation to the basal regions of striated ducts. A positive AChE reaction was also present near the walls of some blood vessels. In sections incubated for 4 hours, weak butyrylcholinesterase (BuChE) activity (pseudocholinesterase) occurred at the same sites as those of AChE activity but the density of reaction was much less (Figure 3.6D). If the incubation time was 1-2 hours, BuChE activity was evident only at the sites of main nerve trunks and was very weak. Control sections incubated in incubation medium plus eserine showed no reactions.

In the parotid gland (Figure 3.6A), AChE-positive reaction products were most dense throughout the glandular elements, but were rarely found around the intralobular duct system. Nevertheless, large AChE-positive nerves were seen in the interstices between lobules near excretory ducts.

The reactions were more intense than those about the secretory cells of the mandibular gland. A few AChE-positive reactions were present in the blood vessels of the parotid gland as well but no BuChE activity was observed.

4. Electron Microscopy

a. Secretory Endpieces

(i) Acinar cells

The most prominent feature of acinar cells was their secretory granules. These granules appeared to be of a single type whose membranes coalesced so that, in the resting gland, the cells appeared almost full of clustered and poorly delimited granules (Figures 3.7; 3.8A). The granular content was predominantly electron lucent although frequently the background took on a finely fibrillar appearance and occasionally a small electron dense body (0.1-0.2 μm diameter) was included (Figure 3.8A). Because of the extent of the granules, the ground cytoplasm and cellular organelles were scarce. RER was identifiable only basolaterally, basally and scattered among granules. Typically, the Golgi complexes, which were not well-developed, were located laterally or apically to the nucleus and were surrounded by secretory granules. Mitochondria were evident in close association with the intercellular plasma membranes and occasionally among the granules. The nuclei, which were located at the cell base, were flattened and irregular in outline and contained condensed chromatin (Figure 3.7A).

Microvilli, though present at the apical surfaces, were not conspicuous structures in acinar cells being both few in number and small in size. The junctions between acinar cells at the basal and lateral surfaces were of a simple type with few digitations, and those at the apical region appeared as typical junctional complexes.

In freeze etching studies, deep-etched complementary replicas showed the presence, in acinar granules, of a

small circular area (ca. 0.2 µm diameter) of fine particles which appeared to correlate with the small dense body identified in conventional thin sections (Figure 3.8A-D). The secretory granules of acinar cells also contained a network of small groups of aggregated particles (Figure 3.8 C,D).

(ii) Demilunes

Demilunar cells, as with the acini, were distinguished by their granule content when in the resting state. Here, however, granules were smaller, discrete and of spherical shape (ca. 0.6-1.2 µm diameter). They were delimited by distinct membrane boundaries and internally displayed varying electron density and what appeared to be separate substructures (Figure 3.9). Three regions in the granules could usually be distinguished - (a) a fine granular matrix of moderate electron density, (b) a dense, finely granular region, and (c) a small heterogeneous substructure containing an inclusion of dense strands and fine particles within an electron lucent background. This substructure was oval (ca. 0.2-0.4 µm diameter) and seemed to be enclosed by a membrane-like boundary (Figure 3.10). The inclusions of the substructures in some glands tended to be easily extracted.

Apart from the above tripartite granules which were the major population in the cell, there were others which were either of uniform internal structure (electron moderate or dense appearance), or of bipartite appearance consisting of any two of the regions of the tripartite granules. Considerable variation in the above morphological appearance of demilune granules was evident in different cells of any one secretory endpiece (see Figure 3.10).

In freeze etching studies the areas of differing structure in the cross-fractured granules of demilune cells were so variable that no correlation with the tripartite structures seen in thin sections was possible.

At the basal region of the cells prominent parallel arrays of RER were present along with mitochondria which were also found in close association with the plasma membranes of intercellular canaliculi. Golgi apparatus was not usually prominent, but lysosomes were common in the basal cytoplasm adjacent to the nuclei and basement membranes (Figures 3.9A; 3.10C). Long microvilli projected into lateral intercellular canaliculi (Figure 3.9B) and basolateral canaliculi (Figure 3.10C).

b. The Duct System

(i) Intercalated Ducts

The cellular transition between acini and intercalated ducts was always relatively abrupt and there was a similar abrupt change at the junctions of intercalated and striated duct cells. The cytoplasm of intercalated duct cells (Figures 3.11; 3.12) contained no secretory granules but short profiles of RER and numerous free ribosomes were present. A Golgi complex was not always evident: when present it was limited and typically located in a juxtanuclear position. Mitochondria were similarly sparse. The shape of the nuclei of intercalated duct cells ranged from ovoid to irregular and they occupied a comparatively large part of the cell. At the membrane boundaries of intercalated duct cells there were tight junctional complexes and desmosomes were common at apical interphases but otherwise the membranes of these cells showed little evidence of specialization. The lateral intercellular membranes exhibited some folding and interdigitation (a feature that was most obvious in basolateral areas) but basal infolding was absent. A few short microvilli were present at the apices of the cells. Fine fibrils (tonofilaments) were found in association with desmosomes and were in abundance throughout the apical cytoplasm.

Basal cells were seen occasionally in intercalated ducts in the position their name implies. They were of small size with large nuclei and apparently lacked cyto-

plasmic connection with the ductal lumen (Figure 3.12B).

(ii) Striated Ducts

Complex foldings of basal membranes and vertically oriented mitochondria characterized the striated duct cells. Four distinct cell types could be identified in the ducts. As outlined below, these were classified simply as type I-IV according to the electron density of their cytoplasm and their position in the ducts.

Type I (Tall Light Cell) constituted the principal cell type (Figures 3.13; 3.14 ; 3.15A). These tall columnar cells had a large centrally placed oval or spherical nucleus and elaborate infoldings of their basal membranes with associated rod-shaped mitochondria. These foldings and the electron dense mitochondria occupied about one-third of the cell area. In the apical regions there were scattered mitochondria, short RER cisternae and numerous cytofilaments, as well as small dense bodies (ca. 40-170 nm diameter) that were only found close to the apical membrane. The apical surface itself was organized into short microvilli. Laterally and toward the apex, adjacent striated duct cells were attached by tight junctions and desmosomes. Occasional blebbing occurred apically in some type I cells (Figure 3.14). Numerous polyribosomes were distributed throughout the cytoplasm while a few Golgi complexes with dilated cisternae were present in juxta-nuclear regions. Long RER cisternae and mitochondria were normally arranged in the perinuclear region and lysosomes were common. Electron lucent vesicles of varying sizes (similar in size to mitochondria) were present in some cells (Figure 3.14).

Type II (Tall Dark Cell) shown in Figures 3.14 and 3.15A, was a columnar narrow cell of relatively high electron density which corresponded to the darkly toluidine blue-stained cells described earlier with the light microscope. These also had large central nuclei and, at their basal regions and apical surfaces, exhibited the general

features described for the type I variety. Unlike these, however, mitochondria and empty vesicles were present in larger numbers.

Type III (Apical Cell). As can be seen in Figure 3.15A these apically located cells possessed no basal striations. They were connected to the basement membranes by a narrow cytoplasmic process and could be distinguished from type I by their irregular shape, more apically placed nuclei and more electron dense cytoplasm. This darker cytoplasm appeared to be due to the presence of abundant cytofilaments. Mitochondria of this cell type were small in size and few in number and dense apical granules were rare. The lateral plasma membranes exhibited the narrow plication typical of all striated duct cells. The distribution of type III cells was less frequent than type II - usually as a single cell. In rare cases, an apical cell with electron lucent cytoplasm was evident (Figure 3.15B).

Type IV (Basal Cell). Basally positioned type IV cells were encountered in striated ducts in a very occasional fashion. Their appearance resembled that of the basal cells of intercalated ducts in that they lacked basal striations (Figure 3.13) and had no contact with the lumen.

The fine structure of the excretory ducts was not systematically studied because these were rarely observed in ultrathin sections obtained by random sampling.

c. Myoepithelial Cells

Myoepithelial cells lay within the basement membranes that enveloped the demilunes and acini, and their processes could be seen to terminate at the cell bases of intercalated ducts. The general arrangement of myoepithelial cells was multipolar, with the processes that invested the ducts being more slender than those about the endpieces. The nuclei of myoepithelial cells were generally ellipsoidal (Figures 3.1C; 3.11A). Connection between the visceral surface of a myoepithelial cell and the base of duct cells or endpiece

secretory cells was made by several desmosomes. The finger-like processes of the myoepithelial cells terminated on the basolateral interdigitation of intercalated duct cells (Figures 3.11; 3.12A). No myoepithelial cells were observed on striated ducts.

d. Innervation

Large nerve bundles were identified in the interstitial spaces as shown in Figure 3.16A and smaller groups of unmyelinated fibres were located outside the basement membranes of a variety of parenchymal cells (e.g. Figure 3.16B,C). Thus unmyelinated axons were in the proximity of acinar cells, demilune cells, myoepithelial cells of demilune or acinar cells, capillaries, striated duct cells, interstitial plasma cells and intercalated duct cells. Close approximations were most frequently seen, however, with secretory cells and myoepithelial cells, and less frequently with striated duct cells. In these latter sites the distance between an axon and an effector cell was rarely less than 0.2 μm. These interstitial unmyelinated nerves appeared as varying numbers of axons enwrapped by a single Schwann sheath. They contained small vesicles both granular and agranular (about 40 nm diameter) and also a few large granular vesicles (80-100 nm diameter), as well as small mitochondria. Axons containing numerous large granular vesicles (ca. 80 nm diameter) were occasionally found (Figure 3.16A).

Hypolemmal axons were observed occasionally beneath the basement membranes of striated ducts. They were found among the basolateral intercellular membranes between type-I cells (Figure 3.18A) and between type-I and type-IV cells (Figure 3.18B). In one case, a small hypolemmal axon with dense cored vesicles (70-100 nm diameter) was seen apically, between the lateral intercellular membranes of a mucous cell and an intercalated duct cell (Figure 3.17). No hypolemmal axons were observed either between adjacent secretory cells, myoepithelial cells, or blood vessels. No definite identification of nerve types was possible with the hypolemmal axons.

Figure 3.1: Light micrographs of the resting mandibular gland from a sheep whose food had been withheld for 20 hours.

A. Paraffin section (6 µm thick), stained with H&E. The secretory endpieces (SEP) consist of acinar cells (A) and demilunes (D), and the intralobular duct system of intercalated ducts (ID) and striated ducts (SD). Excretory (extra-lobular) ducts are not shown. x250.

B-D Araldite-embedded sections (0.5 µm thick), stained with toluidine blue.

B. The varying staining intensities in the SD cells and the SEP, and a myoepithelial cell (arrow) within the basement membrane of an intercalated duct are seen. x500.

C. A myoepithelial cell with an ellipsoidal-shaped nucleus at the base of a secretory endpiece (arrow), and the apical extension of a demilune cell between two acinar cells to reach the acinar lumen (L). x1,250.

D. High magnification of an intercalated duct (ID), which shows a basal cell (b) in addition to the ordinary intercalated duct cells and a myoepithelial cell (arrow). x1,250.

(Other abbreviations: P-interstitial plasma cell, c-capillary).

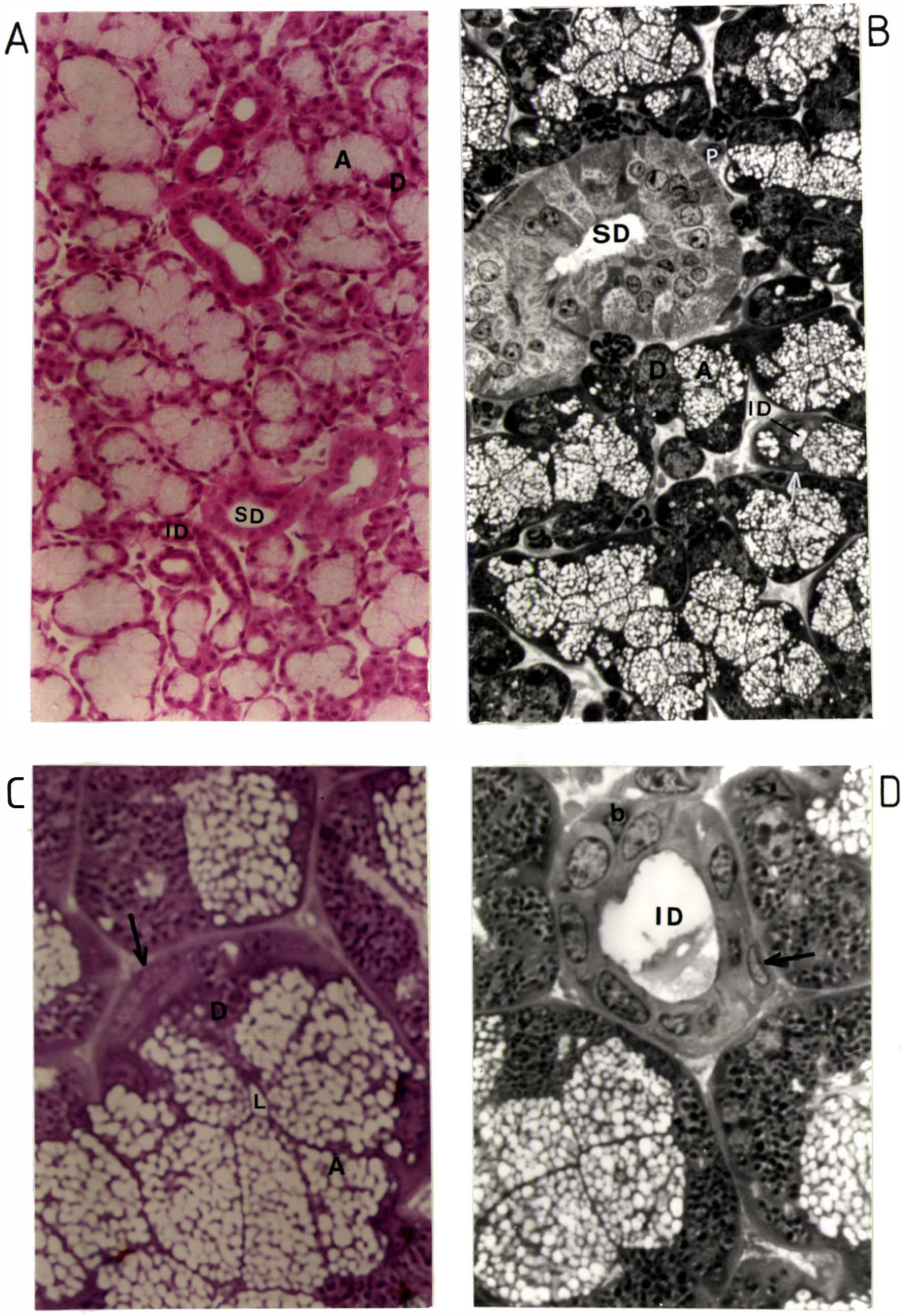

A
A
D
SD
ID
B
P
SD
D
A
ID
C
D
L
A
D
b
ID

Figure 3.2: Light micrographs of the resting mandibular gland stained to demonstrate carbohydrate.

A comparison of tissues fixed and stained in:

A. Bouin's fluid; PAS/H.

B. Bouin's fluid; diastase, PAS/H.

C. Karnovsky's fixative; PAS/H.

D. Karnovsky's fixative; diastase, PAS/H.

Note the empty areas (arrow) within striated duct cells in sections fixed in Bouin's fluid. Sections incubated with diastase show a depletion of PAS staining in the acinar cells. (6 µm sections). x250.

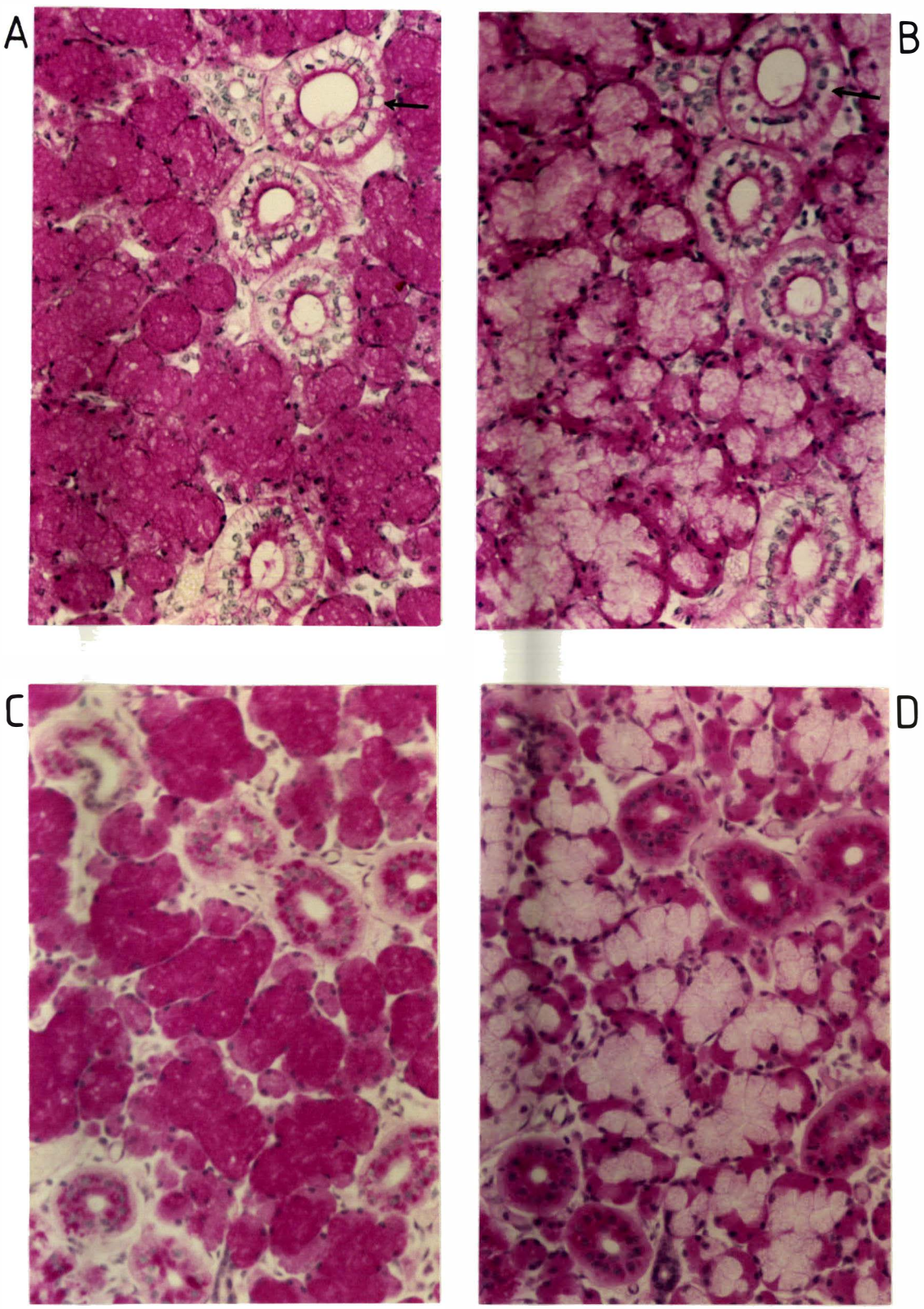

A
B
C
D

Figure 3.3: The resting mandibular gland, half-strength Karnovsky's fixed paraffin sections, stained for carbohydrates. (Details of staining characteristics are given in text). x250.

A. Alcian Blue (pH 2.5)/ H&E

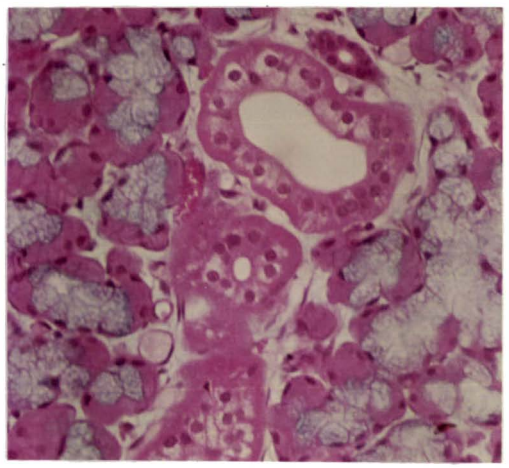
B. Alcian Blue (pH 1.0)/ Alcian Yellow (pH 2.5)/ H&E

C. Alcian Blue (pH 2.5)/ PAS/H

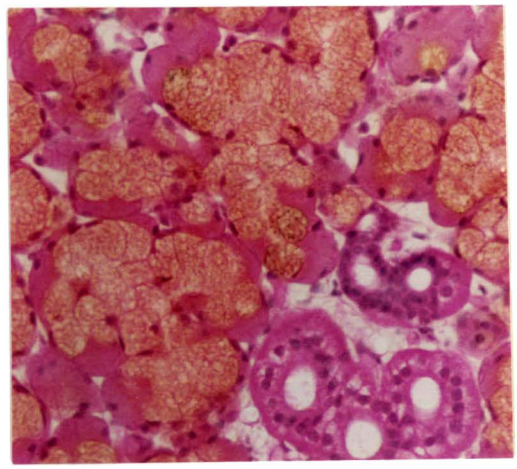
D. Alcian Blue (pH 2.5)/ Schiff's reagent/ H

E. Schiff's reagent/ H

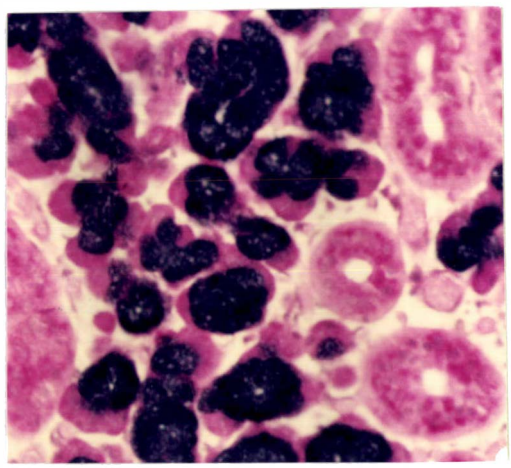
A



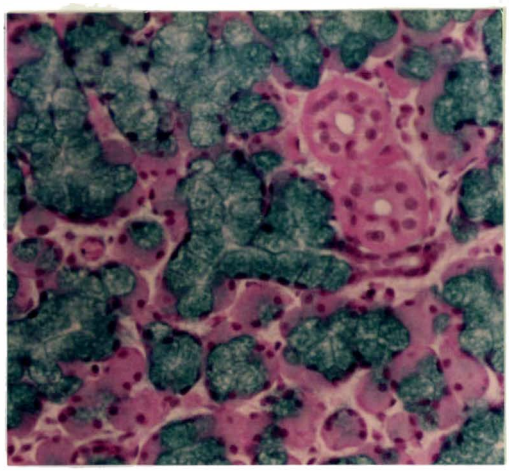
B



C



D



E

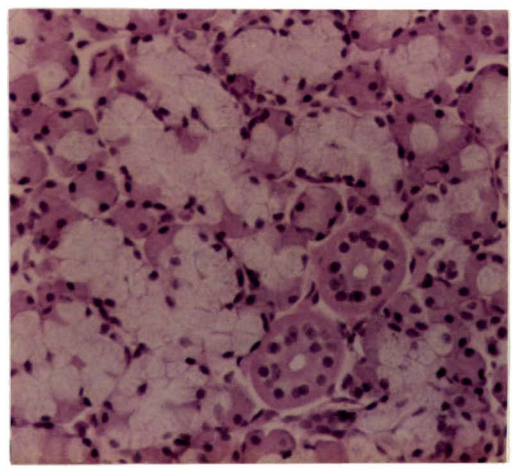


Figure 3.4: Light micrographs of sections stained for alkaline phosphatase activity in the mandibular gland (A-C), and the parotid gland (D). Without counterstaining.

A. Strong alkaline phosphatase reaction products (dark brown) are evident around the secretory endpieces, and also at the interlobular duct cells (ED), but not the striated ducts (SD). (20 µm-cryostat section; x100).

B. Note the positive-reaction products at the apical surface of the striated ducts. (12 µm cryostat section; x500).

C. Showing the multipolar processes of myoepithelial cells at the bases of the secretory endpieces. (12 µm-cryostat section; x1250).

D. Showing strong positive-reaction products of multipolar cells ('basket-like' cells) in the secretory tissues of the parotid gland. (20 µm-cryostat section; x500).

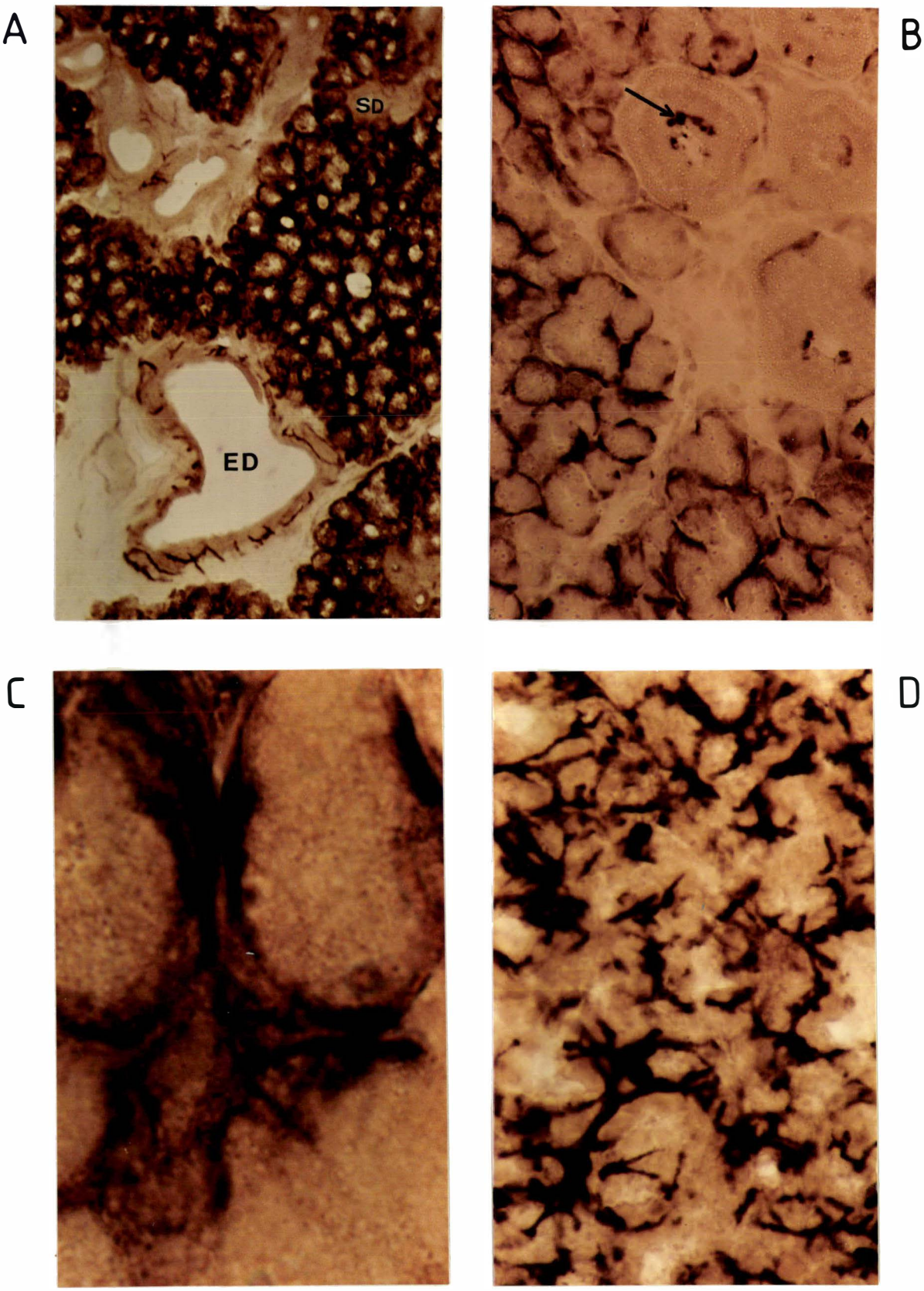

A
SD
ED
B
C
D

Figure 3.5: Light micrographs of biogenic-amine fluorescent fibres (arrow) in 20 µm-cryostat sections of:

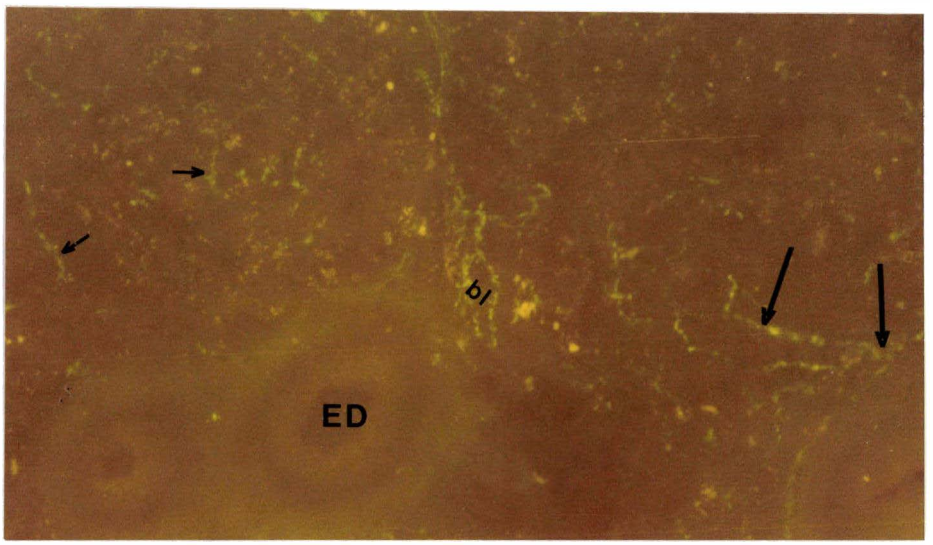
A. Parotid gland. A few adrenergic nerves are shown near the secretory cells (small arrow), as is the course of fluorescent fibres between lobules (large arrow). There is a richer innervation of the small blood vessels (bl). (The autofluorescence showed yellowish orange colour). x100.

B. Mandibular gland, showing a rich adrenergic innervation both around the secretory endpieces (small arrow) and coursing along the lobules (large arrow). Note the relatively poor innervation of the striated (SD) and excretory (ED) ducts. x100.

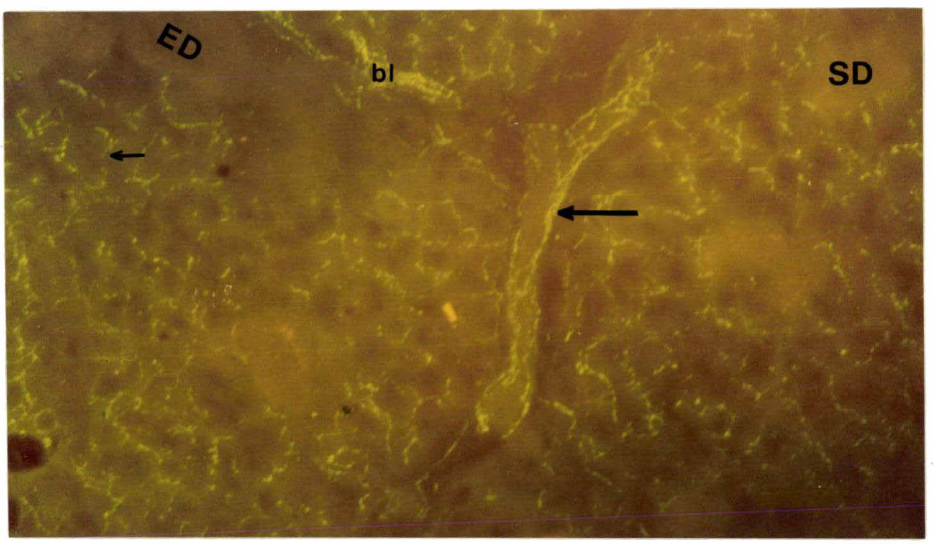
C. Fluorescent fibres in the wall of a blood vessel (arrow) but not in an excretory duct (ED) in a mandibular gland. x500.

Photomicrographs taken from the 400ASA either Fuji (A&B) or Kodak (C) colour negative film.

A



B



C

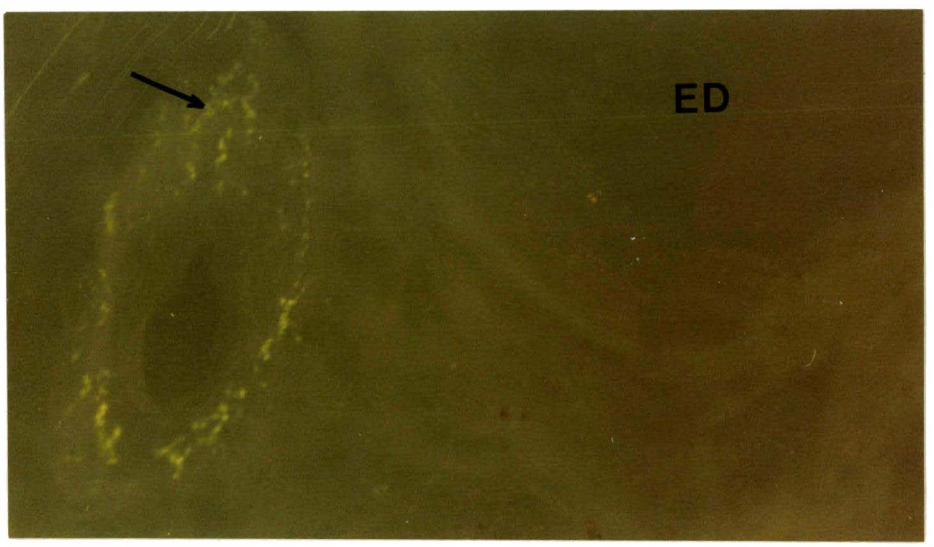


Figure 3.6: Light micrographs of sections stained for cholinesterase activity (brown) in the parotid (A) and mandibular (B-D) glands.

A. AChE-staining (2h incubation) of a parotid gland, showing AChE-positive nerve fibres which are most dense around the acinar structures. Larger positive nerves along the excretory ducts. (20 µm thick cryostat section, counterstained with haematoxylin). x100.

B-C AChE-staining (2h incubation) of a mandibular gland, showing the distribution pattern of the cholinergic nerves around the secretory endpieces, and also alongside the striated and excretory ducts. (20 µm thick cryostat section, without counter-staining). B. x100; C. x250.

D. BuChE-staining (4h incubation) of a mandibular gland, showing a weak non-specific cholinesterase (BuChE) activity (arrow) in the interstices alongside the striated and excretory ducts. A weakly positive reaction is seen at the secretory endpieces. (12 µm thick cryostat section, counterstained with haematoxylin). x100.

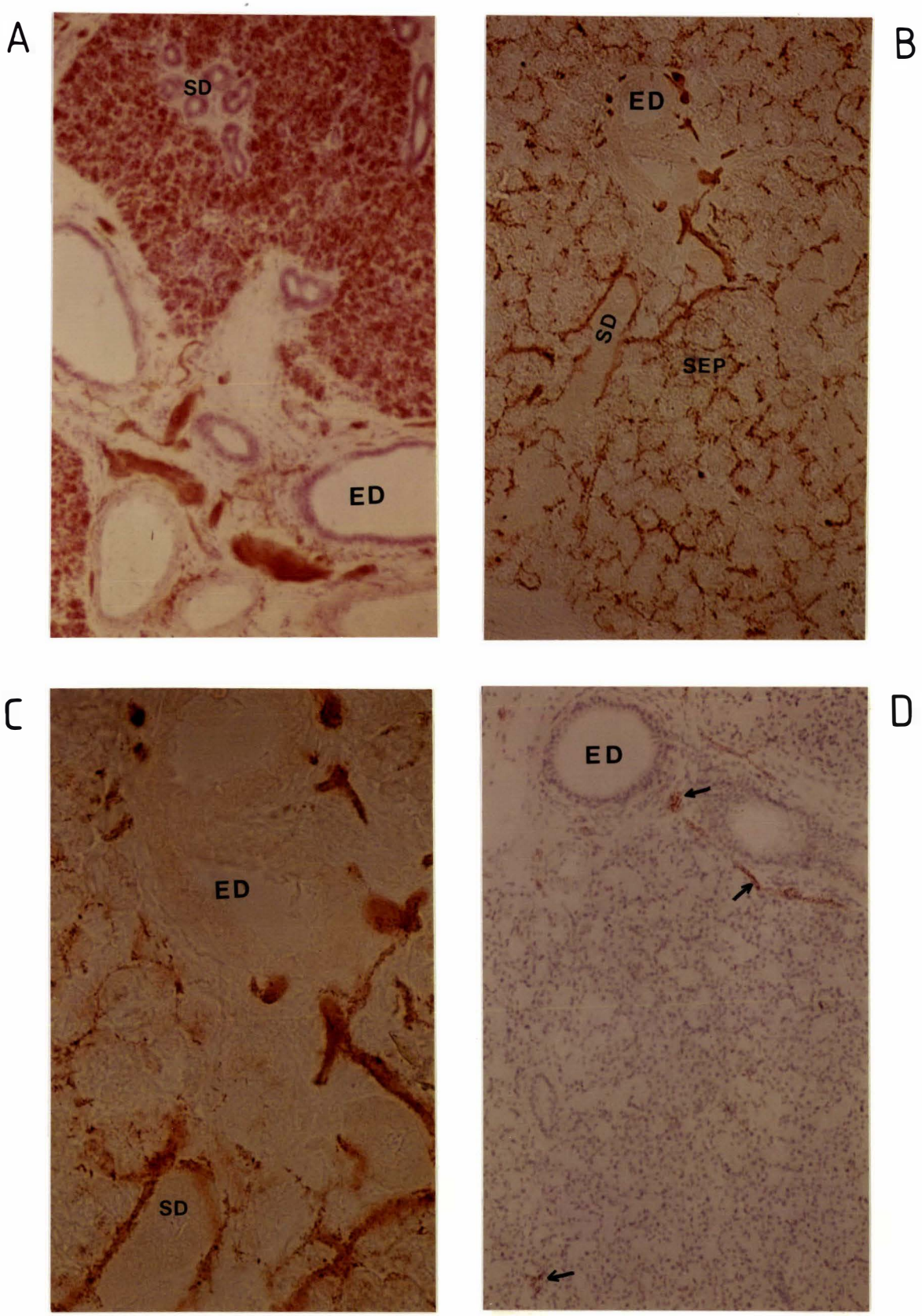

A
SD
ED
B
ED
SD
SEP
C
ED
SD
D
ED

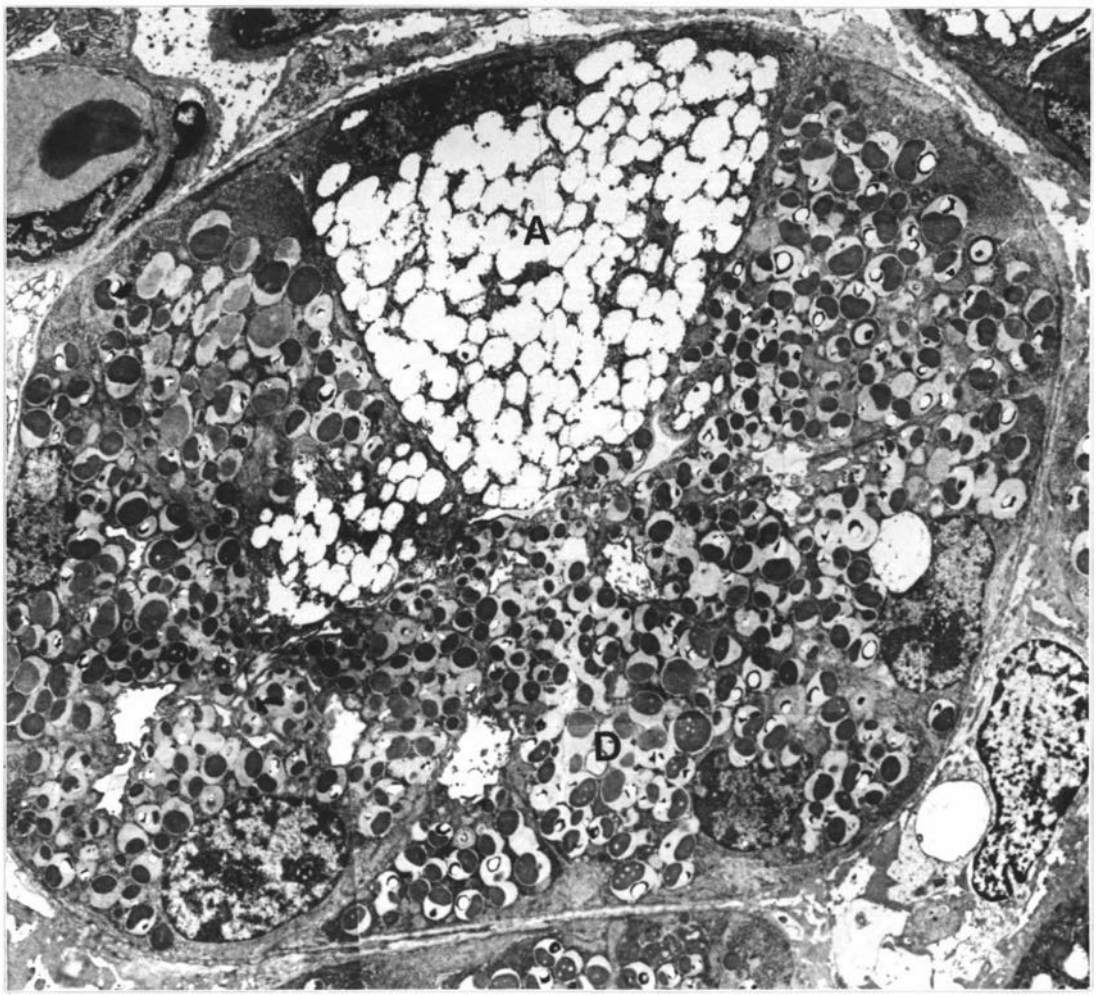
Figure 3.7: The ultrastructure of the resting mandibular gland, showing demilunes (D) and acinar cells (A).

(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; and *en bloc* staining).

A. A secretory endpiece. x4,900.

B. A portion of acinar cells and demilunes, showing the apical extension of demilune cells between acinar cells to reach the acinar lumen (L). x9,000.

A



B

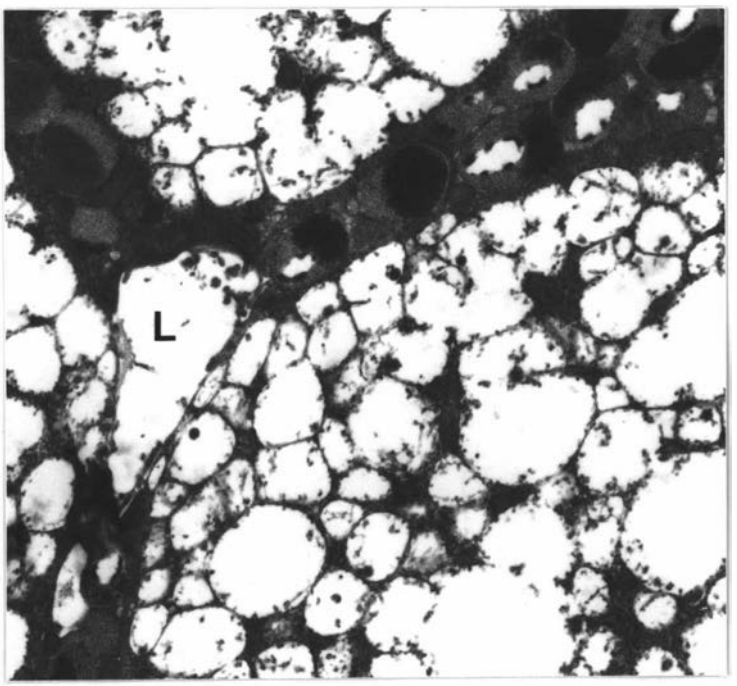
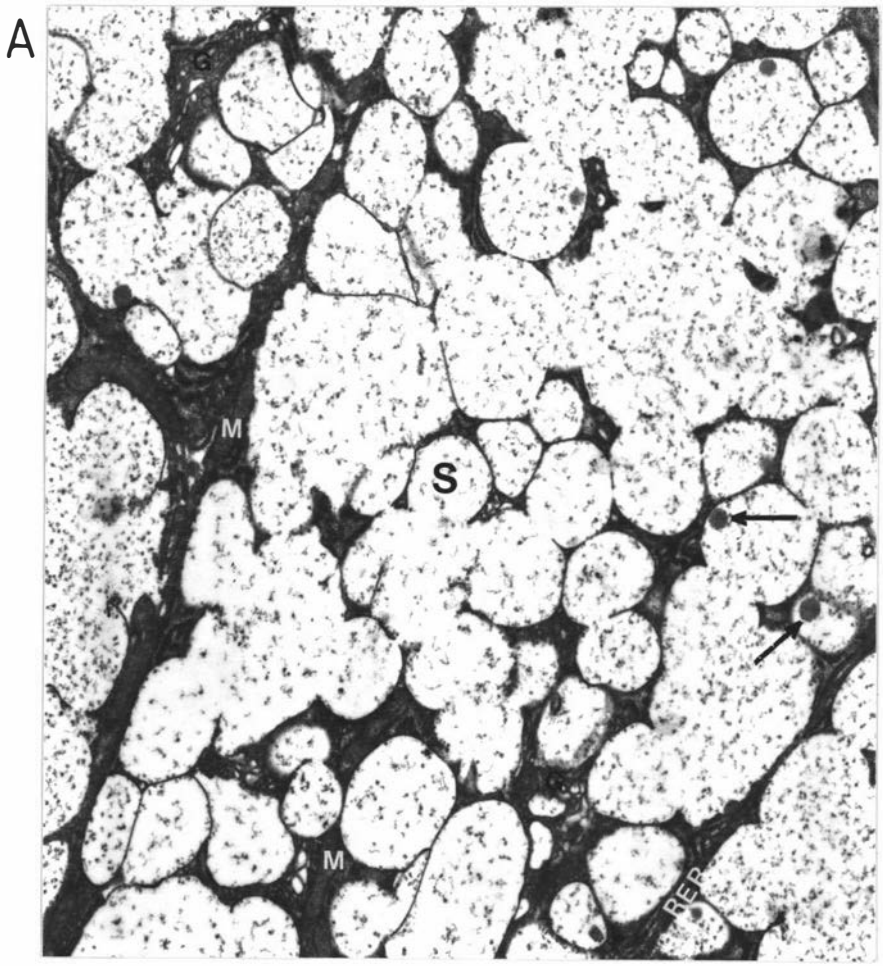


Figure 3.8: The ultrastructure of the acinar cells of the resting mandibular gland.

A. An ultrathin section of acinar cells. *Note* the arrangement of secretory granules (S), small dense bodies (arrow), mitochondria (M), rough endoplasmic reticulum (RER), and Golgi complexes (G). x12,250.

B. A freeze-etched replica of acinar cells (after deep etching). Arrows indicate the small circular areas which correspond to the small dense bodies seen in ultrathin section (see A). x8,100.


A
M
S
M
REP

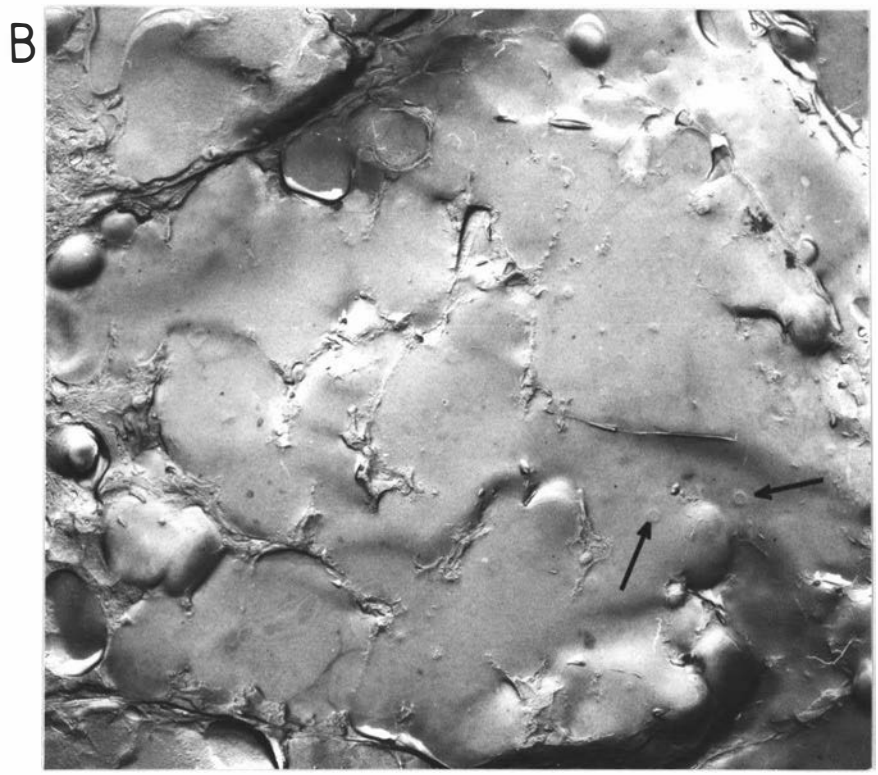
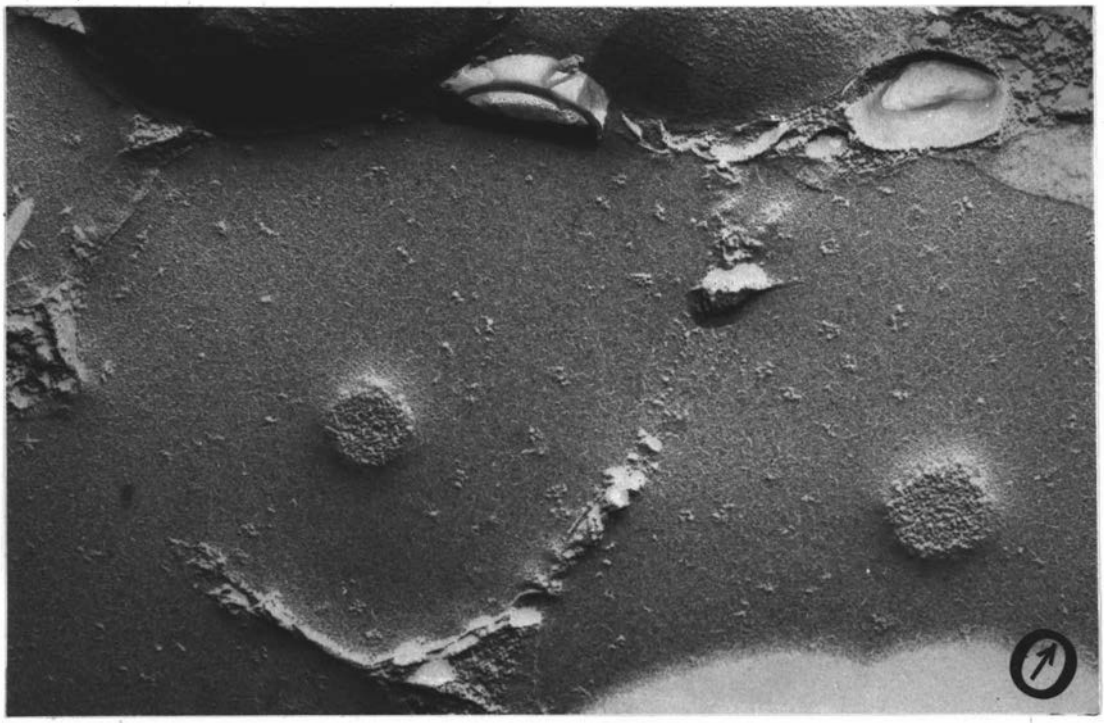

B

Figure 3.8:

C-D The complementary replicas of an acinar cell after deep-etching showing the small circular areas of fine particles within the secretory granules. (Higher magnification of B). x60,000.

C



D

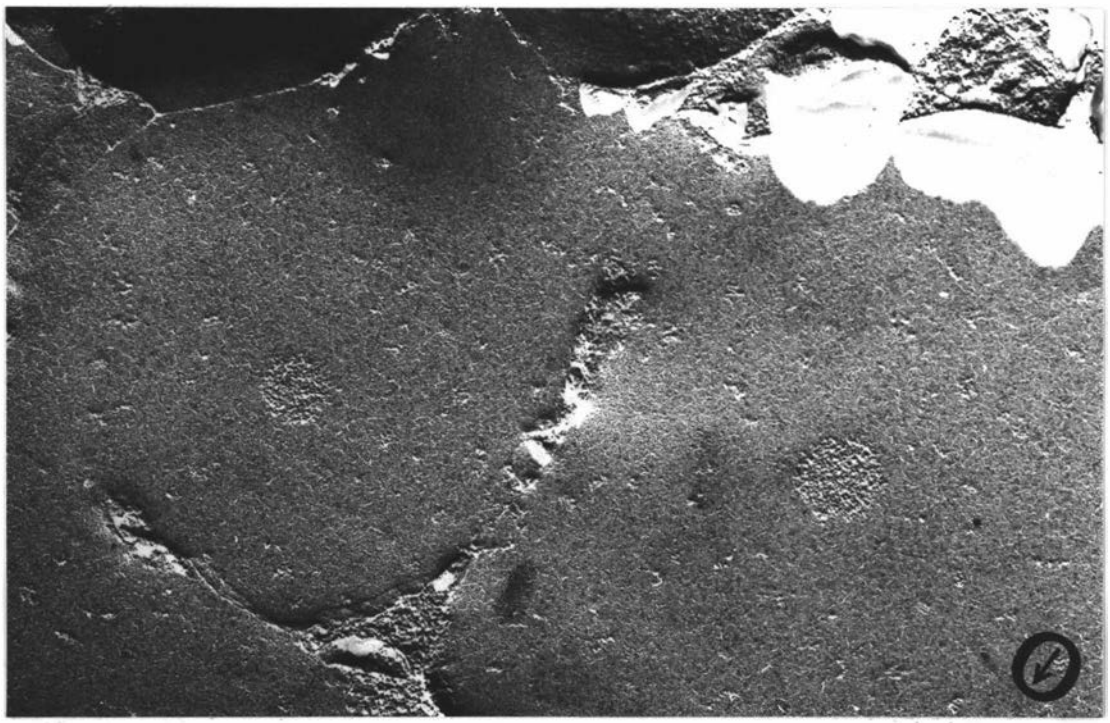


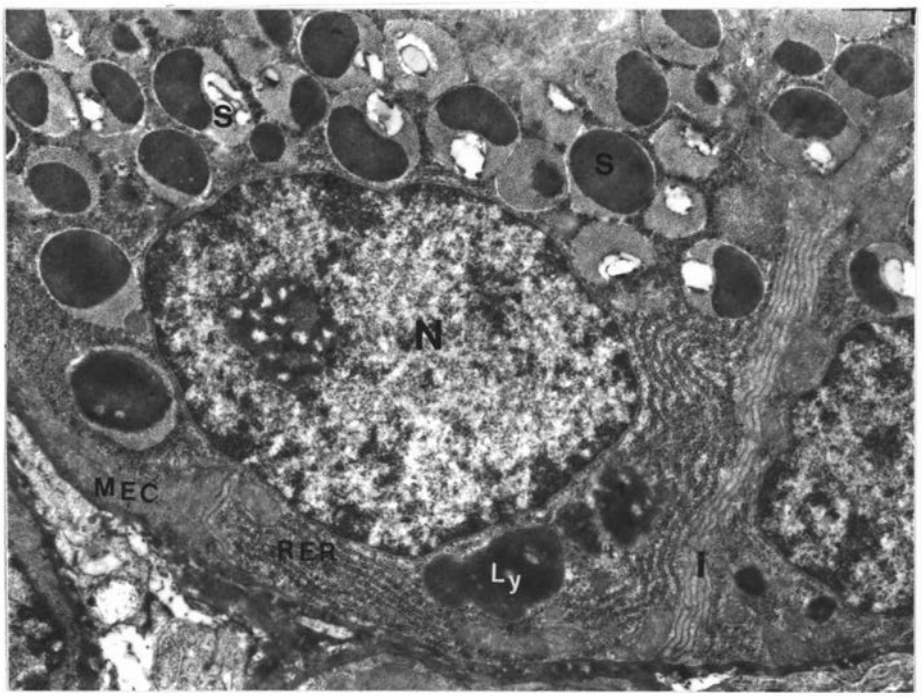
Figure 3.9: The ultrastructure of demilune cells.

A. *Note* the presence of secretory granules (S), RER, lysosome-like structures (Ly), intercellular membrane folds (I), nucleus (N) and a myoepithelial cell process (MEC).

B. The numerous microvilli within an intercellular canaliculus (IC) are shown, along with mitochondria (M) in close association with the luminar membrane. A mucous acinar cell adjoins on the right. x17,500.

(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative: OsO_4 postfixation; *en bloc* staining).

A



B

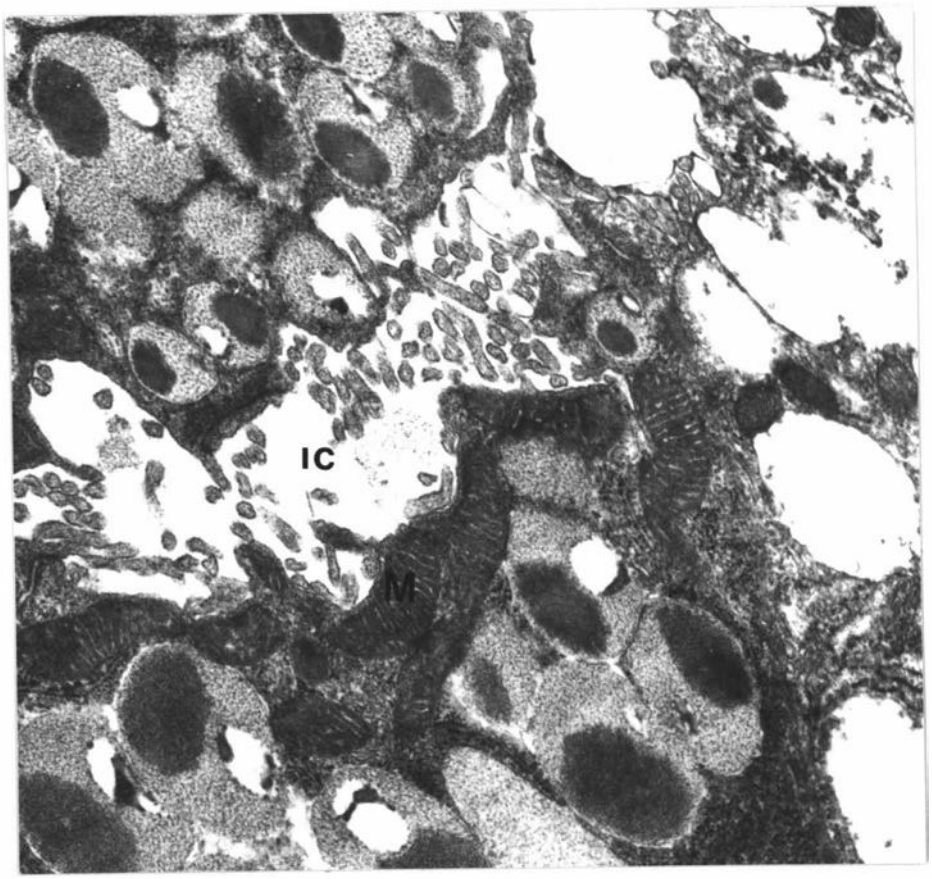


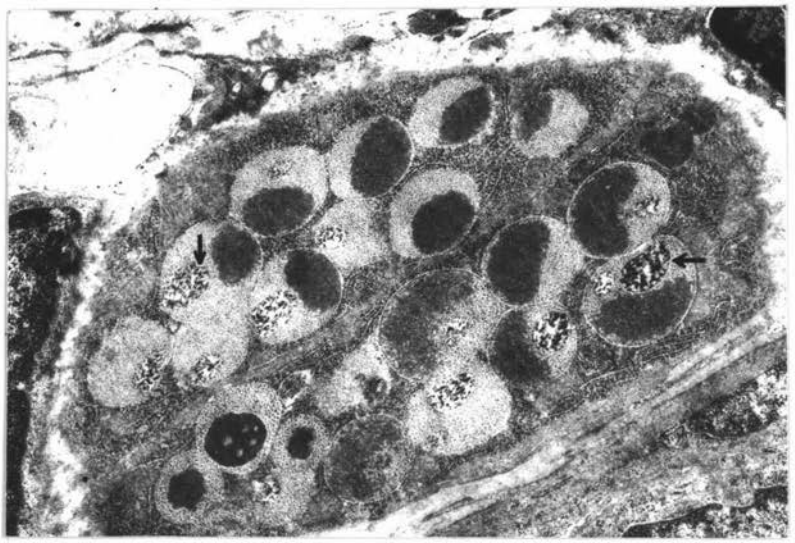
Figure 3.10: The ultrastructure of secretory granules of demilunes.

A&B Show the typical tripartite characteristics of secretory granules.
Note the small heterogeneous substructure (or inclusion) with a membrane-like boundary (arrows).
A. x11,800. B. x14,875.

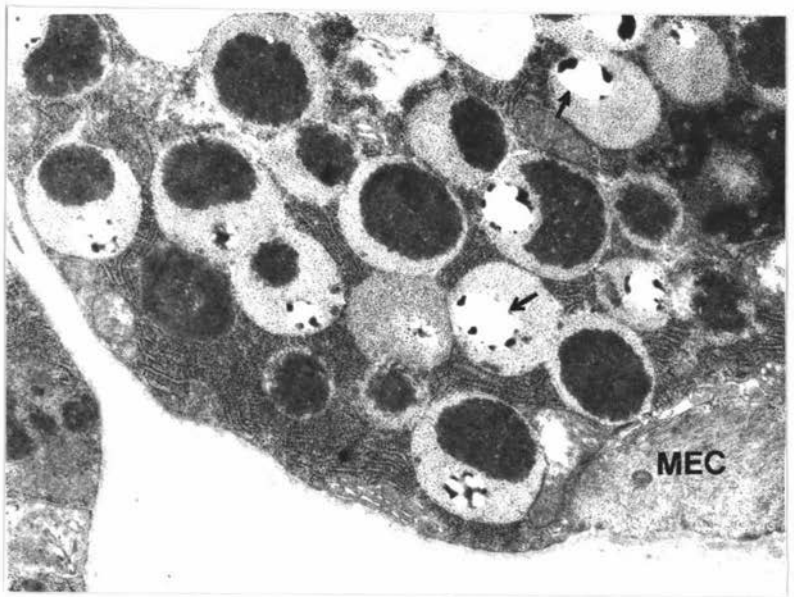
C. Lysosomes (Ly) in the basal region of the cells adjacent to the basolateral canaliculus (BLC). x13,500.

(All Araldite Sections.
A. Immersion fixation: 6.25% glutaraldehyde; OsO_4 postfixation.
B&C. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A



B



C

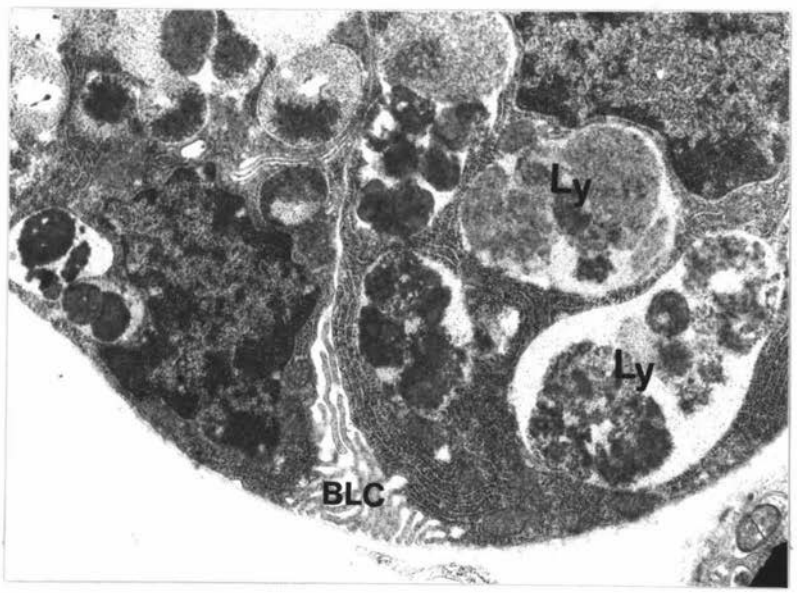


Figure 3.11: The ultrastructure of the junction between mucous and intercalated duct cells. The processes of myoepithelial cells (arrows) are present within the basement membranes of both structures.

A. *Note* the nucleus of a myoepithelial cell (large arrow) within the basement membrane of an intercalated duct cell and the adjacent mucous cell. x8,100.

B. The contents of an acinar granule have apparently been discharged into the lumen of the duct. x4,700.

C. Higher magnification of part of B. x17,500.

(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

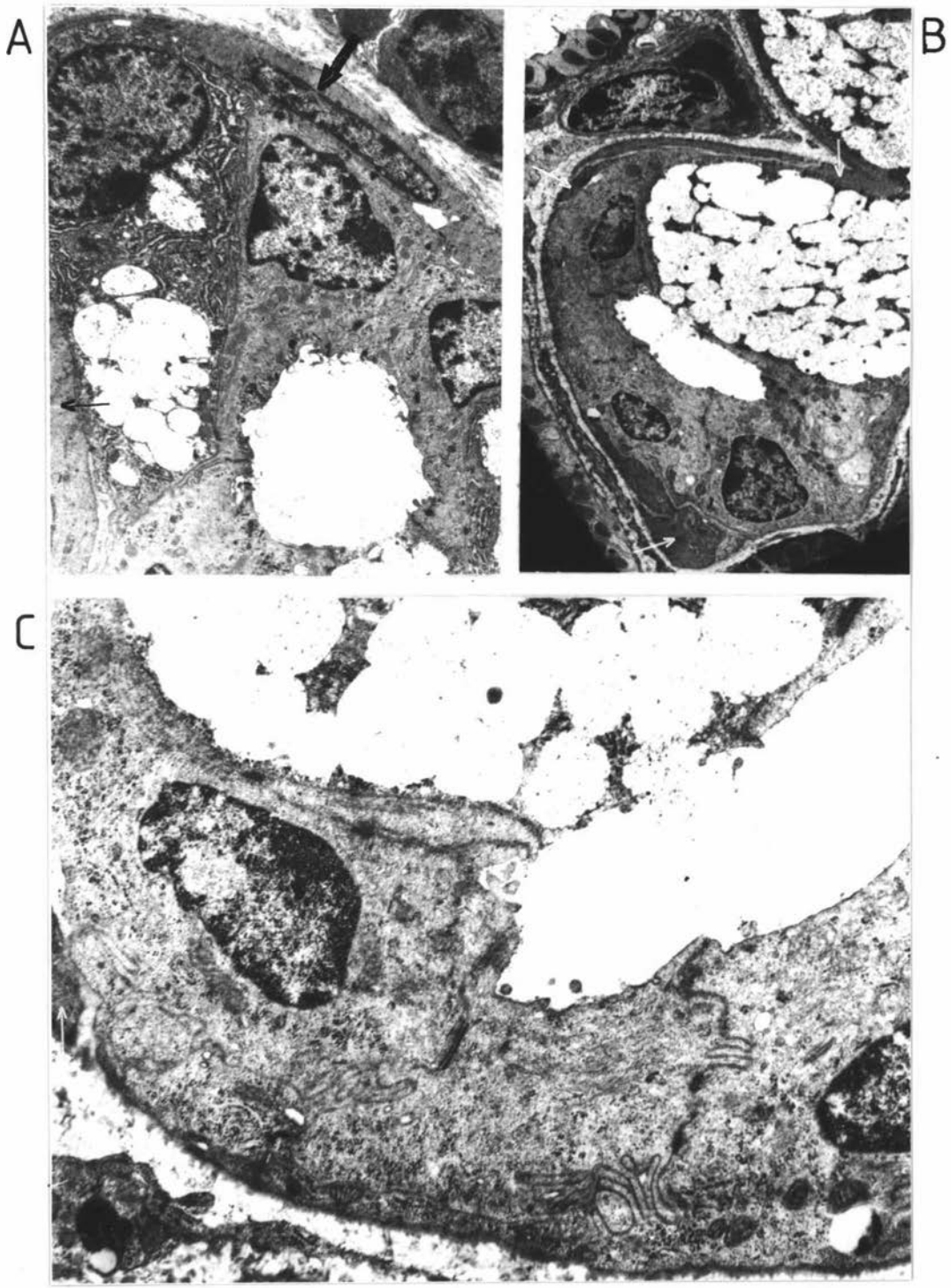
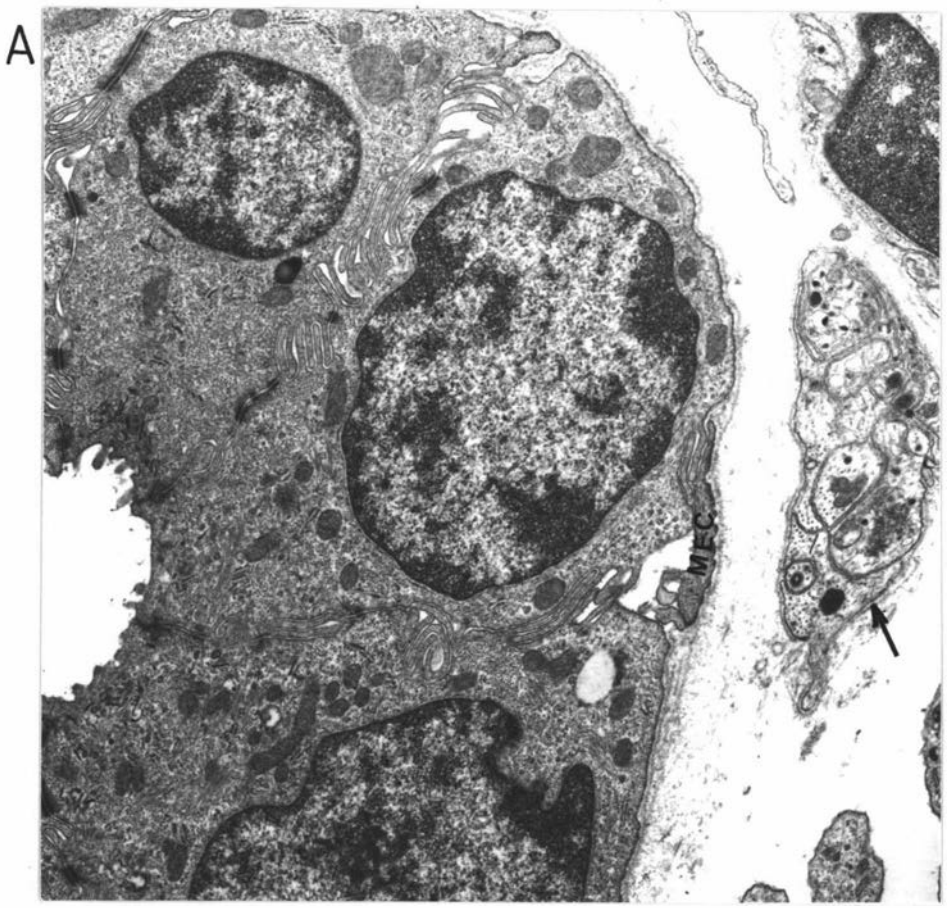

A
B
C

Figure 3.12: The ultrastructure of intercalated duct cells. x13,500.

A. A portion of intercalated duct, showing axons of an interstitial unmyelinated nerve (arrow) adjacent to the base of an intercalated duct cell and the myoepithelial process.

B. A basal cell (b) of an intercalated duct and other intercalated duct cells. *Note* the abundant cytofilaments (arrow) in the apical cytoplasm.

(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).


A
MEC

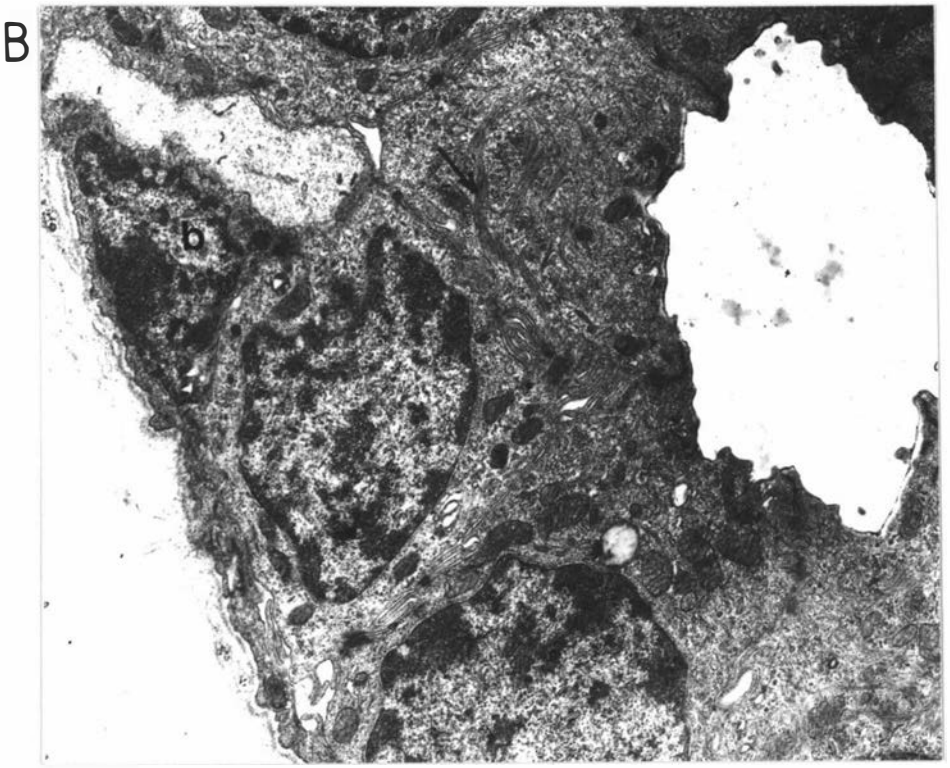

B
b

Figure 3.13: The ultrastructure of type I and type IV striated duct cells. The basal membrane foldings with association mitochondria in a type I cell, and the lack of basal striation in a type IV cell are shown. *Note* also the presence of small dense bodies (arrow) in the apical region of the type I cell. x13,500.

(Araldite section. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining.
In Figures 3.13 to 3.15 and 3.18, Roman numerals indicate the corresponding cell types).

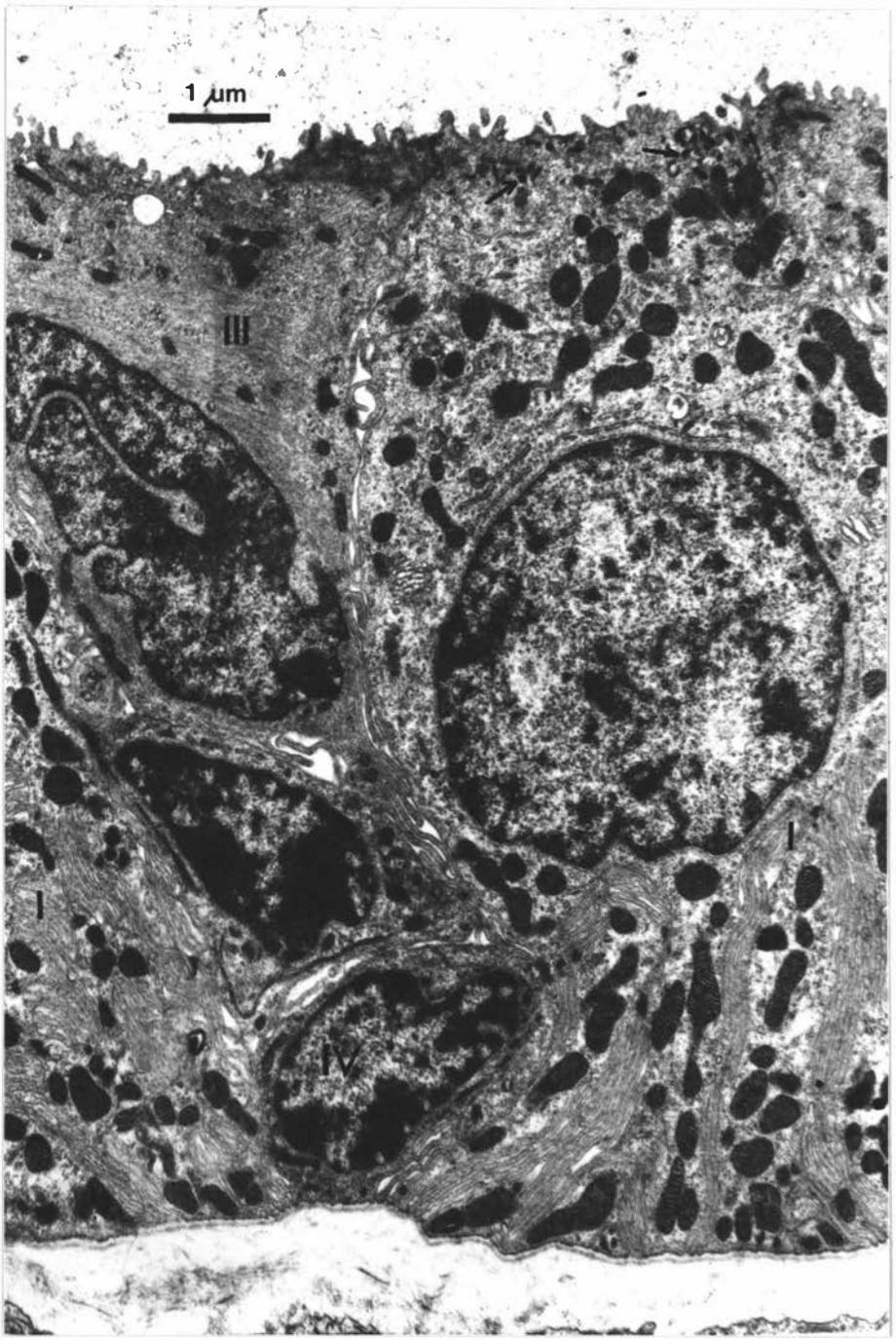

1 µm
III
I
I
IV

Figure 3.14: The ultrastructure of type I and type II striated duct cells.
(Araldite section. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

Note the apical blebbing (arrowed) in the type I cell, and the presence of many mitochondria, distended RER and empty vesicles in both type I and type II cells. x10,500.

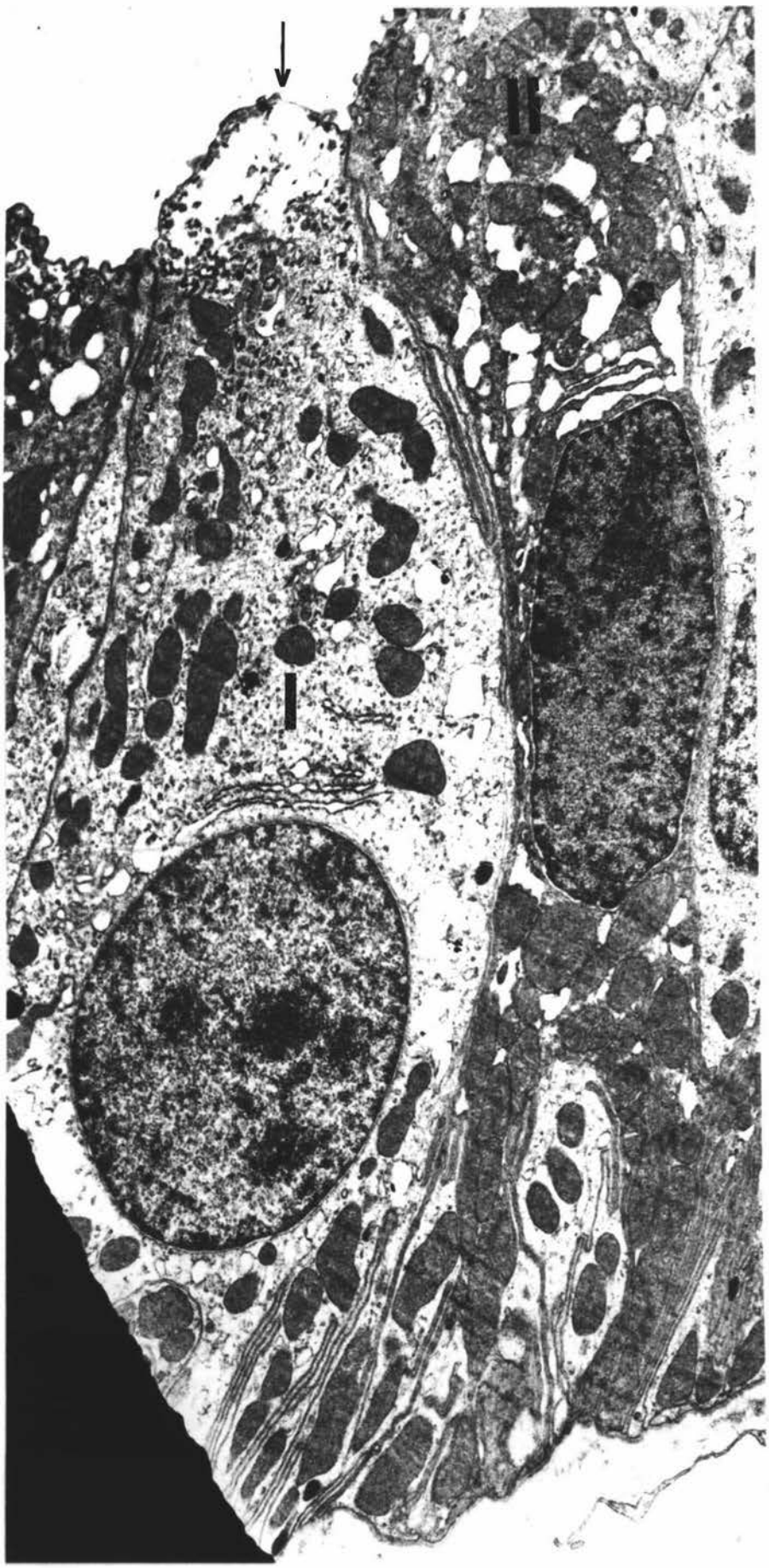

I
II

Figure 3.15: The ultrastructure of type III striated duct cells.

A. A type III cell between a type I and a type II cell.
Note the narrow basal cytoplasmic process of type III cell. x6,400.

B. An unusual apical cell with very clear cytoplasm and a striking paucity of cytoplasmic organelles. x10,500.

(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; post-fixation: OsO_4; *en bloc* staining).

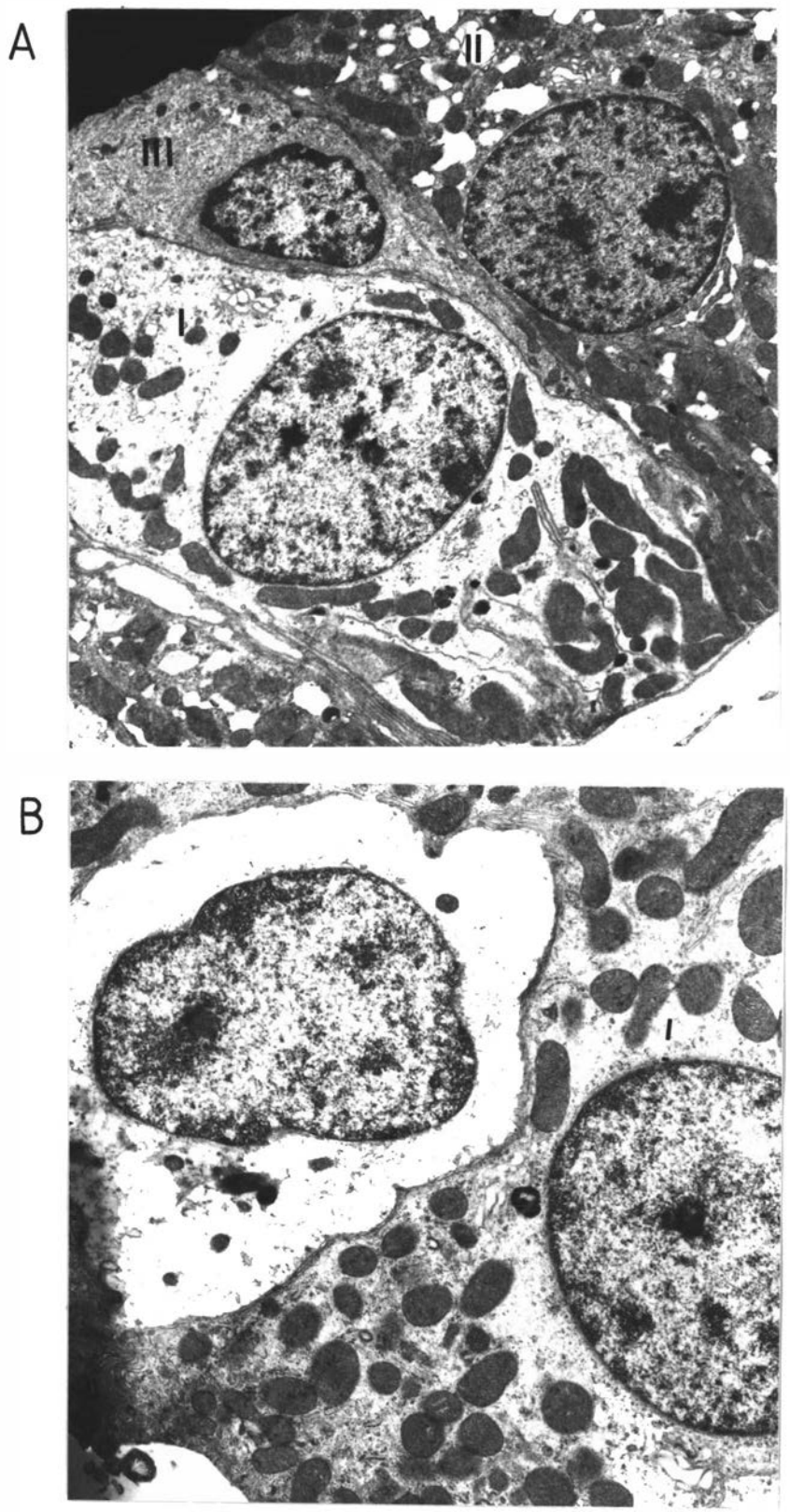

A
II
III
I
B
I

Figure 3.15:

C. Higher magnification of a portion of a type III cell. This shows the presence of abundant cytofilaments (arrowed) which appear to account for the electron density of the cytoplasm in type III cells. A type I cell (light cytoplasm) adjoins. x34,000.

C

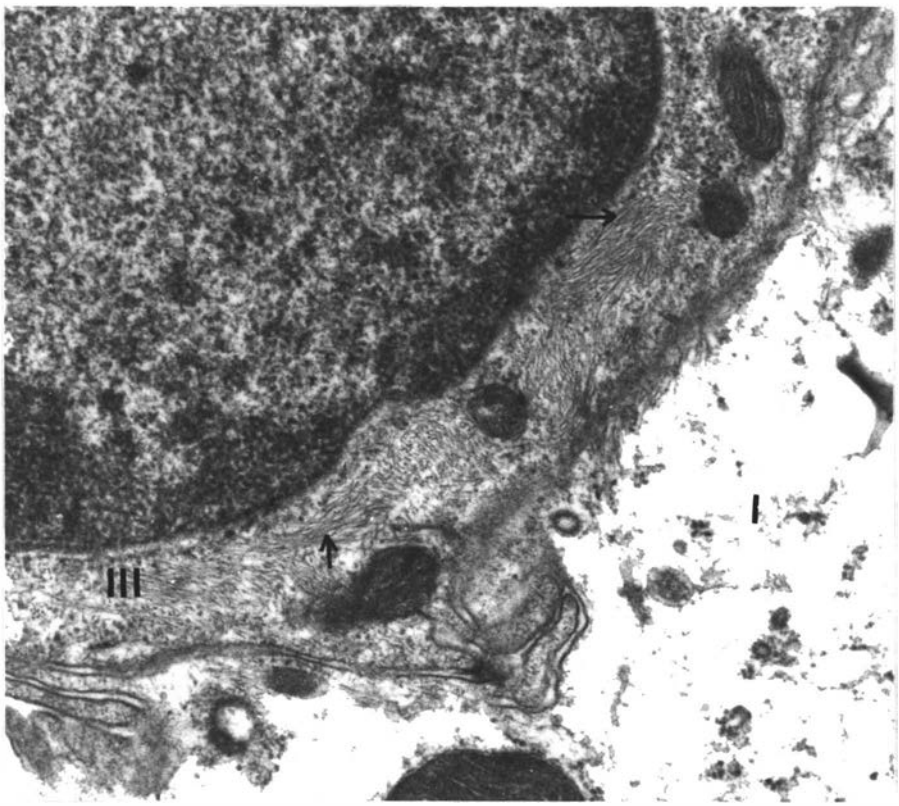


Figure 3.16: Electron micrographs showing different nerve types in the sheep mandibular gland. (Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A. A large unmyelinated nerve bundle in the interstitial space that contains axons with small agranular vesicles (SAV), small granular vesicles (SGV), and large granular vesicles (LGV). x25,000.

A

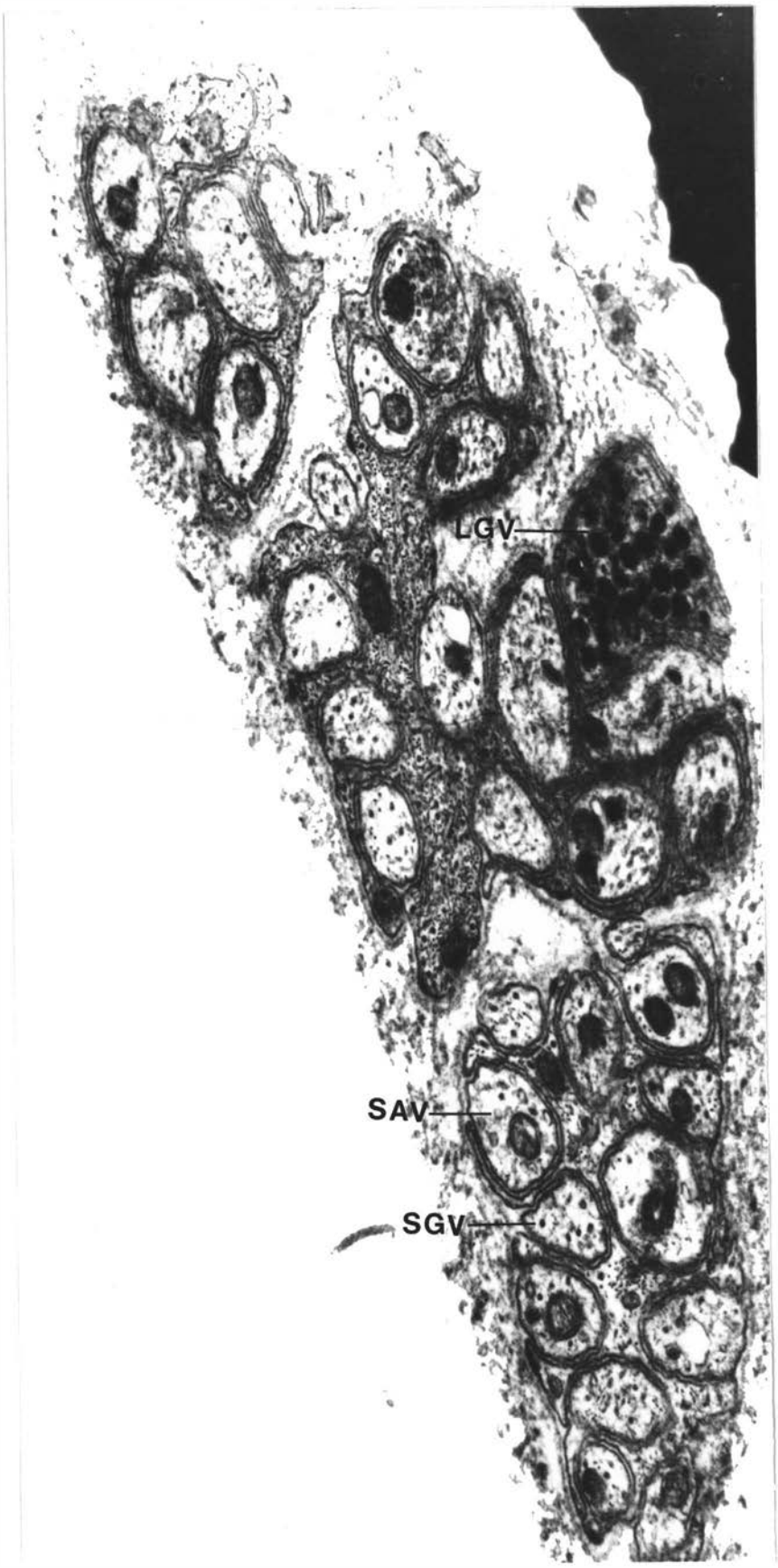


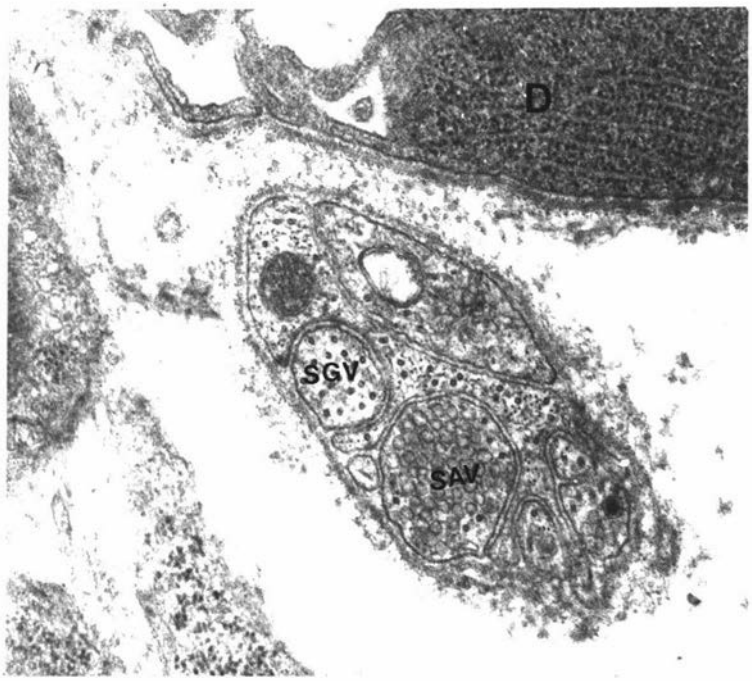
Figure 3.16:

B. A transverse section through an unmyelinated nerve with axons in relatively close proximity to the base of demilunes (D). x34,000.

C. Another unmyelinated nerve in close relation to a capillary (C), myoepithelial cell process (MEC) and demilune cell. x17,500.

Note the presence in a typical unmyelinated nerve, of axons containing SGV and SAV within the same Schwann sheath.

B



C

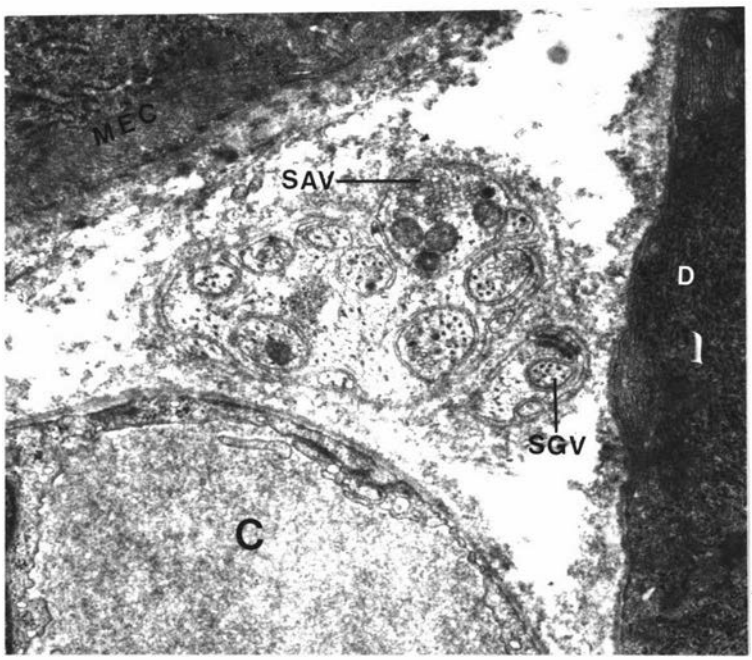


Figure 3.17: A hypolemmal axon located between the intercellular plasma membranes of a mucous cell and an intercalated duct cell.

Araldite sections. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).
A. x8,100; B. x34,000.

(Abbreviations: d - desmosome,
M - mitochondria).

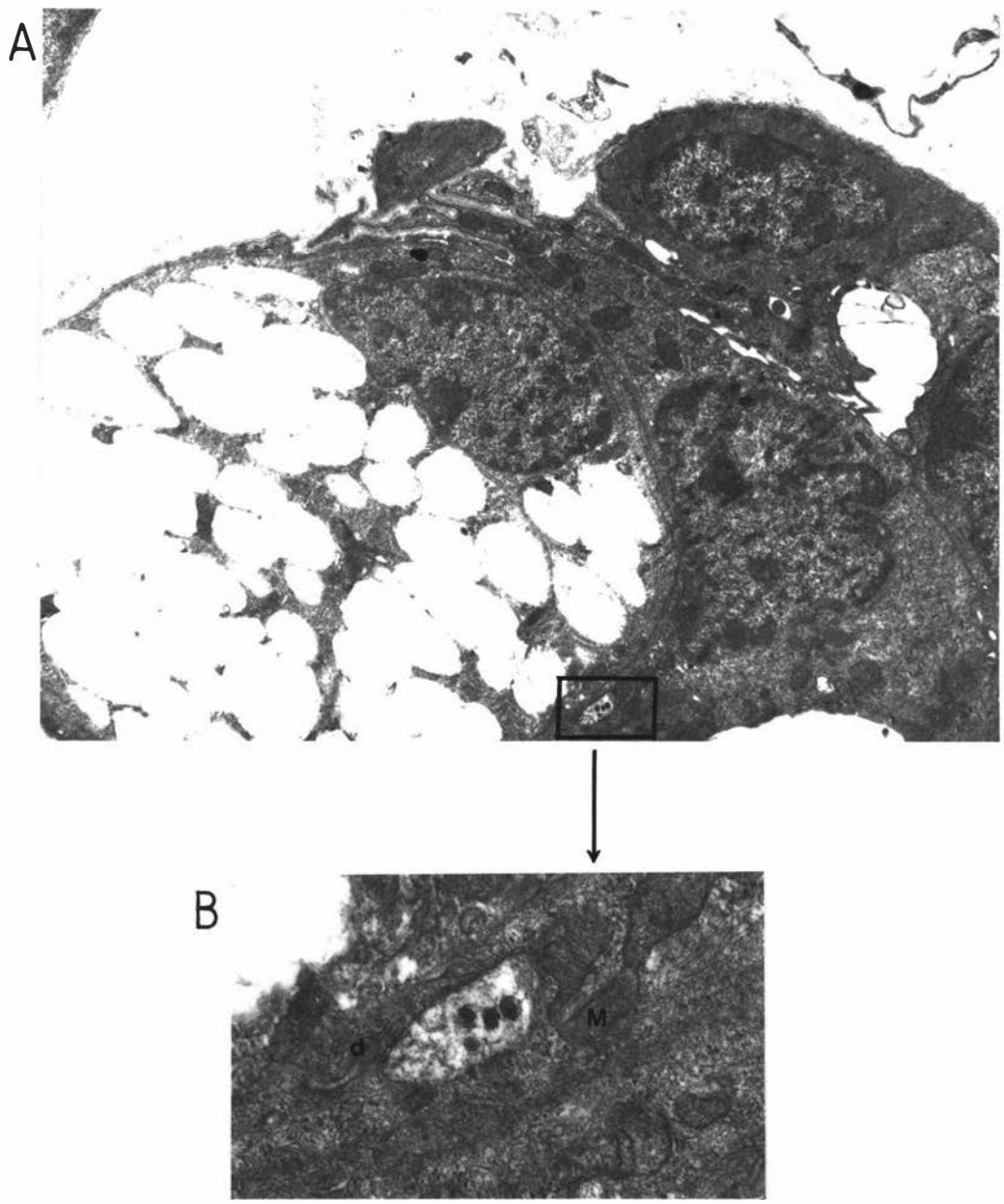

A
B
M
d

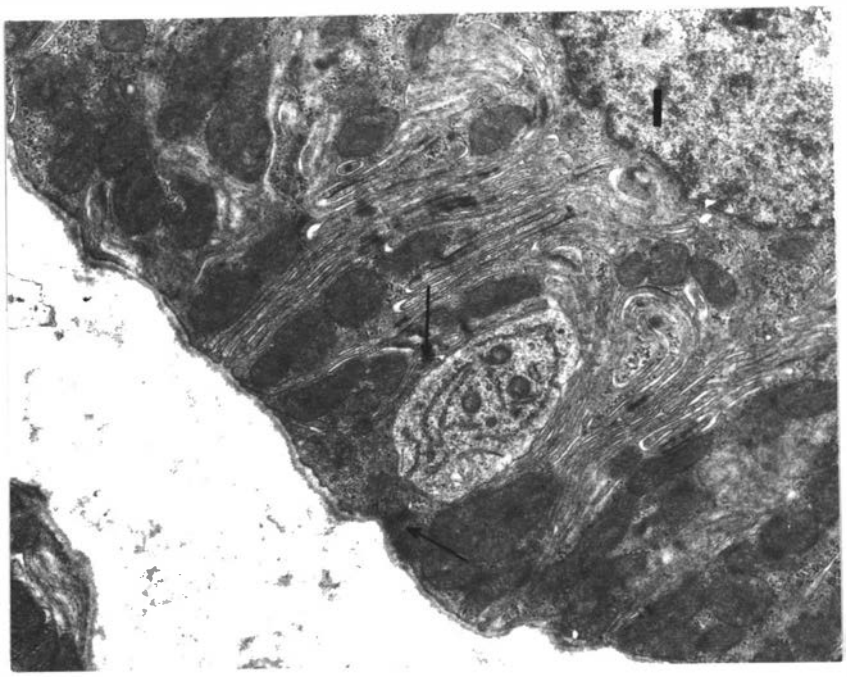
Figure 3.18: Hypolemmal innervation of striated duct cells.

A. A hypolemmal axon located basally between the intercellular plasma membranes of two type I cells.
Note the presence of desmosomes (arrow) nearby. x13,500.

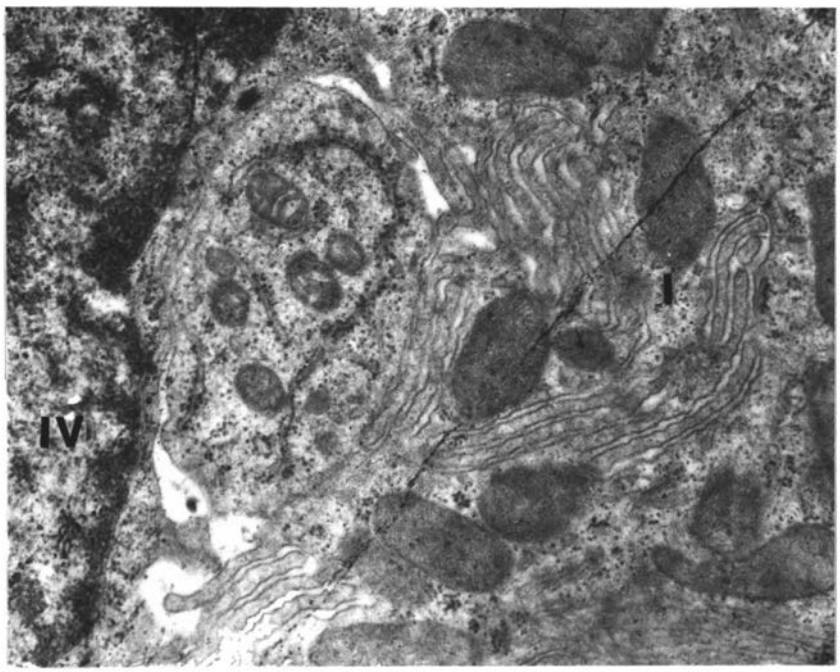
B. A hypolemmal axon between the intercellular plasma membranes of cell type I and IV. x25,000.

(Araldite sections. Perfusion fixation half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A



B



B. DISCUSSION

This discussion of the morphology of the mandibular gland considers classification, the secretory endpieces, duct system, myoepithelial cells and innervation to provide a basis with which changes described in subsequent chapters can be compared.

1. Classification of the Ovine Mandibular Gland

Although no system of classifying salivary glands has yet achieved universal acceptance there is a general consensus that four different types of secretory cells occur, viz, serous, mucous, seromucous and special serous (see Garrett, 1976; Young and van Lennep, 1978; Pinkstaff, 1980). Two methods of classification can be considered here.

First, based on the criteria mentioned in Garrett's (1976) review for the histochemical demonstration of carbohydrate, the tubulo-acinar cells in the sheep mandibular gland can be classed as mucous and those of demilunes as seromucous.

The mucous acini appear rich in acidic-nonsulphated (carboxylated) glycoprotein (judged from their intense positive staining with Alcian Blue and Alcian Yellow at pH 2.5, but not with AB at pH 1.0 - Figure 3.3; Lev and Spicer, 1964; Ravetto, 1964), and probably are sialylated-glycoprotein, as was substantiated histochemically in the glands of lambs and ewes (Quintarelli, 1963; Leppi and Spicer, 1967). The evidence from PAS reactions suggests that these cells also contain both glycogen and neutral glycoproteins. Biochemical assays reveal the presence of hexose and deoxyhexose as well, but their levels are very low (Tettamanti and Pigman, 1968) and thus their contribution to PAS reactions is probably small.

In the present study, as in that of Leppi and Spicer (1967), digestion with malt diastase greatly diminished the PAS reactivity of mucous acini (Figure 3.2) and also markedly

impaired affinity for AB at pH 2.5. This decrease in PAS staining may be due to a loss of substances other than glycogen during diastase digestion, possibly sialic acid groups which are known to possess a positive-PAS reaction (Leblond, Giegg and Eidenger, 1957). However, Leppi and Spicer (1967) argued against the decreased PAS reactivity arising through removal of sialic acid groups, and offered, in support of their argument, the observation that neuraminidase treatment did not appreciably alter the PAS reactivity. They suggested that it might instead reflect "the action of an enzyme hydrolyzing the carbon 1 glycosidic linkage of an amino sugar which is bound to carbon 6 to a terminal sialic acid residue". Nevertheless, in Leppi and Spicer's experiments the diastase susceptibility of mucous acini was observed only in the ovine gland, and not in those of the pig and cow (Leppi and Spicer, 1967).

The seromucous demilunes, in contrast, contained only small amounts of acidic-nonsulphated glycoproteins but relatively more neutral glycoproteins (as distinguished by the presence of moderate to strong PAS-staining after diastase digestion).

Secondly, these two cell populations also fall into the same categories if the definition of cell types suggested by Young and van Lennep (1978) is followed. This scheme is based on the histological appearance of cells under both light and electron microscopy, without regard for their histochemical properties and specifies that:

(i) The 'mucous' glandular cells contain large, closely packed granules which are often ill-defined, and poorly stained with H&E in paraffin sections; while with the electron microscope the granules are often fused and contain a homogeneous, relatively electron lucent matrix.

(ii) The 'serous' glandular cells contain small, discrete, refractile granules, usually eosinophilic under light microscopy and homogeneous, and usually electron dense under electron microscopy.

(iii) The 'seromucous' glandular cells are those exhibiting an intermediate appearance between the serous and mucous. Granules may be more discrete and contain either homogeneous or heterogeneous content.

2. Observations on the Secretory Endpieces

Similar conclusions concerning classification into mucous acini and seromucous demilunes have been reached for secretory endpieces in the mandibular gland of other ruminants, e.g. bovine (Shackleford and Wilborn, 1970*a*; Bloom and Carlsöö, 1974).

a. Mucous Acinar Cells

The fine structure of acinar cells in the resting mandibular glands of sheep observed in this study closely resembles the descriptions provided for the 'mucous' cells in the same gland in cattle (Shackleford and Wilborn, 1970*a*; Bloom and Carlsöö, 1974) and in other non-ruminants (Cowley and Shackleford, 1970*b*; Testa-Riva, 1977) in that they are characterized by abundant electron lucent granules with fused membranes, a relatively small amount of RER, Golgi complexes, and basally placed nuclei.

The apparent scarcity of RER and Golgi complexes in mucous acinar cells of resting gland might give the impression of these cells having only a minor role in protein synthesis but this view is not supported by their abundant content of mucus glycoprotein which is packaged in secretory granules (Figures 3.3 and 3.8A; Gallagher *et al.*, 1969).

Although the ultrastructure of mucous cells is similar in many ruminant and non-ruminant species, the cells appear to elaborate a diversity of mucosubstances (Leppi and Spicer, 1967; Shackleford and Wilborn, 1968). For example, bovine mandibular glands produce mainly sialomucins which consist of a mixture of neuraminic acids (see Herp *et al.*, 1979) and also sulphomucins (Quintarelli *et al.*,

1961), while in sheep, it is mainly sialomucins containing only N-acetyl neuraminic acid that are produced (Herp *et al.*, 1979).

The secretory granules of mucous acini in sheep are of a single type consisting of two components: a major component which exhibits features similar to glycoprotein as described by Gallagher *et al.* (1969), and a small dense body. This view is supported by the results both the freeze etching studies and conventional electron microscopy (Figure 3.8A-D). The extrusion of secretory content from mucous granules evidently occurs even in the resting gland (Figure 3.11C) as has also been noted by others (Kramer and Geuze, 1977).

Acinar cells, in addition to secreting glycoprotein, may also be involved in the secretion of water and electrolytes, although the contribution they make to the total saliva produced by the mandibular gland has not been assessed in the present study nor by other authors.

b. Seromucous Demilunes

The most striking difference between cells of the demilunes and those of the mucous acini was related to distribution of organelles. In demilunes relatively large amounts of parallel RER cisternae occupied the basal regions of the cells along with abundant polyribosomes and this suggests the cells have an important role in the synthesis and secretion of proteins. It is possible as suggested by Blomfield, Simson and Martinez (1980) that some fluid may also be secreted in the process of discharge of protein secretory materials by exocytosis but no evidence of this was obtained.

Materials secreted from demilunes appear to pass to the acinar lumen either *via* intercellular canaliculi (Figures 3.7; 3.9B) or directly from the apical extensions of demilunes (Figure 3.7B). These apical extensions have been reported previously in sheep (Young and van Lennep, 1978) and many other species, e.g. calf (Shackleford and

Wilborn, 1970*a*), East African wild ungulates (Kayanja, 1973), cat (Shackleford and Wilborn, 1970*b*) and squirrel monkey (Cowley and Shackleford, 1970*b*).

The presence of lysosomes, usually basally in the cytoplasm of demilunes in the resting glands (Figures 3.9A; 3.10C) may indicate that, physiologically, the excess secretory granules are degraded by lysosomal enzymes. Such a lysosomal degradation has been described in parotid acinar cells of rats after starvation (Hand, 1972) and in parotid acinar cells of fasted sheep (van Lennep *et al.*, 1977). The numbers of lysosomes present have been suggested to depend on the cell type and the need for lysosomes at the time the tissue was taken (Garrett, 1976). Young and van Lennep (1978) postulated that in the sheep parotid gland, where protein secretion is trivial by comparison with water and electrolyte secretion, the presence of numerous lysosomes reflects an in-built system for degradation of excess secretory proteins.

It was a notable feature that mitochondria were found in close association with the plasma membranes of intercellular canaliculi (Figure 3.9B) and the lateral intercellular membranes between both demilune (Figure 3.9A) and acinar cells (Figure 3.8A). This topographic relation between mitochondria and plasma membranes possibly reflects the means of providing energy for the active ion transport system of secretory endpieces as suggested by Sharawy, Schuster, Pashley and Palmer (1978). It is widely accepted that the endpieces are sites of secretion of the primary isotonic fluid which is subsequently modified as it passes along the ductal system (Schneyer, Young and Schneyer, 1972; Young and van Lennep, 1979).

The fine structure of the secretory granules of demilunes is relatively constant in sheep mandibular glands. This type of tripartite substructure (Figure 3.10) does not seem to have been reported before. The granules differ from those of bovine mandibular glands (Shackleford and Wilborn, 1970a; Bloom and Carlsöö, 1974) and although

granules of tripartite substructure have been reported in mandibular glands of a few species, they differ in important details from those described here. For example, in the Japanese horseshoe bat, the granules have an electron dense core, an electron lucent halo and an electron dense marginal zone (Mineda, 1977), whereas the mature granules in the human show a small dense spherule eccentrically with a moderate dense crescent and a pale matrix (Tandler and Erlandson, 1972).

If the demarcation into different zones within the granules in ultrathin sections is genuine, these probably represent their different chemical composition. Histochemical studies provide some support for this notion. They indicate that granules in demilunes of sheep mandibular glands contain a high content of neutral glycoproteins (which stained red with the PAS-reaction after diastase digestion), and some acidic nonsulphated glycoproteins (which are positively stained with AB at pH 2.5 but not at pH 1.0) as in Figure 3.3. The different degrees of positive-staining that were noted may arise from variations in chemical composition among individual cells.

Further support for the concept of regional differences in granule composition comes from the work of Ichikawa and Ichikawa (1977) who have studied salivary glands in the Mongolian gerbil by histochemical, light and electron microscopy and freeze-etching techniques. They demonstrated that the pale peripheral rim of granules was carbohydrate rich (mainly sialomucin) whereas the dense central core was protein rich. Similar findings have been reported in Paneth cells of mouse intestine (Spicer, Stalay, Wetzel and Wetzel, 1966) where the presence of mucosubstances has also been confirmed in the electron-pale peripheral rims of bipartite granules.

It is tempting to believe that the different electron densities of regions within demilune granules observed in the present study do represent different chemicals and thus different secretions. For example, the electron dense

region may be 'serous' and protein rich, while the electron lucent region may be 'mucous' and the moderate region may be 'mixed'.

In the present investigation, varying degrees of density and forms of granules were commonly observed within the same secretory endpiece and even within the same cell (Figure 3.10A,B). Evidence from other organs and other salivary glands suggests that these varying forms represent granules at different stages of formation or maturation. Thus Troughton and Trier (1969) found that secretory granules in the early stages of formation had a small rounded-dense core in the electron lucent matrix while the mature granules were composed of a relatively large electron dense core with a light peripheral rim. Similarly, various types of granules have been observed in the salivary glands of mice examined at different embryonic and neonatal stages (Borghese, Laj and Di Caterino, 1974).

A possibility that has to be considered, however, is that of artifact arising from processing procedures. One single granule component may receive different degrees of extraction of its substances during fixation, as suggested by Wilborn and Shackleford (1969). Additionally, different fixation procedures may influence the morphology and cytochemistry of secretory granules as reported in the rat's salivary glands (Simson, 1977; Simson, Dom, Sannes and Spicer, 1978). In the present study, however, the use of different buffers, either phosphate or cacodylate, caused no substantial difference in the fine structural appearance of granules. This is in agreement with Trump and Ericsson (1965) who reported that the composition of fixative vehicle was less critical with aldehyde fixatives, where active cross linkage with proteins occurs.

The failure of the freeze-etching studies to support the conclusion of a tripartite structure is also open to various interpretations. The granule structure in either thin sections or freeze-etched replicas (or both) may be artifact. However, if the composition of the membrane-like

boundaries around the small substructures differs from the typical unit membrane, freeze-etching techniques may not be appropriate to reveal them.

3. Observations on the Duct System

The complete physiological role of intercalated and striated ducts has yet to be established.

a. Intercalated Ducts

The intercalated duct has been variously considered to act as a passive conduit (Tamarin and Sreebny, 1965; Garrett, 1976); as a source of secretion (Tandler, 1965; Shackleford and Wilborn, 1968); and as seems to be the case in mandibular glands of rats (Chang, 1974; Chang and Barka, 1974; Cutler and Chaudhry, 1975), of pleuripotent cells when the need arises. While they may function in some of these capacities the relatively simple cytological features of the intercalated duct cells with their lack of secretory granules in sheep mandibular glands (Figures 3.11; 3.12) does not support the idea of a substantial contribution to the glandular secretion unlike those of the calf mandibular gland (Shackleford and Wilborn, 1970*a*), the human parotid (Shackleford and Wilborn, 1968) and submandibular (Tandler, 1965) glands whose intercalated duct cells contain secretory granules and may add a significant component to the saliva.

The possibility that intercalated ducts do participate in the secretion of fluid, though, cannot be excluded. As Young and van Lennep (1978, 1979) have emphasized, the ultrastructural identification of secretory apparatus is concerned exclusively with the structures for the secretion of exportable proteins and no ultrastructurally identifiable cell components have yet been associated with water and electrolyte secretion apart from the possible involvement of the secretory canaliculi. Further, micropuncture studies have not been able to reveal whether the "acinar-intercalated duct fluids" are secreted by endpiece cells alone, or by both endpieces and ducts (Young and van Lennep, 1979). There

is evidence to show that secretion can still be evoked by parasympathetic nerve or parasympathomimetic stimulation in glands after endpiece atrophy induced by chronic main duct obstruction (Schneyer and Schneyer, 1961; Emmelin, Garrett and Ohlin, 1974). While such experiments suggest the occurrence of ductal secretion they do not reveal the role of the intercalated ducts in this.

The striking morphological feature of large amounts of cytofilaments in intercalated duct cells (Figure 3.12B) has been reported in many species, e.g. North American opossum (Wilborn and Shackleford, 1969), cow (Bloom and Carlsöö, 1974), calf (Shackleford and Wilborn, 1970*a*) and rat (Kurtz, 1964), but it is not a universal feature of these cells, e.g. cat (Shackleford and Wilborn, 1970*b*) or squirrel monkey (Cowley and Shackleford, 1970*b*) mandibular glands. These cytofilaments perhaps confer rigidity and anchor opposed cell surfaces (Wilborn and Shackleford, 1969).

b. Striated Ducts

In the present investigation, striated ducts were found to contain four cell types distinguished by differences in their electron density and structure (Figures 3.13-3.15). Cellular features that are similar, but not identical, are shared by striated duct cells in mandibular glands of many species and have been recently reviewed by Pinkstaff (1980). Among these species are the bovine (Shackleford and Wilborn, 1970*a*; Bloom and Carlsöö, 1974), the squirrel monkey (Cowley and Shackleford, 1970*b*) and North American opossum (Wilborn and Shackleford, 1969). The principal cell type in sheep (type I) and the other species mentioned above showed similar characteristics - tall columnar cells with well developed basal striations and a cytoplasm of low electron density. The type II cells, except for the presence of small dense bodies near the apical surface, shared more features in common with the dark vesiculated cells of excretory ducts in the rat (Tamarin and Sreebny, 1965)

than with the dark cells of striated ducts in the species mentioned above. Cells resembling type III cells are described by Blood, Kennerson and van Lennep (1977) in striated duct cells of the Australian brush-tailed possum's mandibular glands although they differ in the density of their cytoplasm. Type IV is similar to the basal cell described in the calf (Shackleford and Wilborn, 1970*a*), the squirrel monkey (Cowley and Shackleford, 1970*b*), the rat (Tamarin and Sreebny, 1965), and the 'type-I - basal cell' of the North American opossum (Wilborn and Shackleford, 1969).

Numerous attempts to account for the different cell types in the striated duct have been made and this no doubt reflects the inadequate understanding of the physiology of these cells. Wilborn and Shackleford (1969) suggested that in the North American opossum the dark cell, type I-basal cell and type II-basal cell may represent either different stages in the cell cycle of duct epithelium or, alternatively, genuinely different cell types. Bloom and Carlsöö (1974) also suggest that the 'light cell' that they observed in calf striated ducts may represent late or pre-degenerative stages. (In this investigation the unusual type III cell [light apical cell] shown in Figure 3.15B was structurally similar to the 'light cell' mentioned by Bloom and Carlsöö [1974]). Tamarin and Sreebny (1965) proposed that the 3 cell types in the granular ducts of rat mandibular glands represent different secretory stages of the same cell type. On the other hand, others have ascribed differences, particularly in dark cells, to preparation artifact (Pinkstaff, 1980), or different states of hydration of the cytoplasm (Young and van Lennep, 1979). These latter authors comment that different functional states of the cell or different permeability properties of the plasma membranes at the time of fixation may contribute to the observed differences.

The characteristic infoldings of plasma membranes and the associated mitochondria in the basal regions of striated

ducts are conventionally taken to indicate an important role in fluid and electrolyte transport. Similar features characterize many epithelial cells specialized for active transport of ions and thus water, e.g. kidney tubules, choroid plexus, ciliary body, as well as the striated duct of many mammalian salivary glands (see Berridge and Oschman, 1972; Young and van Lennep, 1978).

The presence of a well-developed striated duct system in the mandibular gland of sheep in the present study seems to be consistent with the composition of saliva secreted from this gland which contains a low sodium and a relatively high potassium content (Kay, 1960). This final hypotonic saliva may be explained by the two stage hypothesis involving "isotonic primary secretion and sodium reabsorption in the duct system with accompanying ductal secretion of potassium" (Thaysen, Thorn and Schwartz, 1954; Young, Frömter, Schögel and Hamann, 1967; Schneyer *et al.*, 1972; Young and van Lennep, 1979). However, because of difficulties of access to this site for micropuncture experiments there is no direct proof of the relative importance of ion transport in the striated duct cells (Young and van Lennep, 1979) and no observations have been reported on the mandibular glands of sheep. Moreover, no direct correlations between osmolarity of final saliva and mass of striated duct can be drawn for salivary glands of the same or different species. For example, isotonic saliva (of high sodium and low potassium content) is secreted from sheep parotid glands (McDougall, 1948) which possess abundant well-developed striated ducts (Shackleford and Klapper, 1962; van Lennep *et al.*, 1977), whereas profoundly hypotonic final saliva is produced by rat sublingual gland in which striated ducts are sparse and granular ducts are lacking (Martin and Young, 1971; Young and van Lennep, 1979).

The presence of small dense bodies in the apical cytoplasm (Figure 3.13) has been reported in striated duct cells of mandibular glands in the calf and cat (Shackleford and Wilborn, 1970*a*, 1970*b*). These apical bodies seem to

have no close relationship with the Golgi apparatus, which is present in only limited amounts in juxtanuclear regions (Figure 3.13). Such apical structures have been postulated to originate from plasma membrane at the luminal surface and to be concerned with the sequestering of ductal contents (Shackleford and Wilborn, 1970*b*). Evidence of pinocytotic activity at luminal surface of striated duct has been noted by Tamarin and Sreebny (1965) and although this was not confirmed in the present study, neither was a systematic investigation made of this point.

An occasional finding, also of striated duct cells, was blebbing of the apical membrane (Figure 3.14). This feature which has been described in striated duct cells of the mandibular and parotid glands of many species (Scott and Pease, 1959; Tamarin and Sreebny, 1965; Caramia, 1966; Shackleford and Wilborn, 1969*a*, 1970*a*; Cowley and Shackleford, 1970*b*; van Lennep *et al.*, 1977) remains to be adequately accounted for. Apical blebs have been variously interpreted as indicative of an apocrine secretion (Wilborn and Shackleford, 1969; Tandler, Denning, Mendel and Kutscher, 1970) and as fixation artifacts (Tamarin and Sreebny, 1965). Young and van Lennep (1978) favour the latter possibility and have argued that this view is supported by the absence of reports of granules in the blebs and the almost invariable electron lucence of the cytoplasm, which militates against a secretory function (Tandler and MacCallum, 1974; and also see Figure 3.14 in this study). They suggest that the blebbing may be attributed to alterations in local hydration of the duct cells.

The presence of many empty vesicles in type II and also occasionally type I striated duct cells (Figure 3.15A) is another phenomenon of unknown significance. It is difficult to be certain whether the empty vesicles are due to inadequate preservation during fixation or represent secretory granules that have lost their secretory materials as suggested in Pinkstaff's review (Pinkstaff, 1980). Tandler and MacCallum (1974) showed that the PAS-positive, electron dense granules

in striated duct cells of the European hedgehog were well preserved only with OsO_4 and appeared as empty vesicles when Karnovsky's fixative or other fixatives were employed. These workers also failed to show PAS-positive reaction in tissue fixed with Bouin's fluid. It is interesting that Ruby (1978) has recently described relatively large areas devoid of membrane boundaries which were PAS-positive in the striated duct cytoplasm of nine-banded armadillo's parotid glands. Neither granules nor vesicles were reported in these striated duct cells.

Histochemical studies of sheep mandibular glands have shown that the striated ducts contain AB-negative, and PAS-positive, diastase-resistant materials (Figures 3.2; 3.3) which may be considered to be neutral glycoproteins (Mowry, 1963; Pearse, 1968). These materials were present in the same regions as the empty areas described in Bouin's fluid fixed sections. Their relationship to the empty vesicles observed at electron microscopic levels is uncertain. It is conceivable that these empty areas represent structures normally containing neutral glycoproteins which have been lost during fixation with highly acidic fixative such as Bouin's fluid or formal saline. Neutral fixatives such as Karnovsky's apparently preserve their content. However, the actual chemical composition of these PAS-positive materials in the striated duct cells of sheep mandibular gland remains to be determined, although evidence is available to suggest that certain glycoproteins such as immunoglobulins and kallikreins are found in striated duct cells of other species (Comoglio and Gulioelmone, 1973; Garrett and Kidd, 1975; Hojima, Maranda, Moriwaki and Schachter, 1977; Schachter, Maranda and Moriwaki, 1978).

4. Observations on Myoepithelial Cells

There is now little debate about the suitability of using histochemical techniques demonstrating alkaline phosphatase activity for the identification of myoepithelial cells in many salivary glands. Alkaline phosphatase

activity has been demonstrated in the parotid glands of sheep (Silver, 1954; van Lennep *et al.*, 1977) and rats (Garrett and Parsons, 1973), in the mandibular glands of the rat (Leeson, 1956), and cat (Garrett and Harrison, 1970), but is apparently unsuitable for use in the parotid glands of cattle (Silver, 1954) or mandibular glands of the dog and man (Garrett and Harrison, 1970). Electron microscopic cytochemistry has shown that alkaline phosphatase reaction products are associated with the plasma membranes of myoepithelial cells (Bogart, 1968; Garrett and Harrison, 1970), as well as capillaries (Bogart, 1968) and basal duct cells (Garrett and Harrison, 1970). However, Silver (1954) noted that at light microscopic levels the alkaline phosphatase activity in capillary endometrium was slight and did not interfere with the demonstration of myoepithelial cells.

The relationship of myoepithelial cells, whether demonstrated histochemically (Figure 3.4) or electron microscopically, (Figures 3.9; 3.10; 3.11 and 3.12) to secretory endpieces, intercalated ducts and excretory ducts is consistent with the idea that they play a role in support during salivary secretion. This view is reinforced by observations on the fine structure of myoepithelial cells - desmosomes provide suitable attachment between myoepithelial cells and underlying cells (Tandler, 1965; Emmelin, Garrett and Gjörstrup, 1977*a*). On the other hand, the contractile nature of myoepithelial cells has received support from their ultrastructural organization, which shares common features with that of smooth muscle (Tamarin, 1966; Tandler and Hoppel, 1970; Leeson and Leeson, 1971).

Physiological evidence for the contractile nature of myoepithelial cells was provided by Coats *et al.* (1956) in their study of secretion in the parotid glands of sheep. They noted a rise of salivary flow followed by a compensatory pause which was consistent with the mechanical expression of saliva by a contractile mechanism. Recently, additional physiological evidence concerning the pattern of salivary

flow and intraluminal pressure changes has confirmed the effects of myoepithelial cell contraction in other species (Emmelin, Garrett and Ohlin, 1968, 1969*a*; Emmelin and Gjörstrup, 1974, 1976*a*; Garrett and Emmelin, 1979) but there is as yet no direct physiological evidence of myoepithelial cell activity in sheep mandibular glands.

5. Innervation

On both histochemical and electron microscopic grounds, the mandibular and parotid glands of sheep receive an innervation from both divisions of the autonomic nervous system. Both AChE-positive nerves (Figure 3.6) and biogenic-amine fluorescent fibres (Figure 3.5) are present around the secretory endpieces. The distribution patterns were similar in both glands though there were differences in the density of the reactions. The mandibular gland exhibited a rich innervation of both cholinergic and adrenergic nerves around the secretory endpieces, while the parotid had extensive cholinergic but a substantially reduced adrenergic innervation. In both glands the adrenergic fibres were dense around the blood vessels and although AChE-positive nerves were also present at this site it was to a lesser extent. Similar patterns have been reported in histochemical studies of bovine mandibular glands (Alm *et al.*, 1973).

In the present investigations weak BuChE activity was present in the mandibular but not parotid glands. The enzyme activity was localized in large nerves and weakly about secretory cells when longer incubation times (4 hours) were used. The occurrence of BuChE in peripheral nerves is not uncommon (Silver, 1974) and has been reported in both serous and mucous glands of guinea pig (Gerebtzoff, 1959), in demilunes and duct cells of sheep mandibular gland (Chauncey and Quintarelli, 1961), but only in duct cells of bovine mandibular glands (Chauncey and Quintarelli, 1961). Observations on cholinergic nerves were, therefore, performed with the use of iso-OMPA, a BuChE inhibitor, which blocks the non-specific cholinesterase activity.

Electron microscopy revealed the innervation of acinar cells, demilunes, myoepithelial cells and blood vessel to be of the epilemmal type (Figure 3.16) where unmyelinated nerve fibres were found outside the basement membranes of effector cells. Axons containing small dense granular vesicles (typical of adrenergic nerve endings), or small agranular vesicles (typical of cholinergic nerve endings) were often present in individual unmyelinated nerves suggesting the presence of both adrenergic and cholinergic fibres within the same Schwann cell. This is a common feature of post-ganglionic autonomic nerve fibres (Garrett, 1975*b*).

Alm *et al.* (1973) similarly reported an epilemmal innervation in the bovine mandibular gland using permanganate fixation which gave better preservation of the small granular vesicles. Unlike the present study, they (Alm *et al.*, 1973) failed to demonstrate hypolemmal axons associated with the acinar and intercalated duct cells (Figure 3.17) or with striated duct cells (Figure 3.18). Similar observations to those for the bovine mandibular gland were also reported in the bovine parotid gland (Shackleford and Wilborn, 1969*a*). In other species, however, hypolemmal axons have been observed, for example, between 'intra-acinar' cells of rat parotid glands (Hand, 1970), adjacent to and between central acinar cells of cat mandibular glands (Garrett, 1976), within intercalated ducts and granular tubules, beneath the basement membrane and in the space between the parenchymal cell and an associated myoepithelial cell of rabbit mandibular glands (Garrett, 1977). The presence of hypolemmal axons in sheep mandibular glands may represent a genuine species difference or simply a failure to undertake an adequate search in the bovine glands.

The presence of intense fluorescence in the muscle walls of arterioles and near small blood vessels in both mandibular and parotid glands, as well as AChE-positive reactions near small blood vessels, supports the concept of a dual vascular innervation in the mandibular glands. These may be the adrenergic vasoconstrictor and cholinergic

vasodilator nerves described by Emmelin (1967). A similar innervation of blood vessels was reported histochemically in bovine mandibular glands (Alm *et al.*, 1973). A more precise localization of the nerve endings which was possible electronmicroscopically indicated them to be of the epilemmal type, similar to that shown near the capillary endothelial cells by Garrett, (1966*b*).

While it is well established that there are sympathetic vasoconstrictor nerves which normally provide a basal vasomotor tone by acting on α-adrenoreceptors (Emmelin, 1972), the concept of vasodilation during the stimulation of cholinergic nerves remains controversial. Although the morphological evidence supports the existence of cholinergic vasodilator nerves, the presence of atropine-resistant vasodilation in sheep mandibular glands induced by parasympathetic stimulation (Beilenson *et al.*, 1968) remains an enigma. Furthermore, recent comparative studies suggest this may be due to the presence of peptidergic nerves which contain VIP-like substances as reported in other salivary glands (Bloom and Edwards, 1979; Bloom *et al.*, 1979). These non-adrenergic and non-cholinergic nerves have been proposed by many workers (Bloom and Edwards, 1979, 1980; Uddman *et al.*, 1980; Lundberg *et al.*, 1980) to mediate the atropine-resistant vasodilation. In the present investigation, axons containing a population of large granular vesicles (ca. 80 nm diameter) similar to the peptidergic type described by Kidd and Garrett (1979), were occasionally found within the same Schwann sheath as those typical of cholinergic axons (Figure 3.16A).

The epilemmal innervation of myoepithelial cells described here establishes that the sheep mandibular gland follows the same general pattern which was reported in cattle (Alm *et al.*, 1973). Both adrenergic and cholinergic types of axons were in close association with the myoepithelial cells but it is not known whether myoepithelial cells in sheep mandibular glands are stimulated to contract by parasympathetic or sympathetic nerves or both. Physiolo-

gical evidence from other species has shown variations of the control of myoepithelial cells. The motor response of myoepithelial cells may be mediated either *via* (i) α-adrenoreceptors as in sheep parotid glands (Patterson and Titchen, 1975) and rat parotid glands (Thulin, 1976); (ii) cholinergic receptors, as in the cat's mandibular glands (Emmelin, *et al.*, 1974); or (iii) both α-adrenergic and cholinergic receptors, as in the mandibular glands of dogs (Emmelin, Ohlin and Thulin, 1969*b*; Emmelin and Thulin, 1973).

The innervation of duct system was of two types. The epilemmal axons were usually present beside the striated ducts and intercalated ducts (Figure 3.16) whereas hypolemmal axons were occasionally observed beneath the basement membranes and between intercellular membranes of adjacent type-I cells, type-I and type-IV cells of striated ducts (Figure 3.18), and also between mucous and intercalated duct cells (Figure 3.17). It was not possible to categorically identify the types of nerves at these sites. However, histochemical observations revealed that the AChE-positive nerves were present alongside the striated ducts (Figure 3.6B,C) and major nerve trunks were in the interstitial connective tissue spaces near interlobular ducts. Biogenic-amine fluorescent fibres, in contrast, were rarely found at striated ducts and none was observed at the excretory ducts (Figure 3.5B,C). Recently, similar striated duct-hypolemmal nerve relationships have been described by Kidd and Garrett (1979) in cat mandibular glands. By the use of 5-hydroxydopamine, these workers were able to demonstrate that most hypolemmal axons are cholinergic, and that others containing large dense-cored vesicles with a clear halo were possibly of the peptidergic type (or at least non-cholinergic and non-adrenergic).

The biological importance of neuroeffector sites has been discussed by Garrett (1972, 1974, 1975*b*, 1976). It is usually considered that when an impulse passes down an axon, neurotransmitter is released from the vesicles by exocytosis and reaches the effector cells by simple diffusion.

Therefore, the activation of adjacent effector cells will depend on the quantum of neurotransmitter released, the distance between the axon and the effector cells, and the sensitivity of individual effector cell membrane (Garrett, 1976). An axon can thus affect several cells along its course, but whether one axon affects different types of effectors or several cells of a single type is unknown. On morphological grounds, the hypolemmal innervation seems more efficient than the epilemmal and present concepts suggest that the electro-physiological and secretory responses in gland cells to single nerve impulses is likely to be related to a hypolemmal innervation whereas multiple impulses may be required for activation of cells with an epilemmal innervation (Emmelin, 1972). However, as Young and van Lennep (1978) have pointed out, either mode of innervation is likely to suffice to permit secreto-motor activity.

6. Limitations of Present Electron Microscopic Techniques for Innervation Studies

The electron microscopic techniques used in the present study have limitations as far as differentiation of types of nerve endings is concerned. With the double-fixation by perfusion fixation in an aldehyde-mixture and subsequent OsO_4 postfixation, it is possible to demonstrate the actual sites of innervation and decide whether it is 'hypolemmal' or 'epilemmal' but no certain identification is possible of nerve types using the criteria based on the presence of small (dense) granular or agranular vesicles. Tranzer and co-workers (Tranzer and Thoenen, 1967; Tranzer and Snipes, 1968) have shown that the preservation of electron dense granules in small vesicles is strongly dependent upon the fixation procedures employed. Although the electron microscopic techniques used in the present study gave better results than fixation by OsO_4 alone (Tranzer and Thoenen, 1967) and the small (dense) granular vesicles remained recognizable, it is not clear whether some small empty vesicles may

result from the poor preservation of their content during processing for electron microscopy. Therefore, a better method with improved preservation of dense granular vesicles is needed for more detailed investigations of adrenergic and cholinergic nerves.

CHAPTER IV

OBSERVATIONS IN ANAESTHETIZED SHEEP: EFFECTS OF AUTONOMIC NERVE STIMULATION

A. RESULTS

The results obtained in this section are divided into two parts. The first part is concerned with the physiological responses of the sheep mandibular gland either to direct stimulation of autonomic nerves or to pharmacological agents that mimic the action of autonomic transmitters, and the second part with the morphological changes that result.

1. Physiological Effects

a. Mandibular Flow

(i) Resting Gland

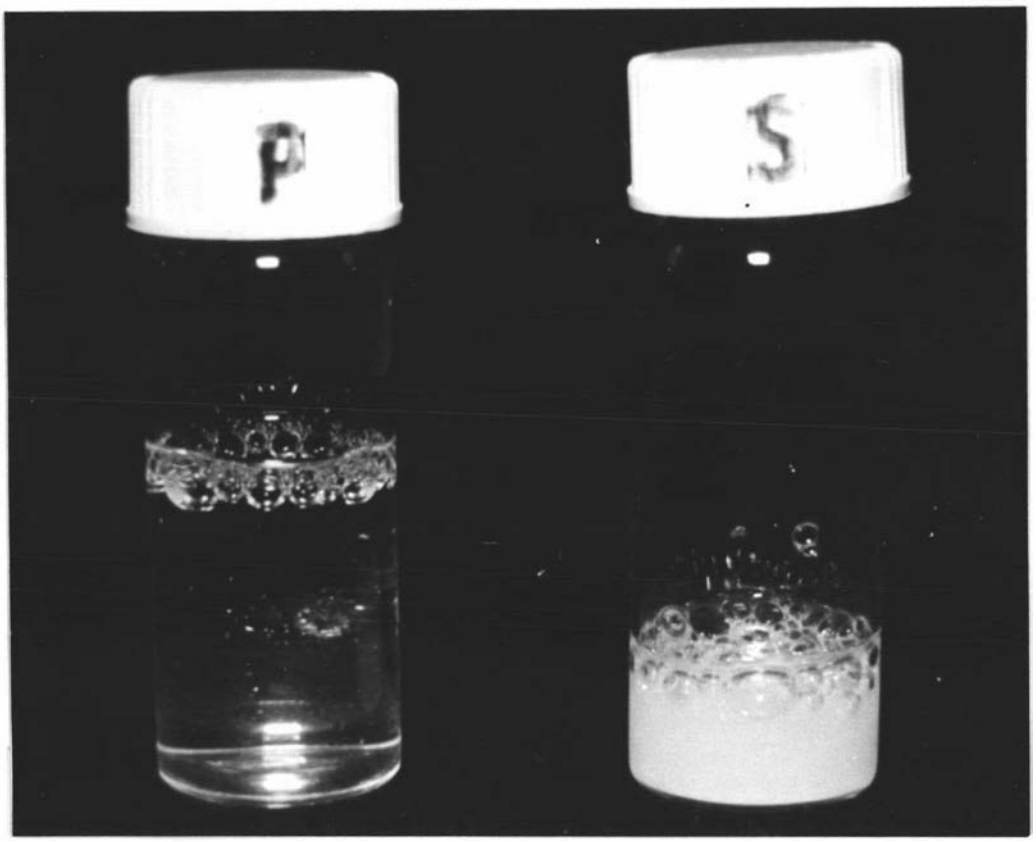
In the absence of direct stimulation of secretomotor nerves, flow from the cannulated mandibular ducts of anaesthetized sheep was either slight or absent (0-0.004 g min^{-1}).

(ii) Parasympathetic Stimulation

Stimulation of the chorda lingual nerve (at 2-8V, 5-10 Hz, 0.2 msec duration) caused the secretion of a clear mucous saliva in relatively copious quantities, i.e. 0.33-0.74 ml min^{-1} (N=10) (Figure 4.1). This secretion appeared after a latency of 10-25 sec (Figure 4.2A), and could be readily maintained over periods of 2-4 hours. Such parasympathetically evoked secretion was blocked by intravenous administration of atropine (0.1 mg kg^{-1}) (Figure 4.2).

Secretory responses similar to those accompanying the stimulation of the chorda lingual nerve were obtained following intravenous injection of carbachol (40 µg kg^{-1}) (Figure 4.2B) and pilocarpine. When pilocarpine was infused

Figure 4.1: Mandibular saliva secreted during electrical stimulation of either the chorda lingual nerve (P) or the cervical sympathetic trunk (S).


P
S

intravenously (5.8-7.4 μg kg^{-1} min^{-1}), the salivary flow persisted at a constant level (0.18-0.45 g min^{-1}, N=5) throughout the infusion period.

(iii) Sympathetic Stimulation

Electrical stimulation of the ipsilateral cervical sympathetic trunk (at 4-8V, 5-10 Hz, 0.2 msec duration) also caused mandibular secretion, though the nature of the response (Figure 4.1) and the secretion itself differed from that evoked parasympathetically. Sympathetically evoked secretion appeared after a longer latency (35-102 sec), occurred at a lower rate (0.01-0.06 g min^{-1}, N=8) and tended to decline or cease with continued stimulation (Figure 4.3B). Flow could usually be maintained, however, by the use of intermittent stimulation (e.g. 30-sec periods of stimulation alternating with 30-sec of rest; Figure 4.3C) or by using lower voltages (e.g. 2-3V; Figure 4.3A). When the α-adrenoreceptor blocking agent, phentolamine (1.0 mg kg^{-1}, iv), was given during sympathetic nerve stimulation, an acceleration of flow occurred in company with a marked fall of the femoral arterial blood pressure (Figure 4.3D). On the other hand, secretory responses to sympathetic stimulation were blocked by the β-adrenoreceptor antagonist propranolol (1.0 mg kg^{-1}, iv; Figure 4.4).

Stimulation of the chorda lingual nerve following a period of sympathetic stimulation resulted (Figure 4.5) in the appearance within 20 sec of mandibular secretion. The rate of flow was initially very slow, but increased progressively over several minutes. In the early stages of the response the drops of saliva did not fall freely from the drop tube, tending to string instead. This saliva, and that obtained during sympathetic stimulation, was opalescent, often with fine white particles visible in it, and was extremely viscous. As the secretion cleared the viscosity decreased and the flow rate increased. In contrast, if the sympathetic nerve stimulation was repeated the latency before any secretion appeared was much prolonged.

(iv) Combined Nerve Stimulation

Because of the difficulties in obtaining a continuing flow of sympathetically stimulated secretion observations were made, in several experiments, on the effects of adding a sympathetic stimulus to a background of parasympathetically evoked flow. The secretion stimulated in this way was always turbid.

In 4 out of 6 experiments, a short-lived increase in flow appeared within 30 seconds of stimulating the cervical sympathetic trunk and only subsequently did a decrease occur (Figure 4.6). Following this the flow either remained at a low rate or, in one experiment, ceased. In this latter experiment, the secretion was very viscous and its protein concentration was 32.28 mg ml^{-1}. Once the sympathetic stimulation stopped the background parasympathetic secretion returned to prior rates (Figure 4.6A). Similar secretory responses - an initial rise of flow at the onset of sympathetic stimulation followed by a prolonged decline - were obtained when the background secretion was stimulated with pilocarpine. Secretion from the contralateral gland, influenced by pilocarpine alone, was at a constant rate throughout the experiment. Infusion of isoprenaline during parasympathetic stimulation did not cause increases in the flow rate above those evoked by parasympathetic nerve stimulation alone.

b. Protein Secretion

(i) Resting Gland

The protein content of saliva secreted by the resting glands of anaesthetized animals was estimated whenever a sufficient volume was obtained, although in only a few of the acute experiments did the flow reach 0.25 g h^{-1}. In three animals the protein concentration in saliva from the resting gland was 3.24, 3.44 and 5.57 mg ml^{-1}* respectively.

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* One ml of mandibular saliva weighed 1 g.

(ii) Autonomic Nerve Stimulation

The effects of autonomic nerve stimulation on salivary protein secretion are summarized in Table 4.1. Saliva obtained during stimulation of the chorda lingual nerve alone always contained less protein than that evoked by either sympathetic stimulation or by combined nerve stimulation - a 3 to 15 fold increase in protein concentration was present in saliva secreted in response to sympathetic nerve stimulation compared with that induced by stimulation of the chorda lingual nerve. However, protein outputs were always greater during combined nerve stimulation than after stimulation of either division of the autonomic system alone (Table 4.1).

In the experiments involving combined nerve stimulation, the salivary protein concentration remained high (3.52-12.63 mg ml^{-1}) following the injection of an α-blocker, phentolamine, but decreased markedly (to as low as 0.47 mg ml^{-1}) following the administration of propranolol. During prolonged sympathetic stimulation (1h) there was a progressive decline in protein concentration.

(iii) Pharmacological Stimulation

The effects of parasympathomimetic and sympathomimetic agents on salivary protein concentrations are presented in Table 4.2. The secretion induced by the infusion of pilocarpine was usually turbid, and of higher protein content when collected from animals in which the superior cervical ganglion was intact than from those in which the ganglion had been extirpated. Intravenous injection of carbachol (40 μg kg^{-1}), however, induced a copious flow with very low protein content.

Since infusion of isoprenaline (0.3 μg kg^{-1} body weight min^{-1}) alone even for 2 hours caused only a very sparse flow (e.g. 0.19 g) estimations of protein content were performed only on saliva secreted in the presence of a parasympathetic stimulus as well. The results showed that protein secretion was increased by the intravenous administra-

Table 4.1: Effects of autonomic nerve stimulation on mandibular flow, protein concentration and protein output.

Site of Stimulation	Mandibular Flow ($g\ min^{-1}$)	Protein Conc ($mg\ ml^{-1}$)	Protein Output ($mg\ min^{-1}$)
Chorda lingual nerve			
range	0.33-0.74	0.44-1.56	0.25-1.02
$\bar{X}\pm SE$ (n/N)	0.56±0.05 (13/10)	1.27±0.06 (13/10)	0.72±0.07 (13/10)
Cervical sympathetic trunk			
range	0.01-0.06	4.02-25.68	0.16-0.63
$\bar{X}\pm SE$ (n/N)	0.03±0.005 (5/5)	14.63±2.84 (8/8)	0.42±0.08 (5/5)
Combined chorda lingual and cervical sympathetic trunk			
range	0.18-0.45	3.95-32.38	1.25-3.89
$\bar{X}\pm SE$ (n/N)	0.36±0.07 (6/4)	8.90±3.02 (9/6)	2.31±0.39 (6/4)

($\bar{X}\pm SE$, mean ± standard error of the mean;

n/N, number of observations/number of sheep).

Table 4.2: Effects of pharmacological stimulation on the protein concentration of mandibular saliva.

Treatment	INTACT GLAND		AFTER GANGLIONECTOMY	
	Protein Conc (mg ml^{-1})		Protein Conc (mg ml^{-1})	
	range	$\bar{X}\pm SE$ (n/N)	range	$\bar{X}\pm SE$ (n/N)
Pilocarpine infusion	0.92-3.47	2.24±0.35 (7/6)	0.63-1.74	0.98±0.06 (24/4)
Pilocarpine infusion plus stimulation of cervical sympathetic trunk	3.73-25.49	10.47±2.92 (7/3)	--	--
Pilocarpine infusion plus single injection of isoprenaline	--	--	2.41-3.90	3.09±0.21 (6/2)
Infusion of both pilocarpine and isoprenaline	--	--	2.65-4.48	3.47±0.29 (6/2)
Chorda lingual nerve stimulation	0.59-0.88	0.77±0.10 (6/1)*	--	--
Chorda lingual nerve stimulation plus isoprenaline infusion	1.47-5.95	3.35±0.69 (9/1)*	--	--
Carbachol injection	0.57	0.57 (2/2)	--	--

($\bar{X}\pm SE$, mean ± standard error of the mean; n/N, number of observations/number of mandibular glands).

* The mean values were calculated from successive 10-minute samples obtained from the same salivary gland.

The dose of drugs given intravenously - infusions: pilocarpine, 5.8-7.4 µg kg^{-1} min^{-1}; isoprenaline, 0.3 µg kg^{-1} min^{-1}
- single injections: isoprenaline, 2-4 µg kg^{-1}; carbachol, 40 µg kg^{-1}.

tion of isoprenaline either as a single injection (2-4 μg kg^{-1} body weight) or an infusion for 1-2 hours. As with sympathetic nerve stimulation it was not possible to sustain high levels of protein output over a prolonged period.

(iv) Gel Electrophoresis

Electrophoretic studies using acrylamide gel (Figure 4.7) demonstrated that the major soluble proteins in saliva produced during sympathetic stimulation were anionic migrating components, which have been termed Band X and Y. Of these the former (Band X) was usually the more intense in its staining. Banding in this region also occurred intensely in samples obtained in response to combined stimulation of the autonomic nerves. In contrast, although present in chorda lingual nerve stimulated secretion, the staining intensity varied and was always much less than that in sympathetically evoked samples. These bands (X and Y) disappeared in chorda lingual nerve stimulated samples from animals that had been treated with both an α and a β-blocker. (Bands X, Y were used as antigen I for the immunocytochemical studies).

The insoluble proteins of sympathetic secretion were examined in a SDS-polyacrylamide gel. The results of this study again showed two intensely staining bands that migrated rapidly toward anode (Figure 4.7E). Such bands remained in the insoluble salivary precipitates after being washed in phosphate buffer and freeze-dried and were used as antigen II for the immunocytochemistry.

2. Morphological Changes

Observations on the morphological changes in mandibular glands induced by electrical stimulation of the autonomic nerves were made in 15 animals. Preliminary experiments revealed that such changes were readily apparent if the glands were stimulated for two hours or longer, while shorter periods of stimulation, e.g. one hour or less, often failed to produce any obvious changes. For this reason the glands

studied in detail were those subjected to the longer periods of stimulation.

The macroscopic appearance of the unstimulated glands was whitish and opaque, whereas the contralateral glands stimulated either parasympathetically or sympathetically, usually had a pinkish and more translucent appearance.

a. Effects of Parasympathetic Nerve Stimulation

Parasympathetic nerve stimulation (at 2-8V, 5-10 Hz, 0.2 msec) resulted in changes confined almost exclusively to the acinar cells, even when stimulation was continued for 4 hours.

(i) Light Microscopy

In sections taken after 2-4 hours of stimulation of the parasympathetic innervation, enlargement of central lumina together with a reduction in the cell size and a more diffuse appearance of stained materials in the acini was obvious in both 5 µm (Figure 4.8) and 0.5 µm sections. Demilunes, intercalated and, usually, striated ducts showed no differences from the contralateral unstimulated glands although in some sections a loss of PAS-positive materials seemed to have occurred from striated duct cells. Purple-blue stained secretion could be seen in the ductal lumina (Figure 4.8C).

(ii) Electron Microscopy

Acini of glands stimulated for 2-4 hours exhibited signs of hyperactivity (Figure 4.9). Exocytosis of secretory granules occurred in acinar cells apparently after the fusion of perigranular and acinar cell membranes at luminal surfaces. Enlargement of both central acinar lumina and basolateral canaliculi was pronounced in these cells and the former commonly contained membrane ghosts as well as the content of acinar granules including their small dark bodies (Figure 4.9A). In the glands examined after electrical stimulation of parasympathetic nerves some variation in the

degree of degranulation of acinar cells within the same secretory endpiece and between endpieces in the same gland was usual.

In stimulated cells, the cisternae of RER became prominent and widely dilated, - particularly those adjacent to the nuclei and the intercellular plasma membranes (Figure 4.9B,C). Membrane-bound vesicles of various sizes, probably the newly formed secretory granules, were present in the vicinity of the dilated RER and Golgi complexes, while nuclei were less flattened in outline than those of resting glands (Figure 4.9C).

The fine structure of demilunes showed no obvious changes following parasympathetic nerve stimulation (Figure 4.10) and thus confirmed the impression gained with light microscopy. There was, however, occasional dilation of intercellular canaliculi among demilune cells, basolateral canaliculi, and lateral intercellular spaces between demilune and the adjacent acinar cells.

b. Effects of Sympathetic Nerve Stimulation

Electrical stimulation of the cervical sympathetic trunk at 4-8V, 5-10 Hz and 0.2 msec for 2 hours caused considerable morphological changes in the fine structure of demilunes and the striated ducts but not in the acinar cells.

(i) Light Microscopy

Light microscopic observations on 5 µm paraffin sections (Figure 4.11) did not disclose any obvious changes of the endpiece secretory cells in sympathetically stimulated glands but there was a loss of PAS-positive materials from all striated duct cells and other changes in the basal regions of these, particularly at their junctions with the intercalated ducts. Other portions of striated ducts were normal. These changes in the basal areas of striated duct cells appeared as numerous vacuoles of irregular shape and size containing PAS-positive and AB-positive materials similar to those present in the ductal lumina.

In addition to those described above, further changes in the demilune cells were visible in 0.5 µm toluidine blue-stained sections. The cells contained less dark-staining granules than in the resting gland and there was the appearance of some lightly-stained granules. Occasionally, large vacuoles were evident in demilunes and there was also widening of the basolateral canaliculi of demilune cells. Acinar cells showed no changes.

(ii) Electron Microscopy

When examined in detail the structure of demilunes was seen to be markedly affected by sympathetic nerve stimulation (Figure 4.12A,B). A major difference could be seen in the granules - particularly the presence of fused granules with an absence of their characteristic substructure. Enlarged intercellular canaliculi which had acquired a scalloped outline (apparently caused by the exocytosis of secretory granules) were conspicuous. The phenomenon of chain exocytosis was occasionally observed.

The electron microscopy suggested that the substructures of demilune granules were discharged from the cell as discrete bodies whereas the other components were dispersed. These substructures and other granule contents, as well as membrane ghosts and some cytoplasmic organelles, e.g. mitochondria, were present in the enlarged basolateral canaliculi (Figure 4.12C), central acinar lumina and striated duct lumina (Figure 4.14). Damage to some demilunes was occasionally evident in sympathetically stimulated glands (Figure 4.13) and masses of cytoplasmic structures and membrane ghosts were discharged. The materials which appeared in the canaliculi and lumina also appeared in the saliva and seemed to comprise the major fraction of the insoluble components of sympathetically evoked secretion (Figure 4.15). Another finding was the presence in demilune cells of large cytoplasmic vacuoles containing a product which resembled the secretion (Figure 4.12B). Such vacuoles were usually found near the nuclei.

Although acini showed no obvious alterations in their fine structure after sympathetic stimulation, secretion of granules from some was a relatively common feature as was also the case in the resting gland. It was unusual, however, to find the secretion of intact secretory granules from the acinar cells though, infrequently, they were observed in the basolateral canaliculus (as in Figure 4.12C) together with the discharged contents of demilune granules.

Ultrastructural changes of the basal regions of striated duct cells are shown in Figure 4.16. Features not evident from light microscopy include dilation of basal cellular membrane folds, vacuoles enclosed by double membranes, multimembrane-bound structures and empty vacuoles without membrane boundaries. These changes in the striated duct cells were not seen in the experiment which the sympathetic nerves were stimulated after injection of phentolamine, although the demilunes did show severe cellular damage. In addition, the type-I and type-II striated duct cells often contained numerous vesicles (as were also occasionally observed in the resting gland).

c. Effects of Combined Nerve Stimulation

The glands examined in this part of the study were from animals that had their cervical sympathetic trunk and chorda lingual nerve stimulated simultaneously (at 2-10V, frequencies of 5-10 Hz, and 0.2 msec duration) for 2-3 hours. After stimulation, both acini and demilunes showed marked changes and the enlargement of basal intercellular spaces was obvious (Figure 4.17B). Although changes in acini were similar to those described previously with parasympathetic nerve stimulation alone (see Figure 4.9), changes in demilunes appeared more pronounced (Figure 4.18A) than with sympathetic nerve stimulation alone.

With dual stimulation for 2 hours there was extensive degranulation in demilunes. The basolateral canaliculi of demilunes remained enlarged (Figures 4.17B; 4.18B), while a diminution of the intercellular canaliculi seemed to occur. Mitochondria, RER and free ribosomes were abundant in the

cytoplasm. Golgi complexes became dilated and some small vesicles were present nearby (Figure 4.18B). After 3 hours of sympathetic nerve stimulation on a background of chorda lingual nerve stimulation (Figure 4.18C-E) the cells which were essentially depleted of their preformed secretory granules were characterized by the presence of many vesicles containing dense and pale matrix, probably representing the newly formed granules. These were confined to the apical regions of intercellular canaliculi. The RER were rearranged into whorl-like structures at the cell base. Dilated Golgi cisternae were accompanied by many small vesicles (Golgi vesicles) and condensing vacuoles were present at the inner surface of Golgi cisternae. There were numerous mitochondria usually located adjacent to the cell membranes, among RER and in the Golgi zone.

Striated duct cells also showed similar changes at their basal regions to those found at light microscopy with sympathetic nerve stimulation alone.

d. Pharmacological Stimulation

Four preliminary experiments were undertaken to enable a comparison of the effects of nerve stimulation with those of parasympathomimetic and sympathomimetic drugs. This was done in an effort to assess whether an effective neural stimulation (particularly sympathetic) was being obtained. In general the structural changes induced by pharmacological agents resembled those arising with electrical stimulation of nerves but differed considerably in their extent.

(i) Infusion of Isoprenaline (0.3 μg kg^{-1} body weight min^{-1})

(a) Light Microscopy

With intravenous infusion of isoprenaline, for 2-2.5 hours, only a scant viscous secretion (ca. 0.19 g h^{-1}) was secreted from the mandibular gland. Paraffin sections with AB/PAS staining showed no changes in either the secretory endpieces or the striated duct cells, despite the fact that a

purple-red stained secretion filled all the dilated ductal lumina. In addition, purple-blue material resembling the secretion of acini could be found in some lumina (Figure 4.19A).

In these experiments the contralateral gland, which received a simultaneous stimulation of the chorda lingual nerve, showed variable changes in both the demilunes and acini. Vacuolation in demilunes was observed in one gland but not in the other. The ductal lumina were less dilated and contained PAS-positive secretions in various combinations, mostly purple-blue (Figure 4.19B). However, in 0.5 μm (toluidine blue) sections, almost no dark-staining granules were present in the demilunes.

(b) Electron Microscopy

Examples of changes in the fine structure of endpiece secretory cells after 2 hours of infusion of isoprenaline on a background flow evoked by the stimulation of chorda lingual nerve are shown in Figure 4.20B as compared with the contralaterally isoprenaline stimulated gland (Figure 4.20A). With both types of stimulation (Figure 4.20B), demilune cells were almost completely degranulated, intercellular canaliculi remained prominent and large vacuoles were observed in some cells. Acinar cells also showed depletion of their secretory granules and numerous dilated RER. The fine structure of the contralateral gland which was only subjected to isoprenaline's actions showed changes in demilunes which included fusion and discharge of secretory granules. Large cytoplasmic vacuoles containing granule components were occasionally evident (Figure 4.20A). These vacuoles usually occurred in the basal region of demilune cells.

(ii) Infusion of Pilocarpine (5.8-7.4 μg kg^{-1} body weight min^{-1})

(a) Light Microscopy

In two experiments in which pilocarpine was infused

intravenously (for 2h or 5h), acini showed wide acinar lumina and depletion of their secretory granules while demilunes showed only slight changes. In striated ducts the PAS-positive reaction appeared confined to the luminal regions and secretion was present in the ductal lumina (Figure 4.21A).

Additional changes apart from described above were found in demilunes and striated duct cells in the glands that received simultaneously a stimulation of sympathetic nerve during pilocarpine infusion. As shown in Figure 4.21B, in the gland receiving 2h sympathetic nerve stimulation during a 5h pilocarpine infusion, demilunes exhibited many empty vacuoles, while PAS and AB positively stained materials were evident at the basal regions of some striated duct cells, particularly those connected with the intercalated ducts.

(b) Electron Microscopy

Similar changes in the fine structure of demilunes and acini were observed after the combinations of pilocarpine and sympathetic nerve stimulation and isoprenaline infusion plus chorda lingual nerve stimulation. However, greater alteration was caused by the former treatment, particularly the occurrence of large vacuoles in the demilune cells.

3. Light Microscopic Observations on the Localization of the Major Proteins in Sympathetically Stimulated Saliva

An attempt was made to define the cellular origins of the major proteins secreted in sympathetically stimulated saliva. Paraffin sections of resting mandibular glands from four sheep were examined, as described in chapter II, using the immunocytochemical (PAP) method and the antisera raised against the major bands of soluble and insoluble proteins of the saliva.

a. Soluble Proteins

Soluble proteins were located mainly in the demilune cells, some striated duct cells and occasionally in the

excretory duct cells (Figure 4.22). In demilunes, the strong reaction products were present in the granules, whereas the acinar cells and intercalated ducts exhibited no positive reactions.

b. Insoluble Proteins

Insoluble proteins were distributed less specifically than their soluble counterparts. Intense positive reaction products appeared in the demilune cells, but the acinar cells in some secretory endpieces showed very mild reactions throughout the cells. Intense product was observed in some striated duct cells (Figure 4.23). The control sections which were treated either with rabbit non-immune immunoglobulin or with Tris-phosphate buffer instead of the rabbit primary anti-sheep immunoglobulins showed a negative reaction.

Figure 4.2: Effects of parasympathetic stimulation on mandibular flow.

In these records and those that follow, the traces are (from above downwards):

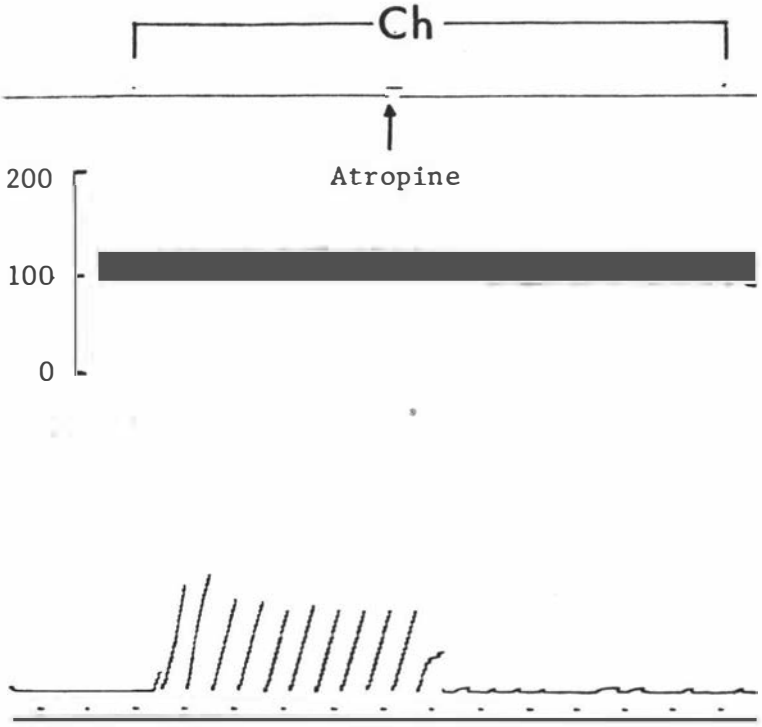
event marker
arterial blood pressure in mmHg
drops of saliva per 30 sec
time marker (one-minute intervals)

Atropine (0.1 mg kg^{-1} body weight) was given intravenously at the time indicated by the arrow.

A. The chorda lingual nerve was stimulated electrically at 4V, 5Hz and 0.2 msec duration; ⌐—Ch—¬). The protein concentration of the secretion was 1.52 mg ml^{-1}.

B. Carbachol (40 µg kg^{-1} body weight) was administered intravenously at the double arrows. The protein concentration of the secretion collected before injection of atropine was 0.57 mg ml^{-1}.

A.



B.

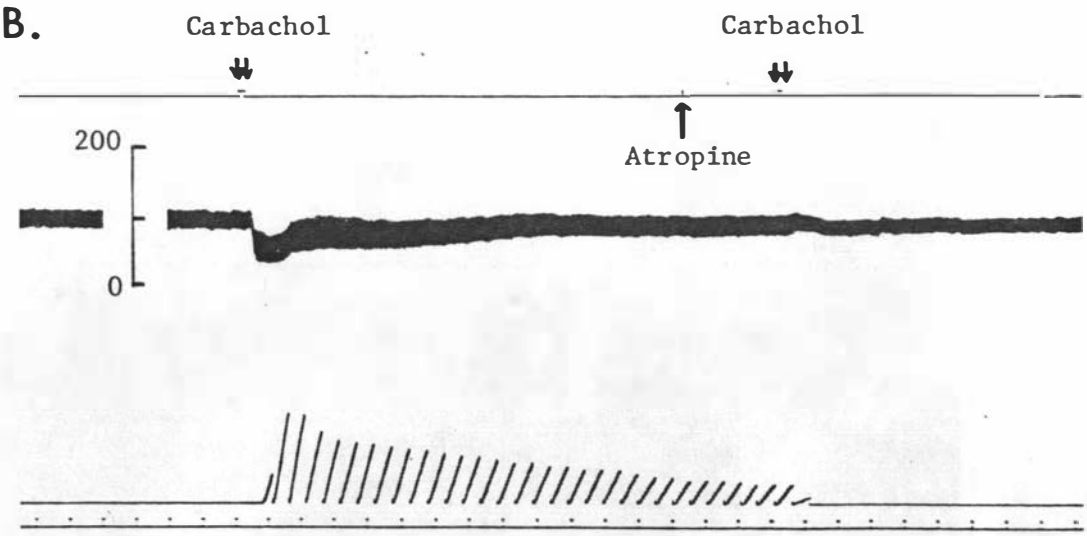
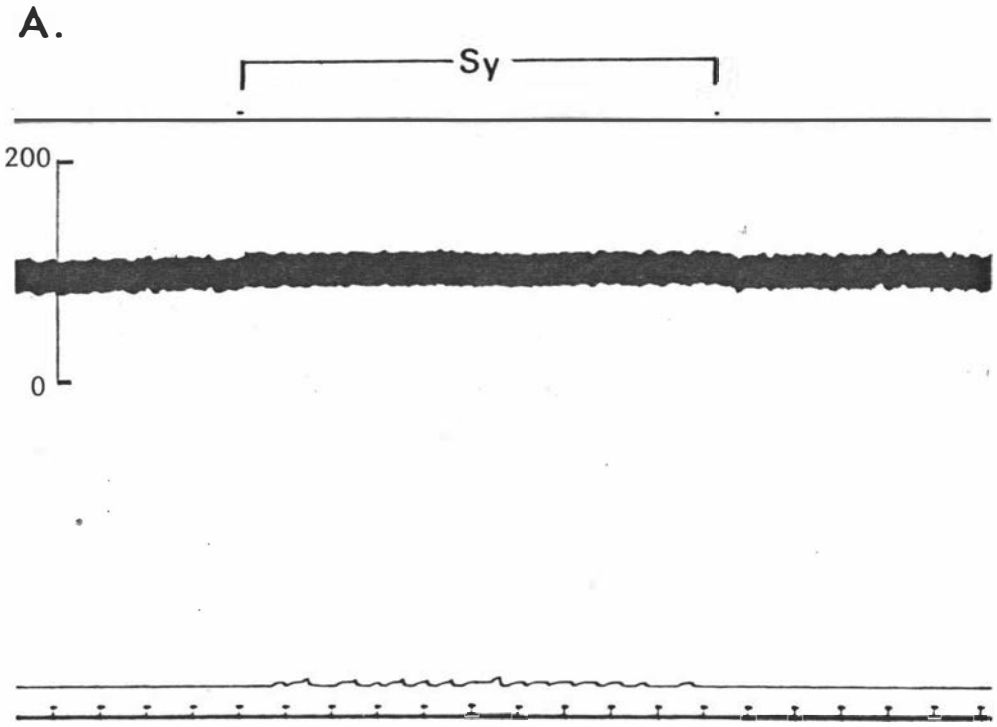


Figure 4.3: Mandibular salivary flow during electrical stimulation of the peripheral cut end of the left cervical sympathetic trunk for the period indicated by ⌐—Sy—¬. Records A-D are from the same sheep, an animal in which the left cervical vagus nerve had also been sectioned.

A. Ten minutes of continuous stimulation (3V, 5Hz and 0.2 msec).

B. Five minutes of continuous stimulation (5V, 5Hz and 0.2 msec).


A.
Sy
200
0

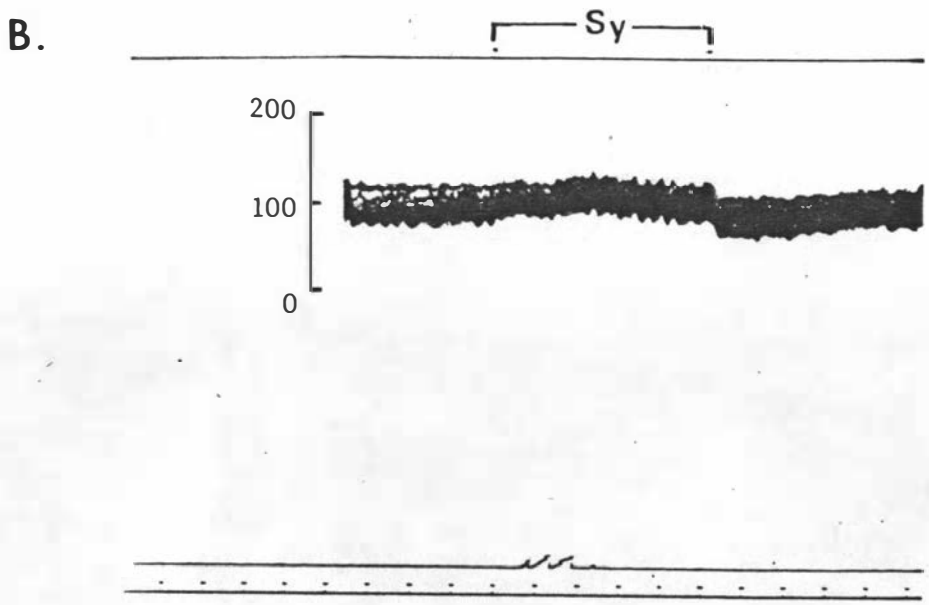

B.
Sy
200
100
0

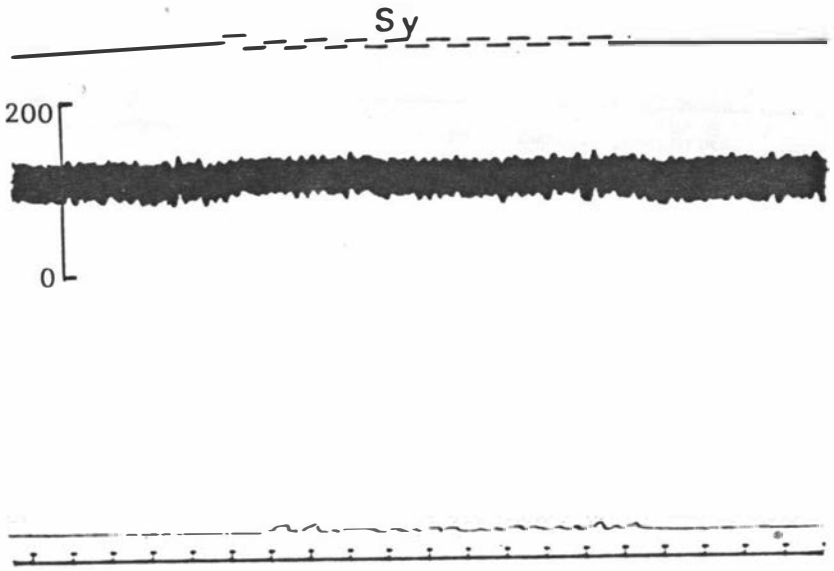
Figure 4.3:

C. Intermittent stimulation with same stimulus parameters as in B, for a total of five minutes.

D. Effects of the α-blocker phentolamine (1.0 mg kg^{-1} body weight, iv) which was given at the arrow during the sympathetic stimulation (5V, 10Hz and 0.2 msec).

Latencies of onset of secretion in A to D were 42, 35, 45 and 65 sec, respectively. The protein concentrations in saliva samples of A, C and D were 4.03, 10.91 and 12.63 mg ml^{-1}, respectively.

C.



D.

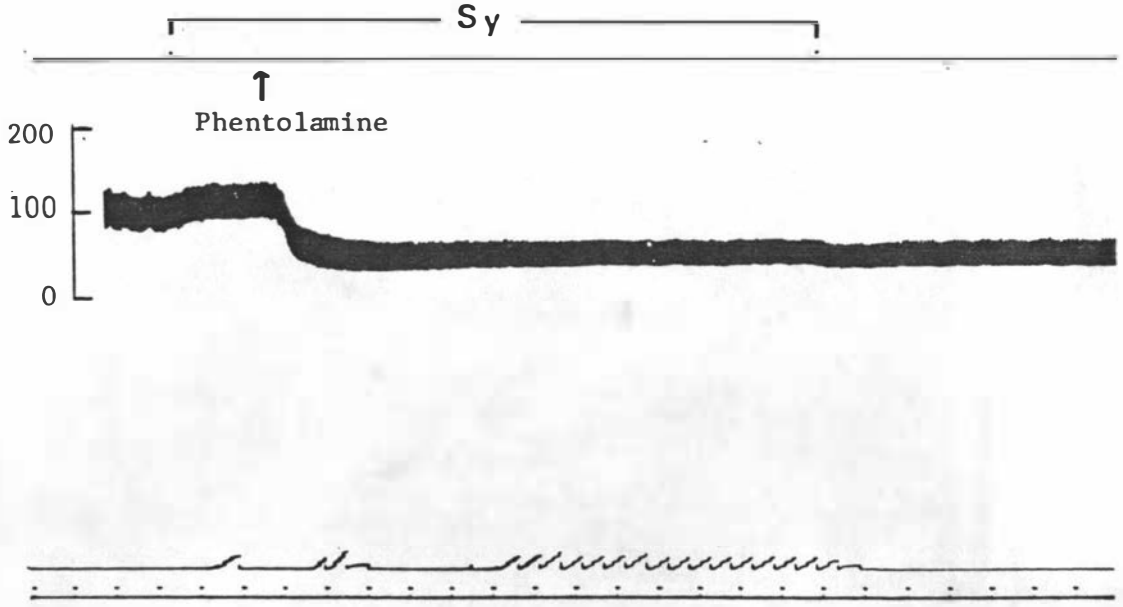


Figure 4.4: Effects of the β-blocker propranolol on mandibular flow. The cervical sympathetic trunk was stimulated (5V, 10Hz and 0.2 msec) 20 minutes after an injection of the α-blocker phentolamine.

Note the long latency before sympathetically evoked secretion appeared (4 min).

Then propranolol (1.0 mg kg^{-1} body weight) was administered intravenously (at the arrow) during the sympathetic nerve stimulation. (Records of different sheep from that for Figure 4.3).

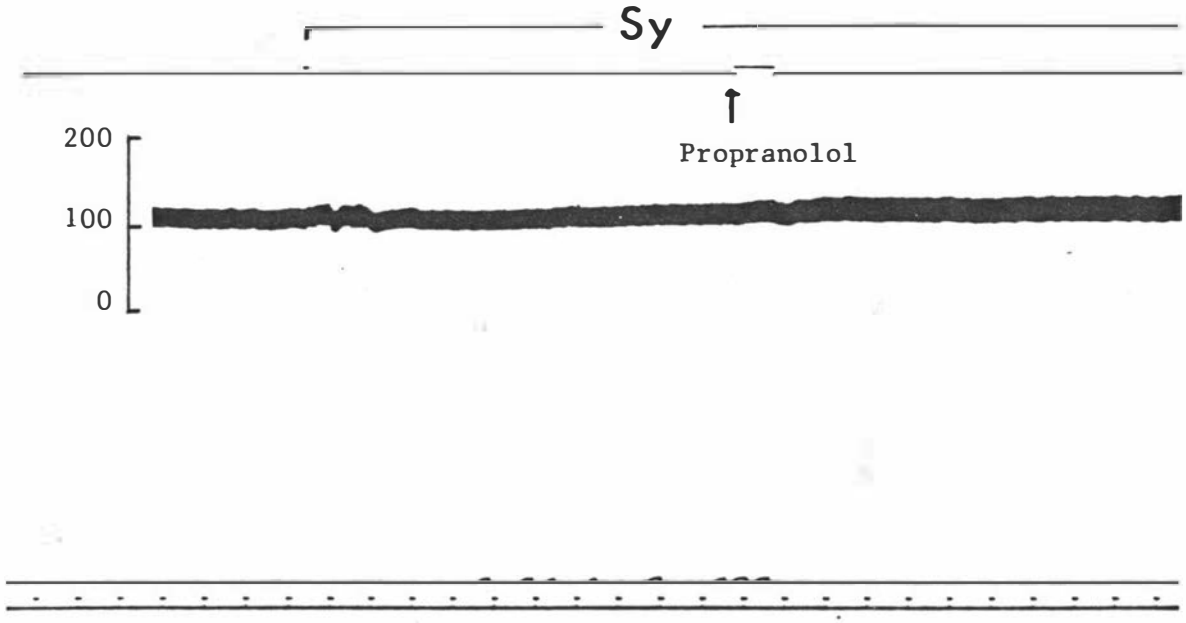

Sy
Propranolol
200
100
0

Figure 4.5: Effects of electrical stimulation (4V, 5Hz and 0.2 msec) of the chorda lingual nerve (⌐——Ch——¬) after a period of sympathetic nerve stimulation (⌐——Sy——¬ ; same stimulus parameters).

The latency to the onset of the flow for sympathetically evoked secretion was 62 sec, and 18 sec for parasympathetically evoked secretion. The saliva secreted during sympathetic stimulation was opalescent and contained fine white particles, as was also the case in the initial period of stimulation of the chorda lingual nerve.

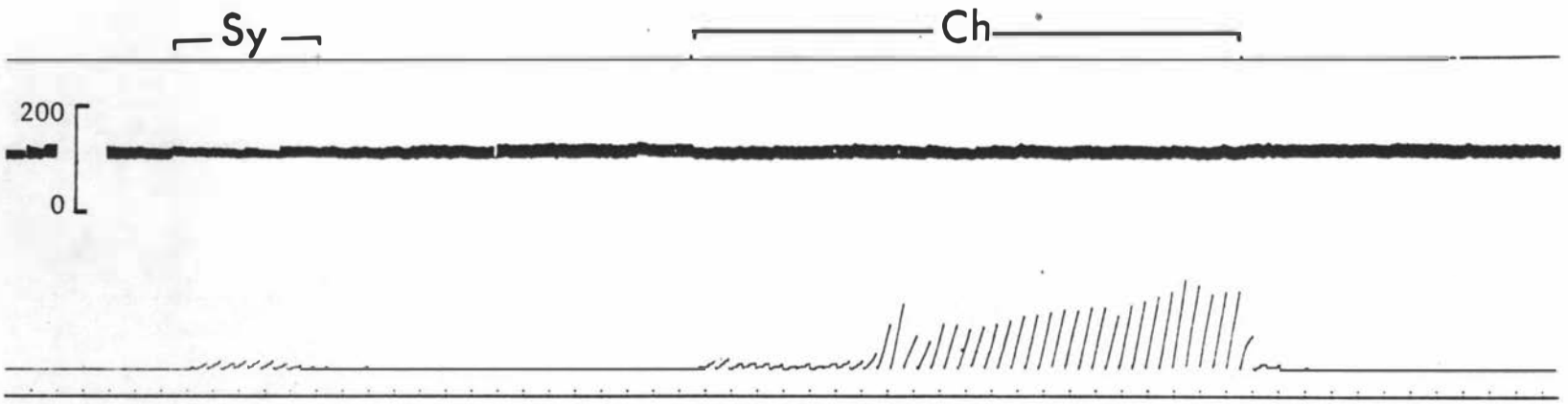

Sy
Ch
200
0

Figure 4.6: Effects of sympathetic nerve stimulation on mandibular secretion during a background flow induced by stimulation of the ipsilateral chorda lingual nerve.

A. The peripheral end of the ipsilateral cervical sympathetic trunk was stimulated for the period Sy.

B. Phentolamine (1.0 mg kg^{-1} body weight) was given at the small arrow.

Note the initial rise in secretion when sympathetic was superimposed on the para-sympathetic background activity (large arrow).

The protein concentration of saliva evoked by the stimulation of the chorda lingual nerve was 1.38 mg ml^{-1}, whereas that obtained after the dual nerve stimulation was 4.44 mg ml^{-1}.

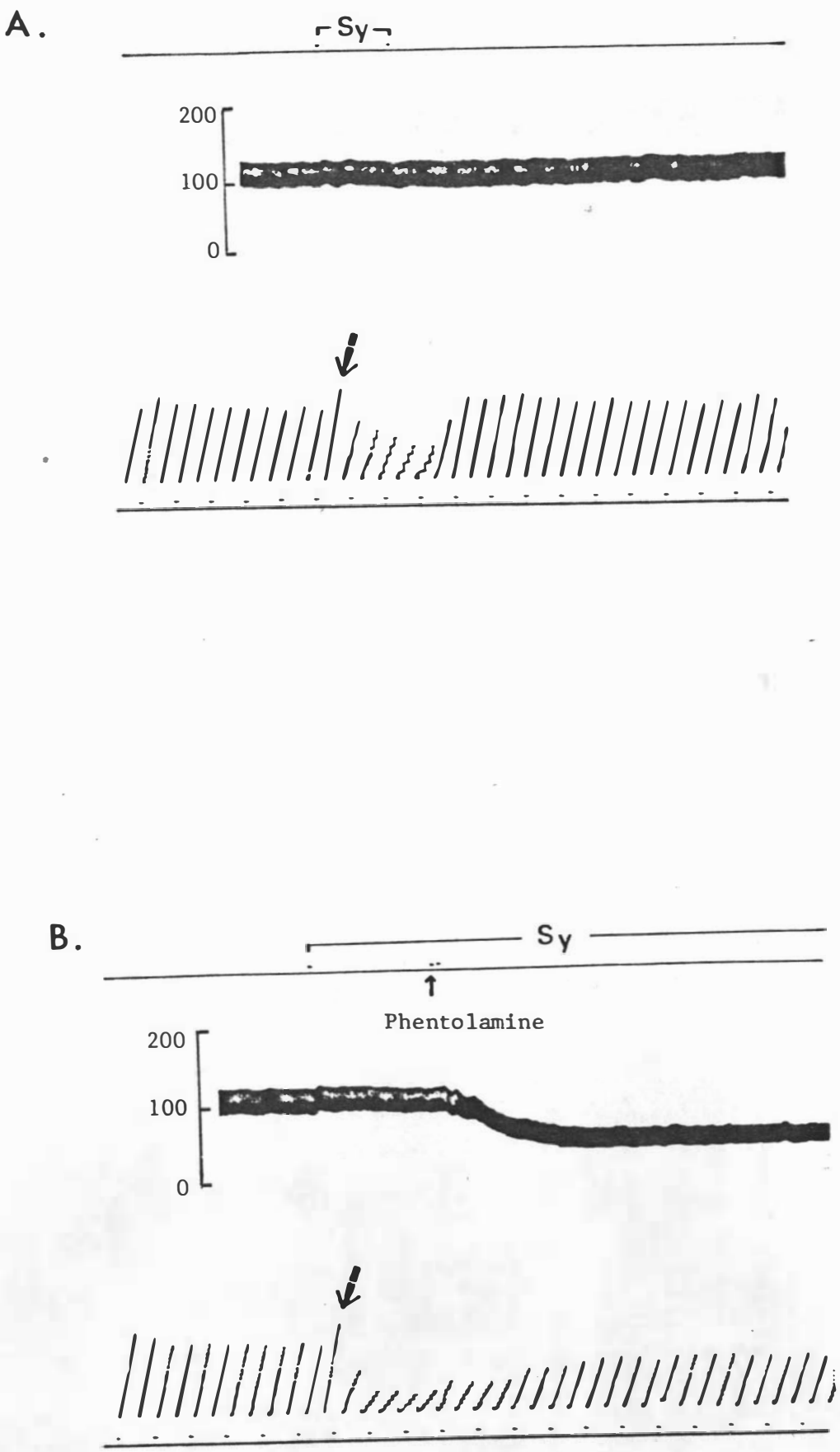

A.
Sy
200
100
0
B.
Sy
Phentolamine
200
100
0

Figure 4.7: Electrophoresis of mandibular saliva. The acrylamide gel has been stained with Coomassie Brilliant Blue to demonstrate protein bands.

A-D Polyacrylamide gel (7.5%). This shows the major soluble protein bands (X&Y) in samples of saliva obtained under various conditions of autonomic nerve stimulation:

- A & B Chorda lingual nerve stimulation in two sheep (5 µl sample, each).
- C Both sympathetic and chorda lingual nerve stimulation (5 µl sample, 1:2 dilution).
- D Sympathetic nerve stimulation (5 µl sample, 1:10 dilution).

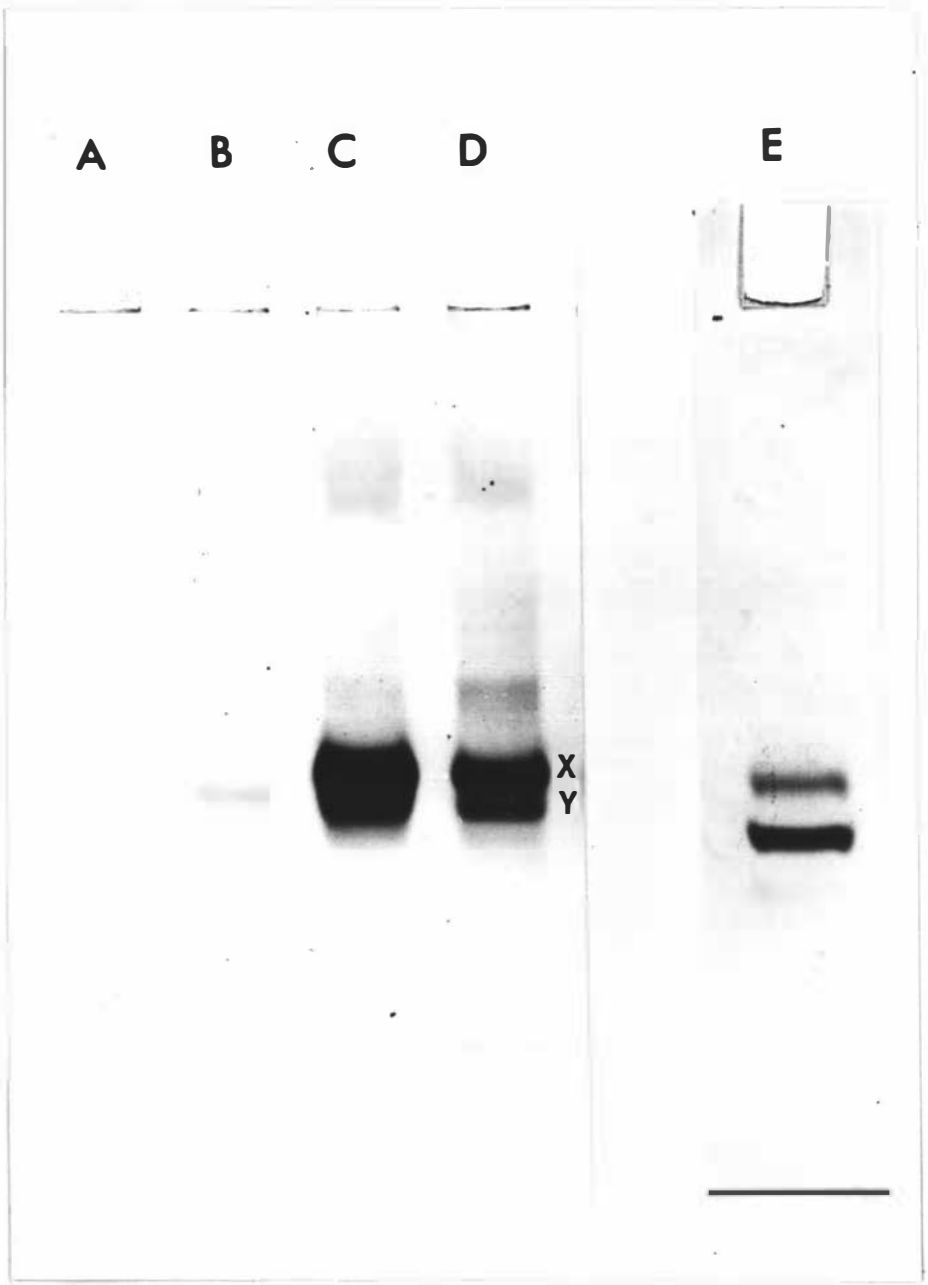
E The major insoluble protein components in the sediment of sympathetically evoked saliva. (1.0% SDS-polyacrylamide gel).

Figure 4.8: Light micrographs of mandibular glands showing the effects of electrical stimulation of the ipsilateral chorda lingual nerve for 4 hours. x250.

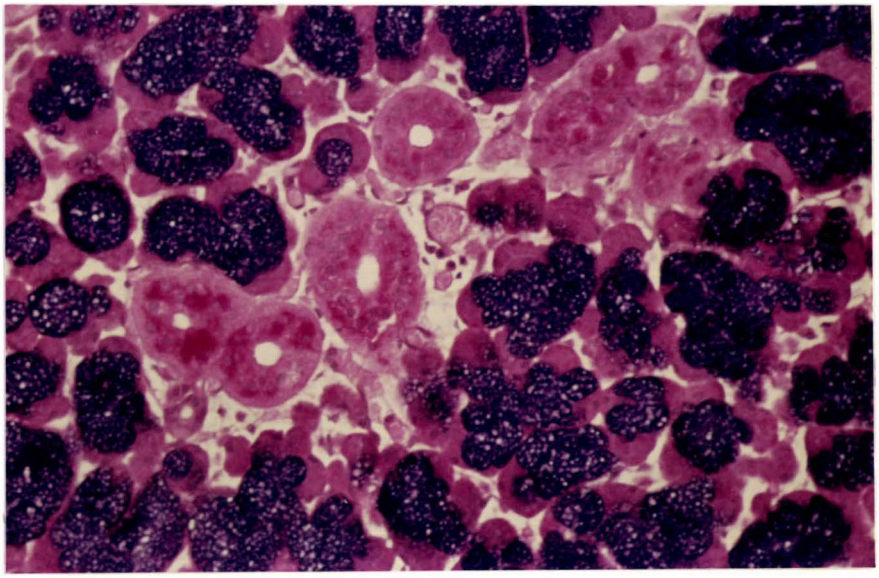
A. *Right (control) gland.* Only the contralateral chorda lingual nerve was stimulated.

B&C. *Left gland.* After stimulation of the ipsilateral chorda lingual nerve. *Note* the presence of conspicuous central acinar lumina, dilated blood vessels (see bv in B) and secretion of the lumina of some ducts (see C).

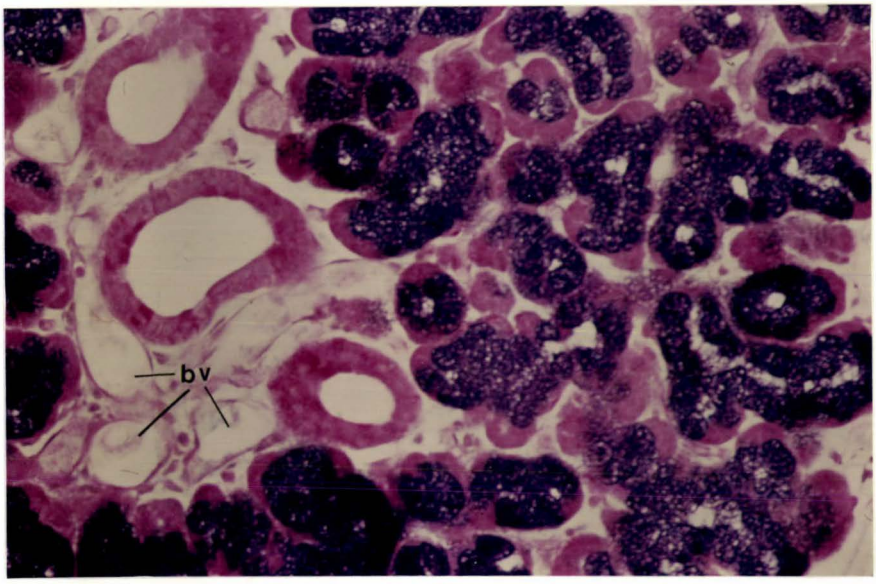
(A&B, Karnovsky's fixative; C, neutral formal saline fixative; and AB/PAS/H staining).


A
B
C
D
E
X
Y

A



B



C

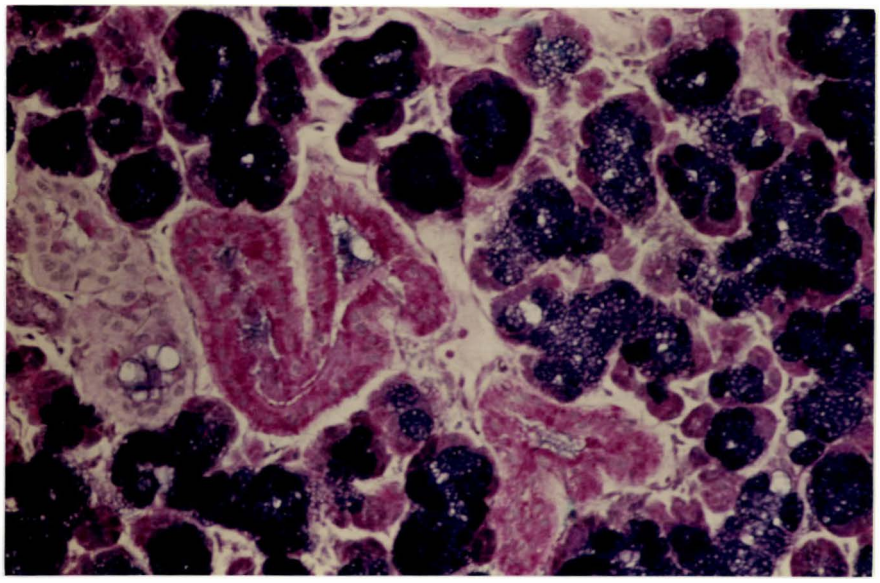
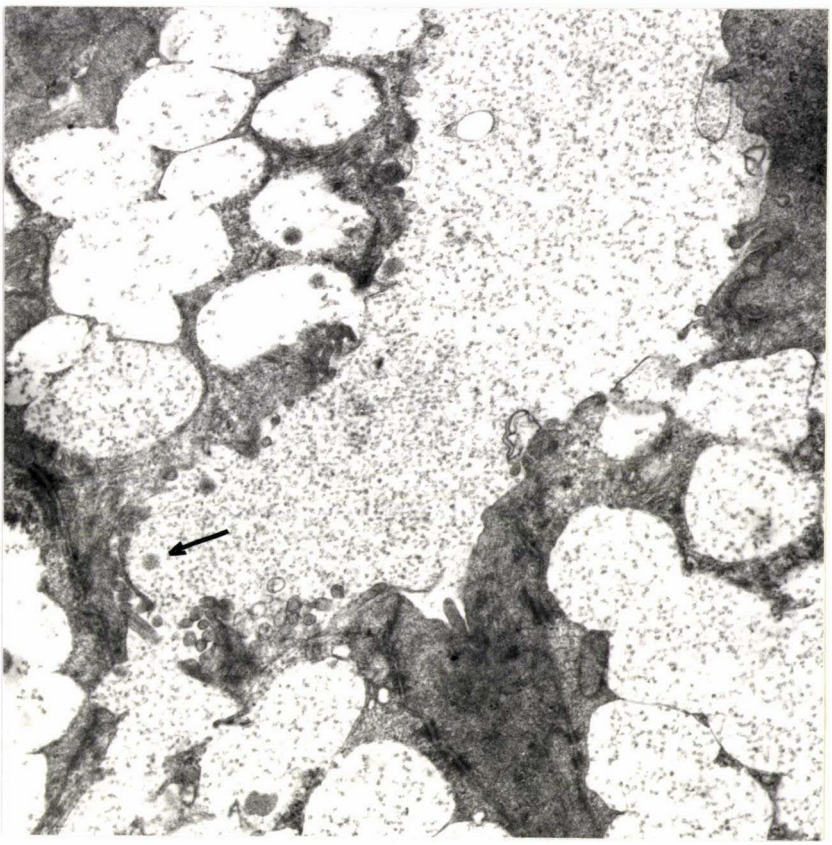


Figure 4.9: Electron micrographs of mucous acinar cells after stimulation of the ipsilateral chorda lingual nerve for 2h. x17,500.

A. Discharge of secretion including small dense bodies (arrow) into the central acinar lumen.

B. The region between two acinar cells, showing Golgi complexes (G) and dilated RER adjacent to the lateral intercellular membrane.

A



B

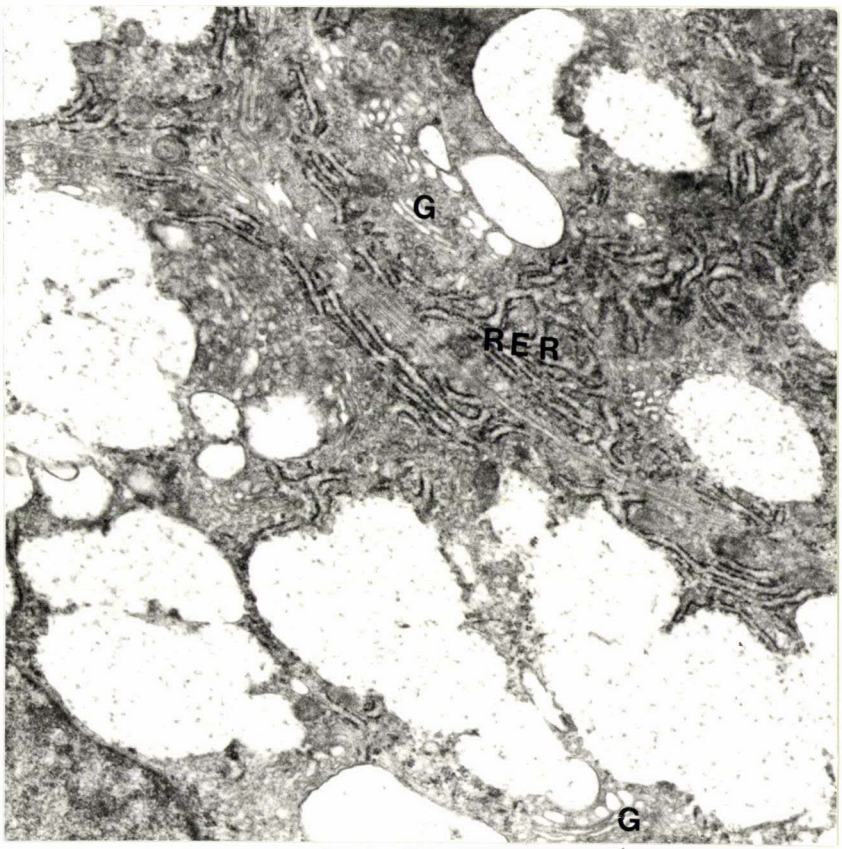


Figure 4.9:

C. The basal region of acinar cells, showing dilated RER. x8,100.

(All Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

C

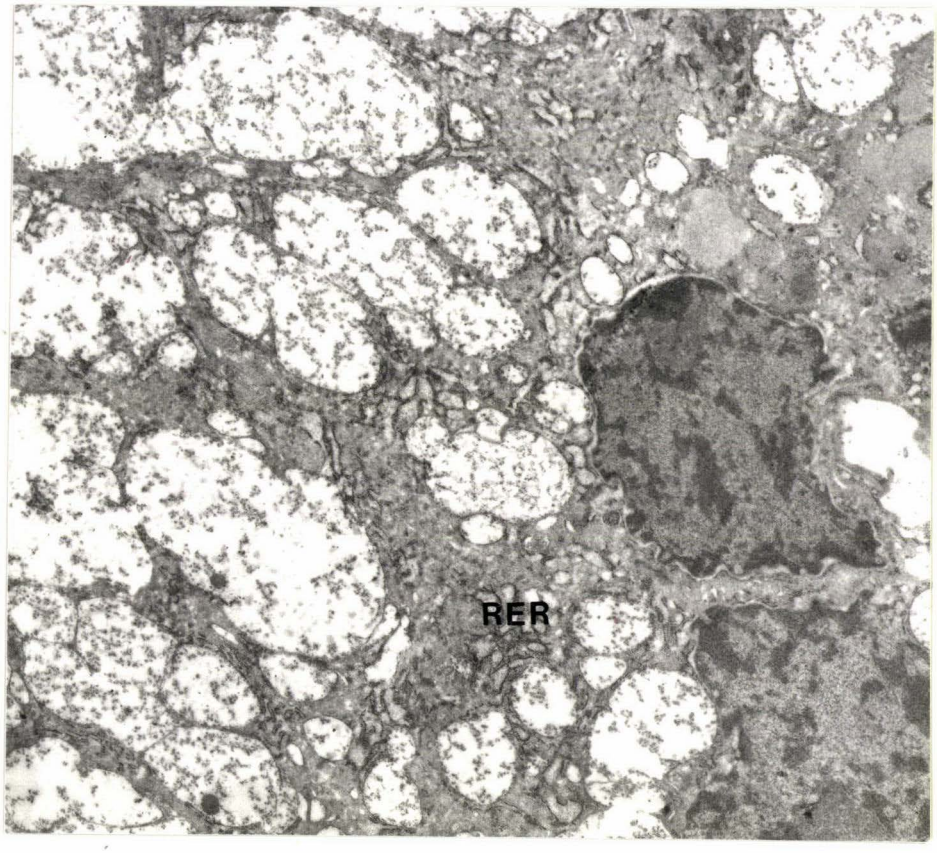


Figure 4.10: An electron micrograph of demilune cells after stimulation of the ipsilateral chorda lingual nerve for 4h, showing apparently unchanged demilune cells, and two intercellular canaliculi (arrow) which connected to the widened central acinar lumen (L).

Araldite section. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 post-fixation; *en bloc* staining). x8,100.

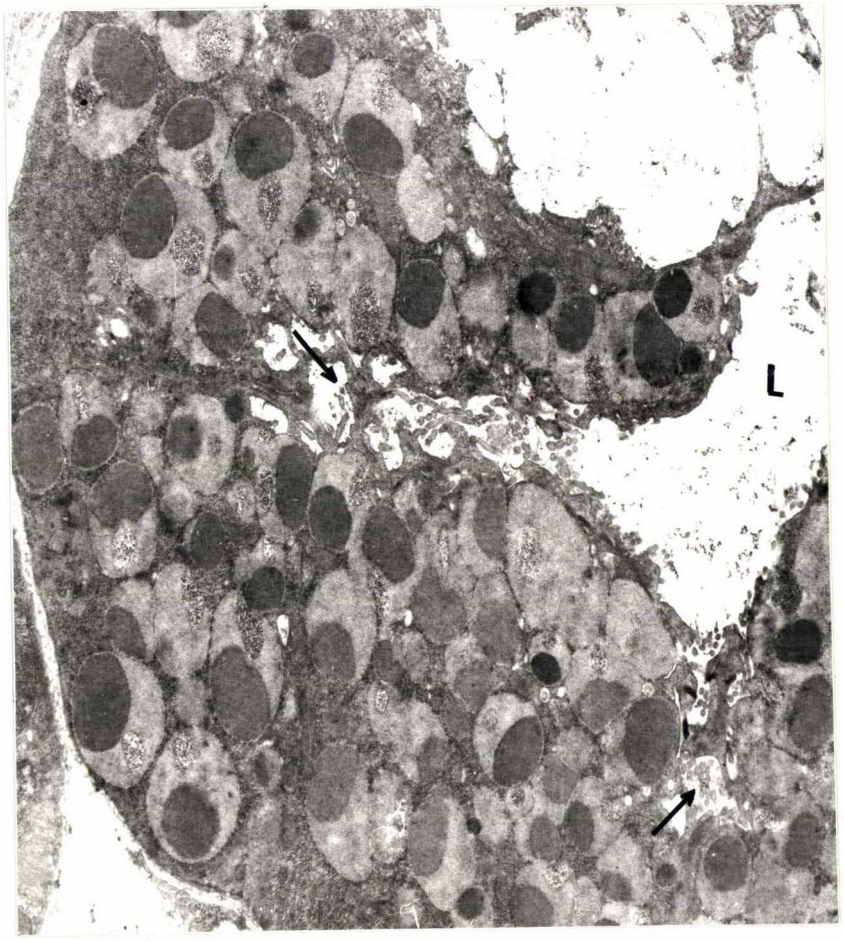

L

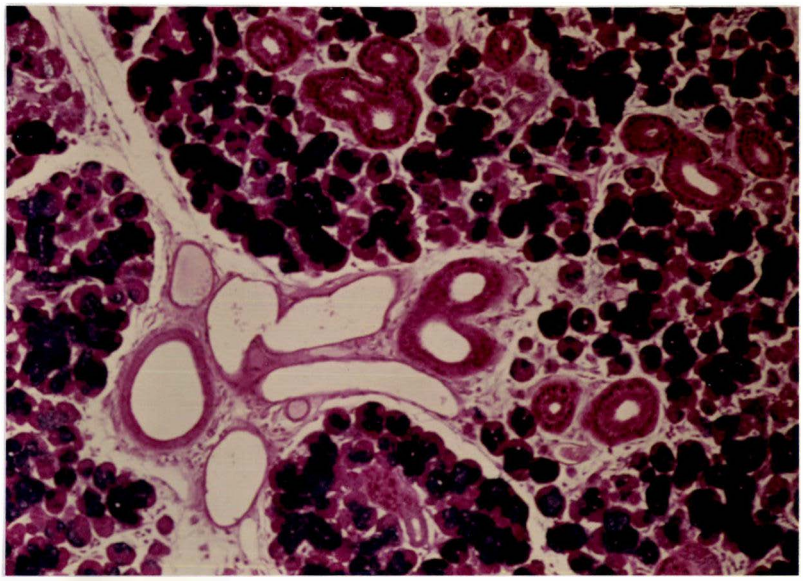
Figure 4.11: Light micrographs of mandibular glands showing the effects of electrical stimulation of the cervical sympathetic trunk for 2h. (Karnovsky's fixed paraffin sections, stained with AB/PAS/H).

A. *Right gland*, unstimulated nerve. x100.

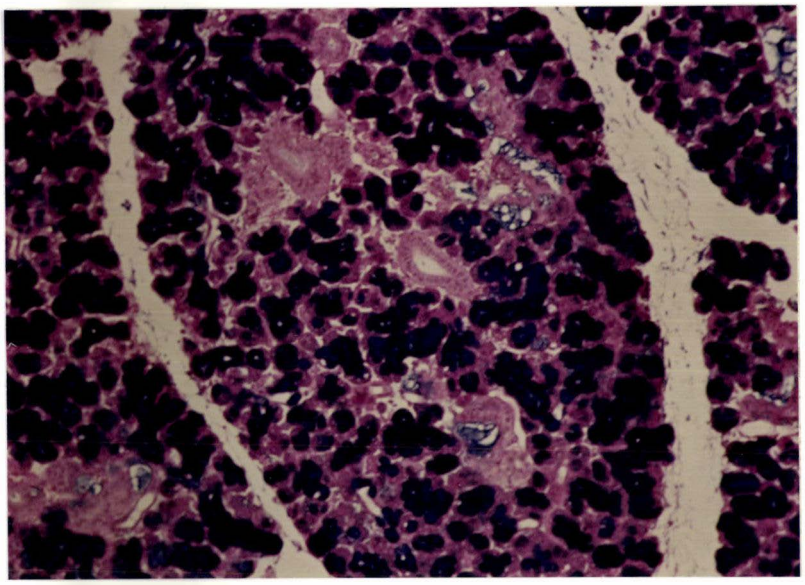
B&C *Left gland,* stimulated nerve. Magnification x100, and x620, respectively.

Note the presence of purple blue to purple red secretion in the ductal lumina and the striking changes of the striated duct cells - a loss of PAS-positive materials in most, and changes in the basal regions of many striated duct cells (arrow). Acinar cells show no obvious changes.

A



B



C

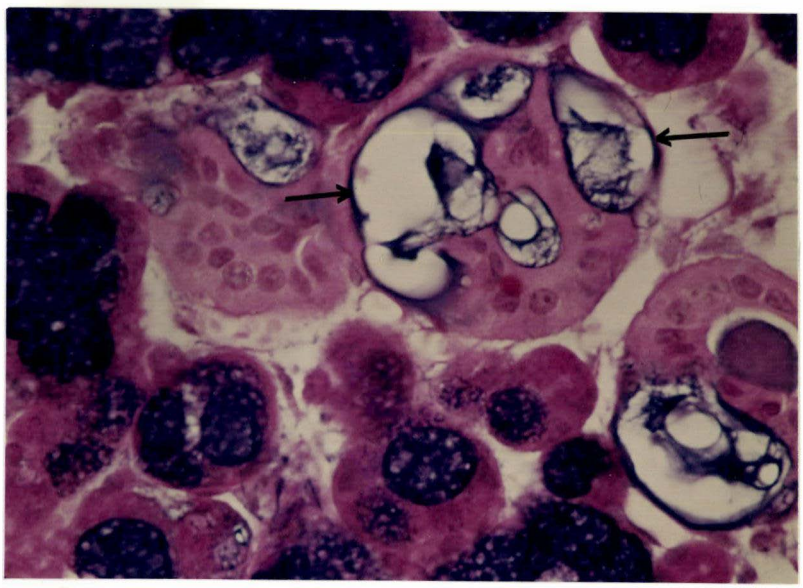


Figure 4.12: Electron micrographs of demilunes after stimulation of the cervical sympathetic trunk for 2h.

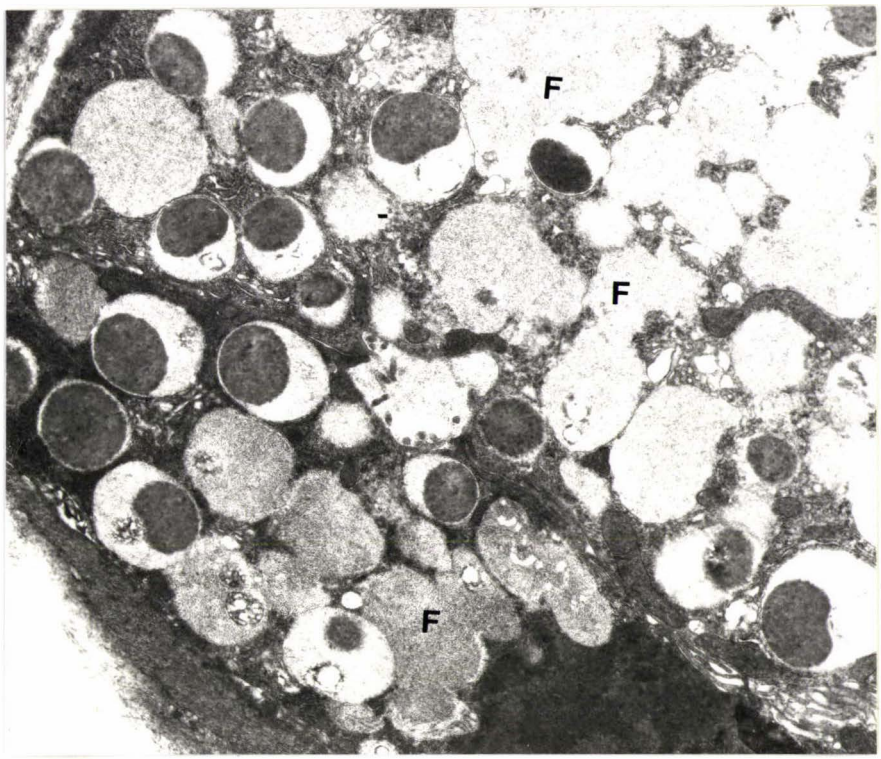
Attention is drawn to:

A. The presence of fused granules (F). (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining. Araldite section). x9,700.

B. The presence of a large cytoplasmic vacuole (V) and the intact substructures of secretory granules (arrow) in the intercellular canaliculus.

(Immersion fixation: 6.25% glutaraldehyde in sucrose phosphate buffer; OsO_4 postfixation, Araldite section). x6,400.

A



B

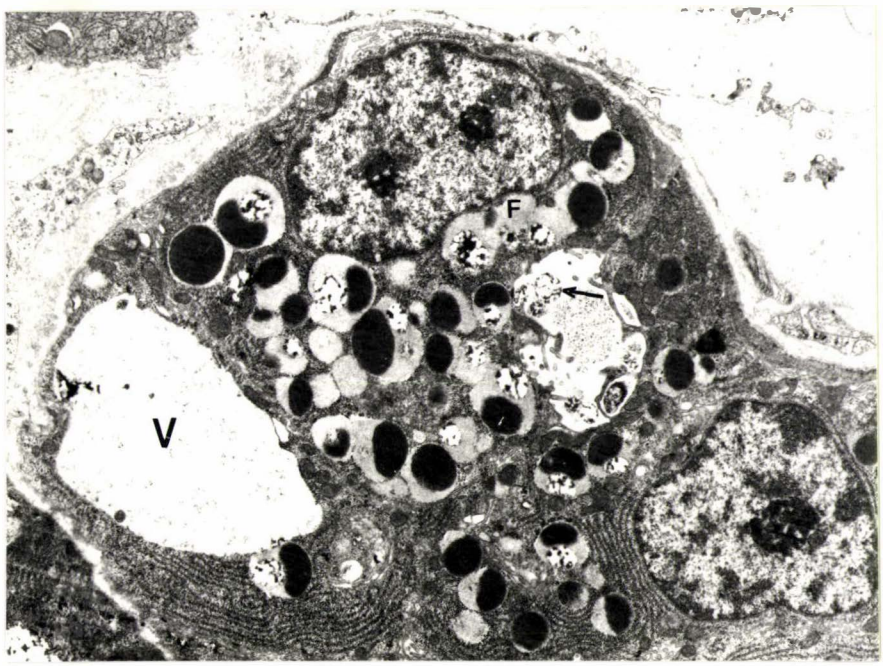


Figure 4.12:

C. The presence in the basolateral canaliculus of whole substructures of demilune granules (arrow) and the seldom-observed feature of an intact acinar granule (g).

(Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; Epon-Araldite section). x10,500.

(Other abbreviations:
MEC - myoepithelial process
n - nerve)

C

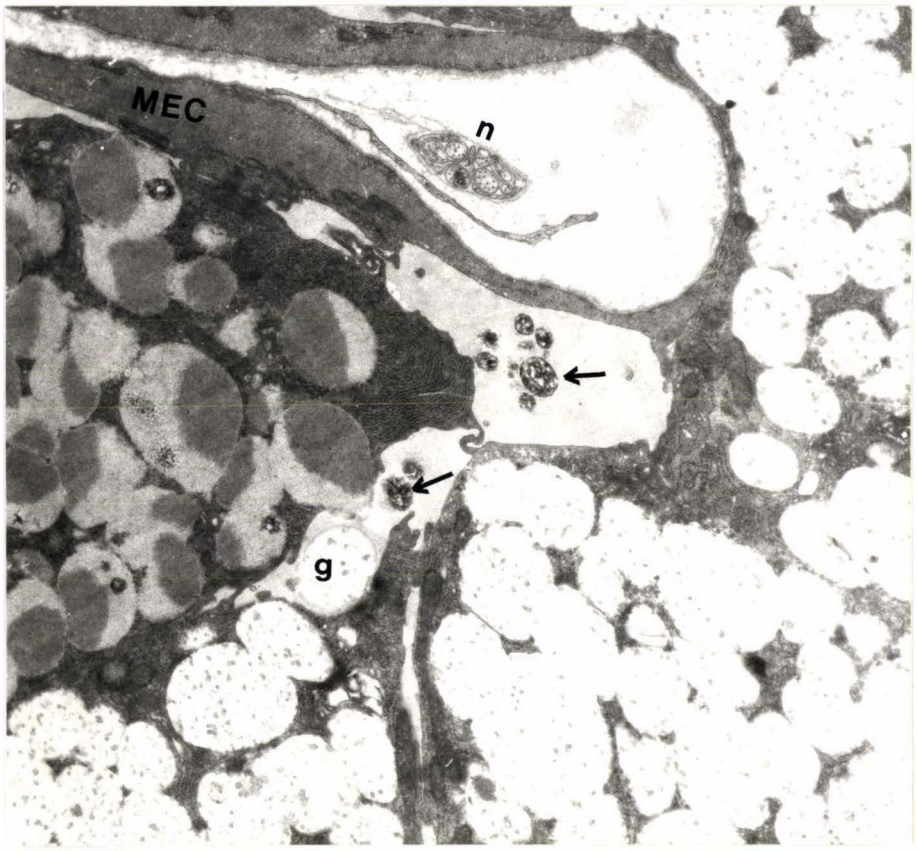


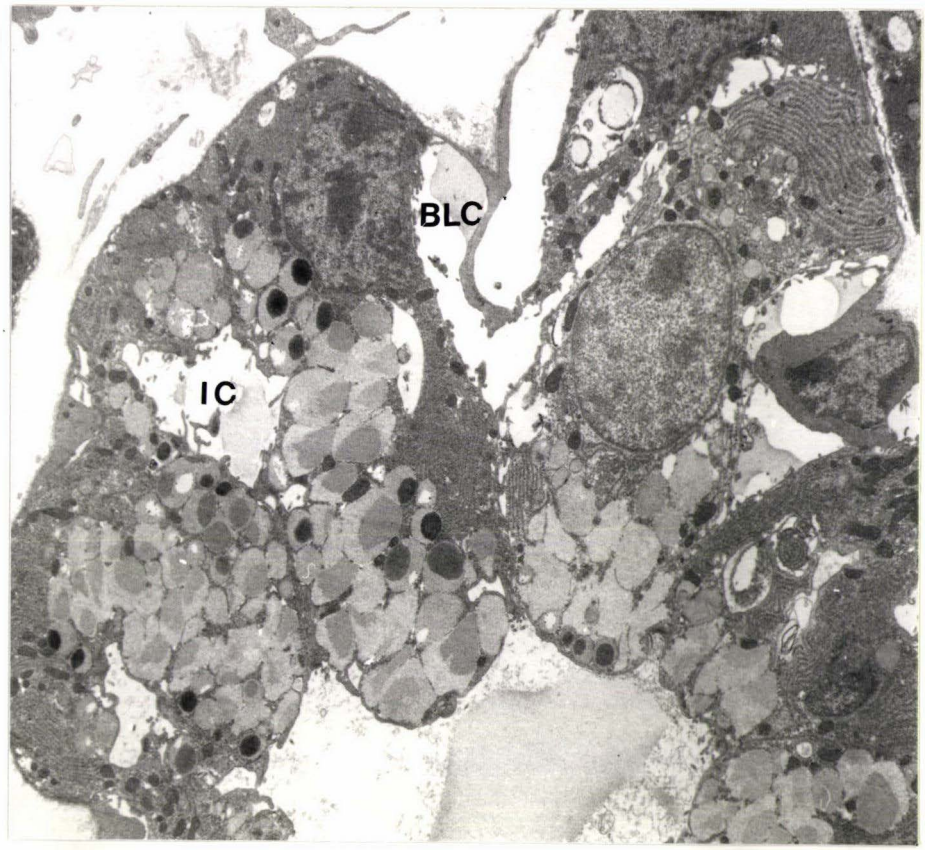
Figure 4.13: Damaged demilune cells after 2h of sympathetic nerve stimulation.

Features include the loss of what appears to be cytoplasmic organelles from demilune cells, the presence of secretory content into the basolateral canaliculi (BLC), and apparently intact basal membranes. (Other abbreviation: IC - intercellular canaliculus).

A. Araldite section. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 post-fixation; *en bloc* staining. x4,700.

B. Epon-Araldite section. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation. x8,100.

A



B

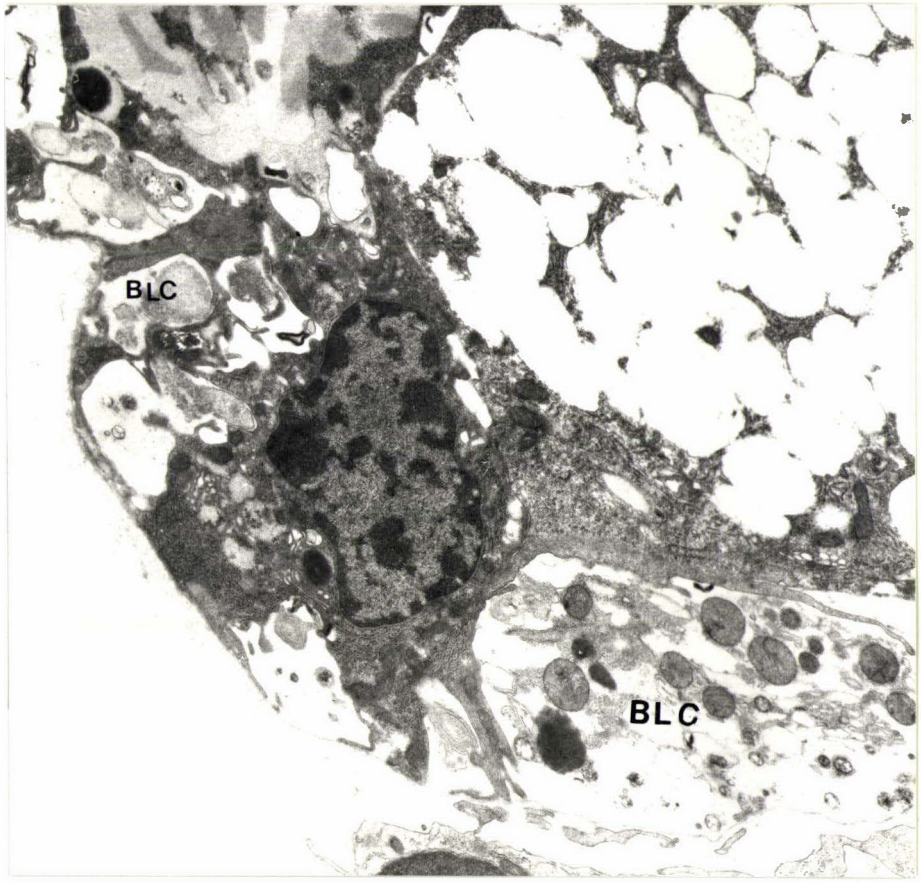


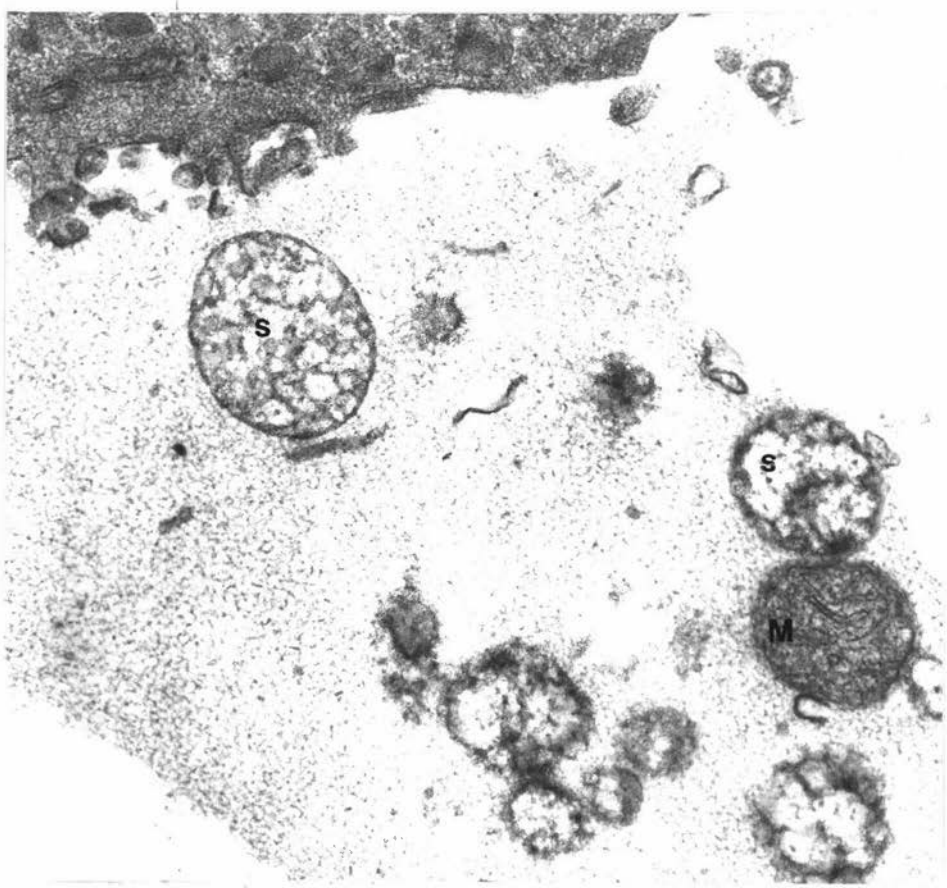
Figure 4.14: Secretion in the lumen of a striated duct following 2h sympathetic nerve stimulation.

This contains intact substructures (s) from the secretory granules of demilunes as well as other granule contents and a mitochondrion (M; see A).

A. Epon-Araldite section. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation). x46,000.

B. Araldite section. (Immersion fixation: 6.25% glutaraldehyde; OsO_4 postfixation). x25,000.

A



B

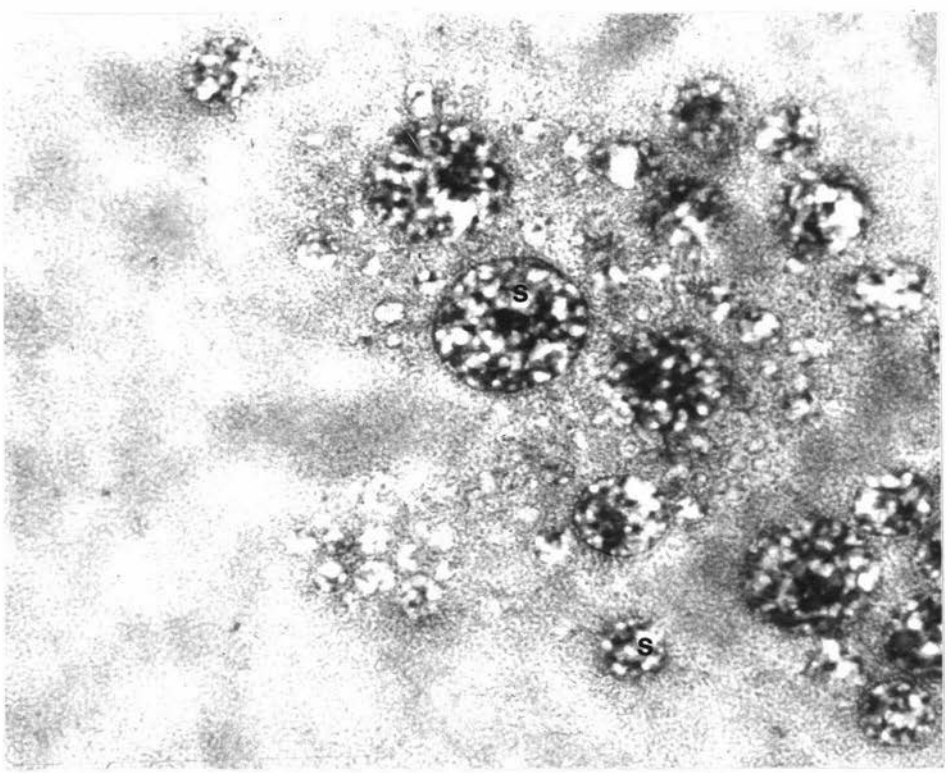
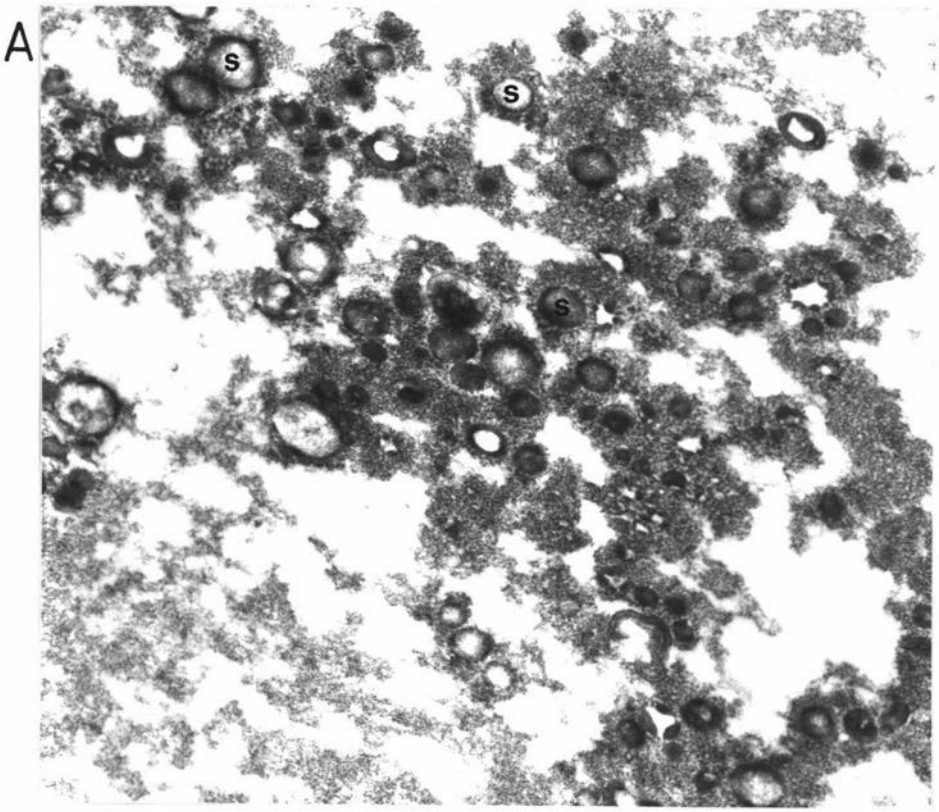


Figure 4.15: Electron micrographs of salivary sediment from sympathetically evoked secretion.

A. Showing what appear to be the substructures (s) and other components of demilune granules. x17,500.

B. Cell debris (membrane ghosts) and granule inclusions in the sediment. x25,000.


A
S
S
S

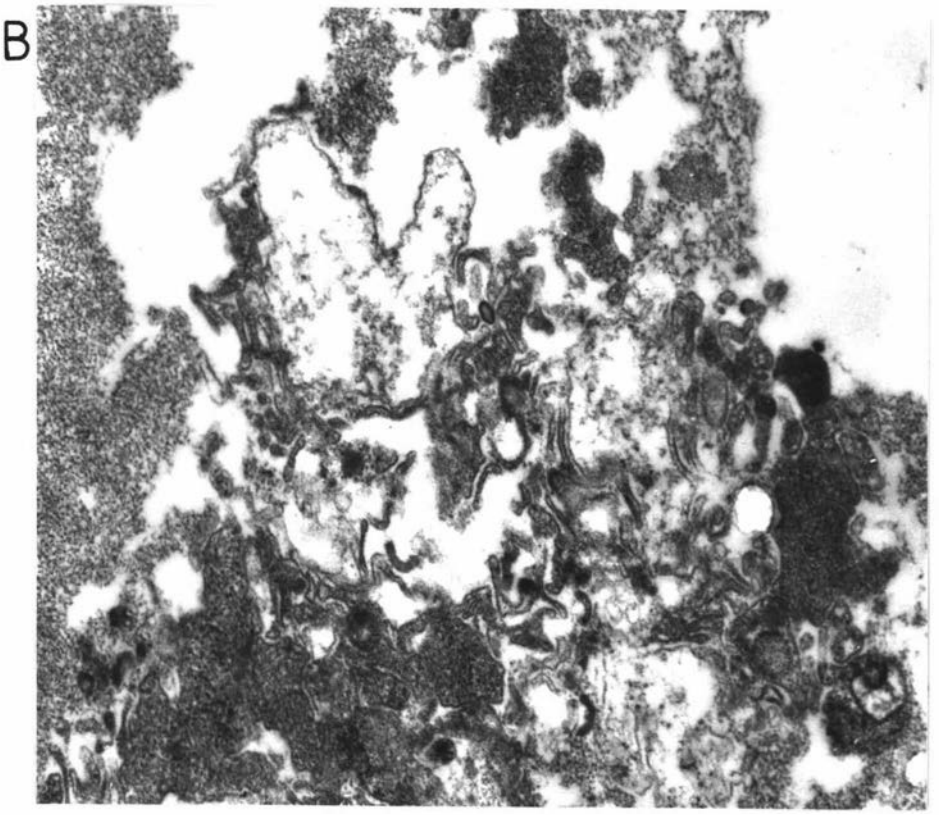

B

Figure 4.16: Electron micrographs of the basal region of striated duct cells after 2h of cervical sympathetic trunk stimulation. x17,500.

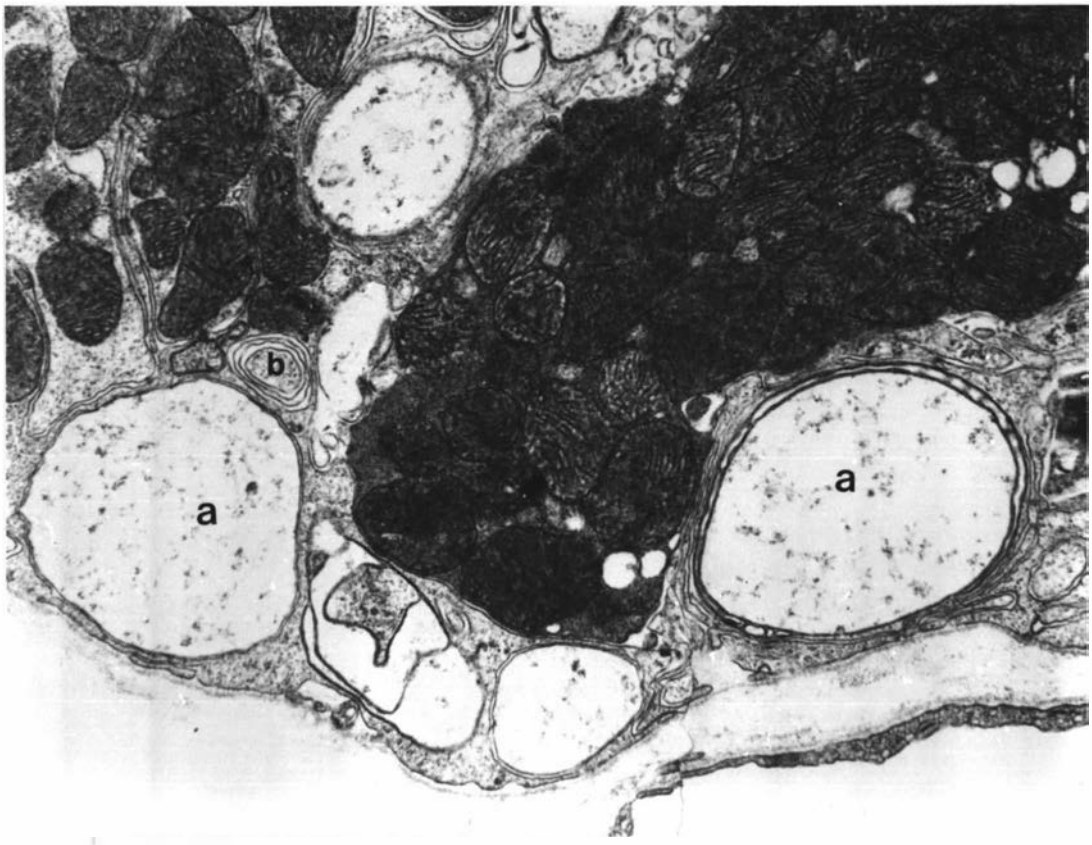
A. *Note* the large vacuoles with double or multi-membrane boundaries (as in a and b).

Araldite section. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

B. Multivesicle-like structures are indicated by the arrows.

Epon-Araldite section. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation).

A



B

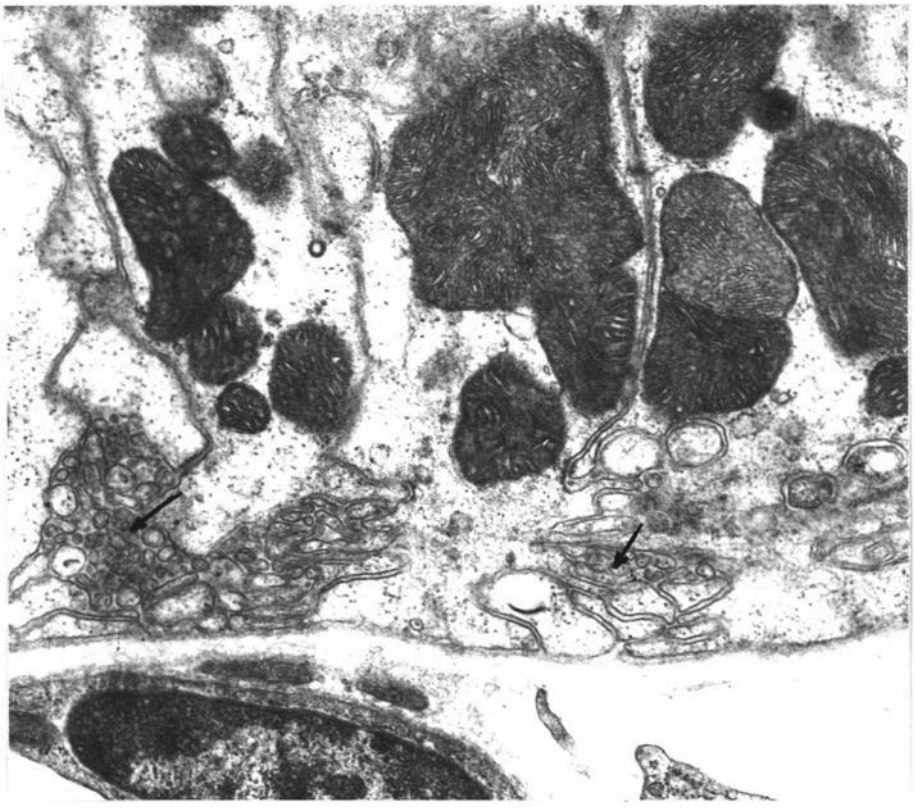


Figure 4.17: Light micrographs of 0.5 µm (toluidine blue stained) sections from the mandibular glands of two sheep after combined nerve stimulation. x980.

A. The control resting gland (nerves not stimulated).

B. Stimulated gland - 2h of sympathetic nerve stimulation on a background of chorda lingual nerve stimulation. (Same animal as in A).

C. The control resting gland (nerves not stimulated).

D. Stimulated gland - 3h of sympathetic nerve stimulation on a background of chorda evoked secretion. (Same animal as in C).

(Total mandibular secretion from B was 69.0 g in 2h of stimulation while its unstimulated counterpart A produced none; that from the gland D was 92.5 g).

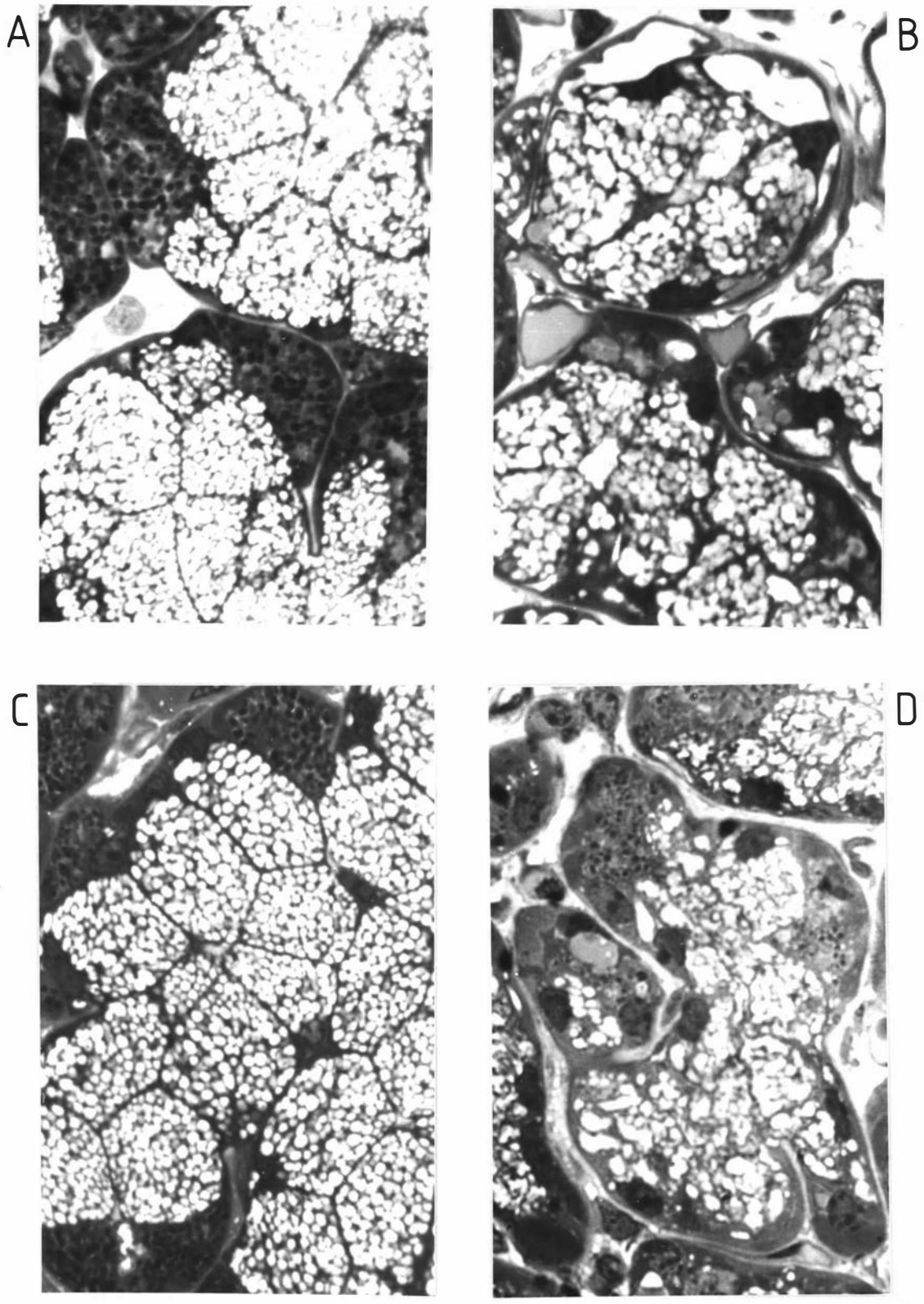

A
B
C
D

Figure 4.18: Electron micrographs showing the effects of combined nerve stimulation.

A. This shows extensive degranulation in the demilunes after 2h of sympathetic nerve stimulation and after 3h, the presence of whorl-like structures of RER (w) at the basal region of the cell and secretory granules which are presented at a higher magnification in Figure 4.18C. x8,100.

1. The control resting gland (same gland as in Figure 4.17A).
2. The contralateral gland, after 2h sympathetic nerve stimulation on a background chorda evoked secretion (same gland as in Figure 4.17B).
3. The gland after 3h sympathetic nerve stimulation on a background chorda evoked secretion. (Same gland as in Figure 4.17D).

(Other abbreviations: A - acinar cell, G - Golgi complex, IC - intercellular canaliculus, MEC - myoepithelial cell process).

A1

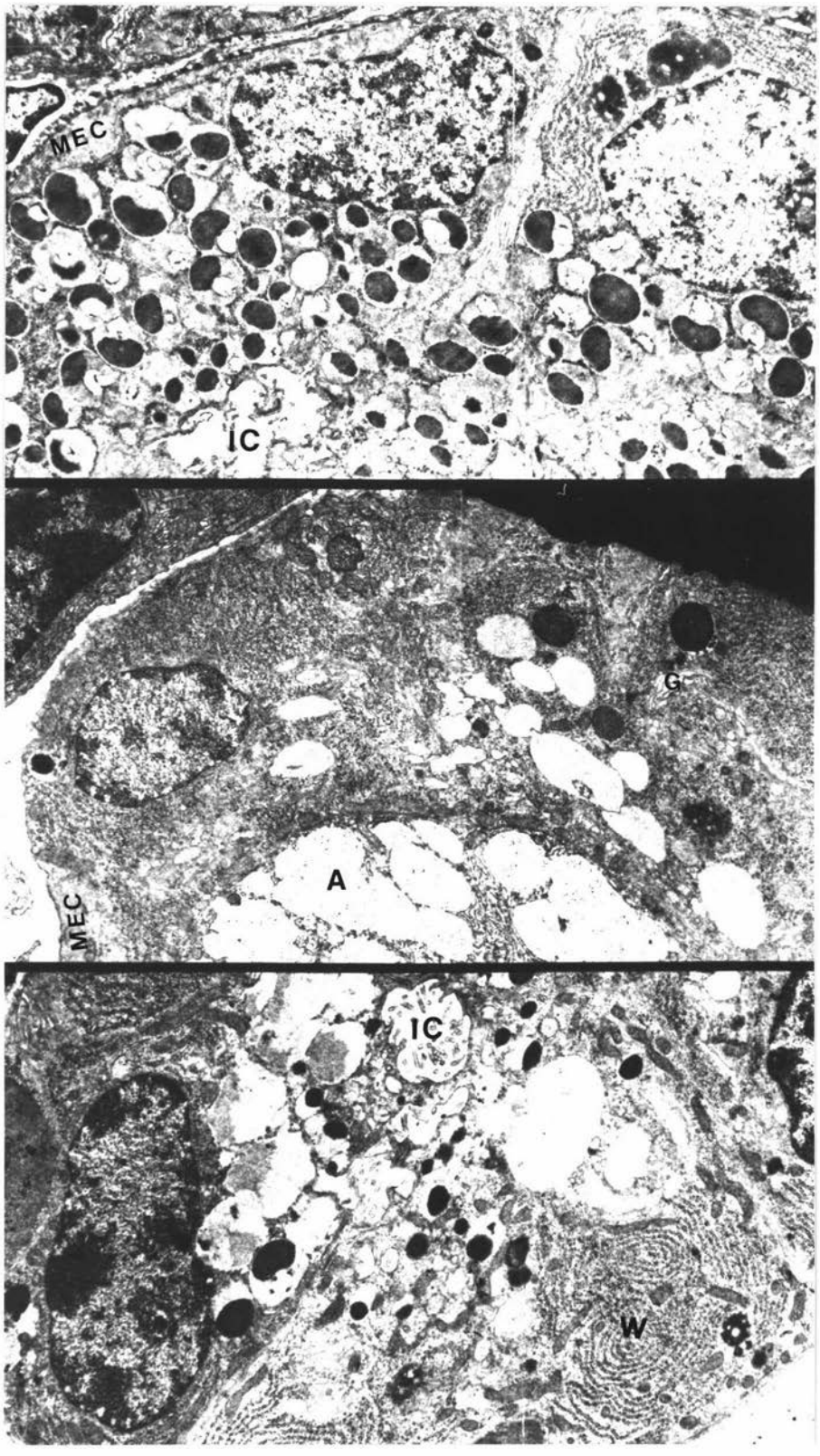


Figure 4.18:

B. Higher magnification of the basal region of a demilune cell after 2h sympathetic nerve stimulation, showing the expanded basolateral canaliculus (BLC), enlarged Golgi complexes (G) and a few small vesicles (sv). x34,000.

C-E Higher magnification of a demilune cell after 3h sympathetic nerve stimulation.

C. Newly formed granules (S) of varying size are present near the luminal surface of intercellular canaliculus (IC), and small vesicles (v) are also evident. x10,500.

D. This shows a well developed Golgi zone with dilated Golgi cisternae, many small vesicles (sv) and the condensing vacuoles (C_v) which were usually present at the inner surface of the Golgi cisternae. x34,000.

E. The Golgi zone adjacent to the luminal surface of an intercellular canaliculus. *Note* the presence of a secretory granule (probably newly formed) in association with condensing vacuoles. x17,500.

Other abbreviations: d - desmosome; M - mitochondria; MEC - myoepithelial cell process; N - nucleus and r-ribosomes.

(All Araldite sections. Perfusion fixation: half-strength Karnovsky's fixation; OsO_4 postfixation; *en bloc* staining).

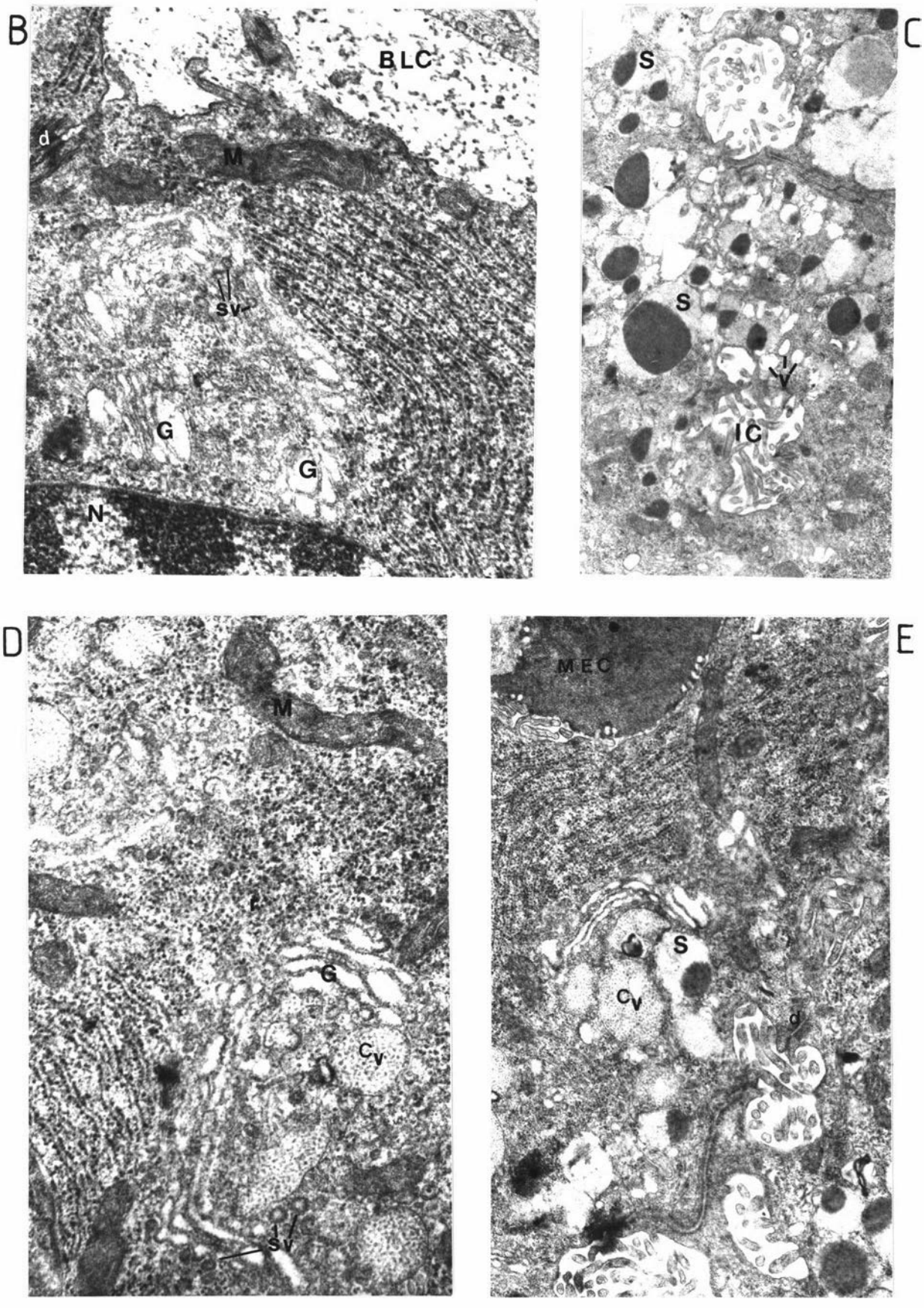

B
BLC
d
M
sv
G
G
N
C
S
S
V
IC
D
M
G
Cv
sv
E
MEC
S
Cv
d

Figure 4.19: Light micrographs showing the effects of a 2h intravenous infusion of isoprenaline (0.3 μg kg^{-1} body weight min^{-1}). x100.

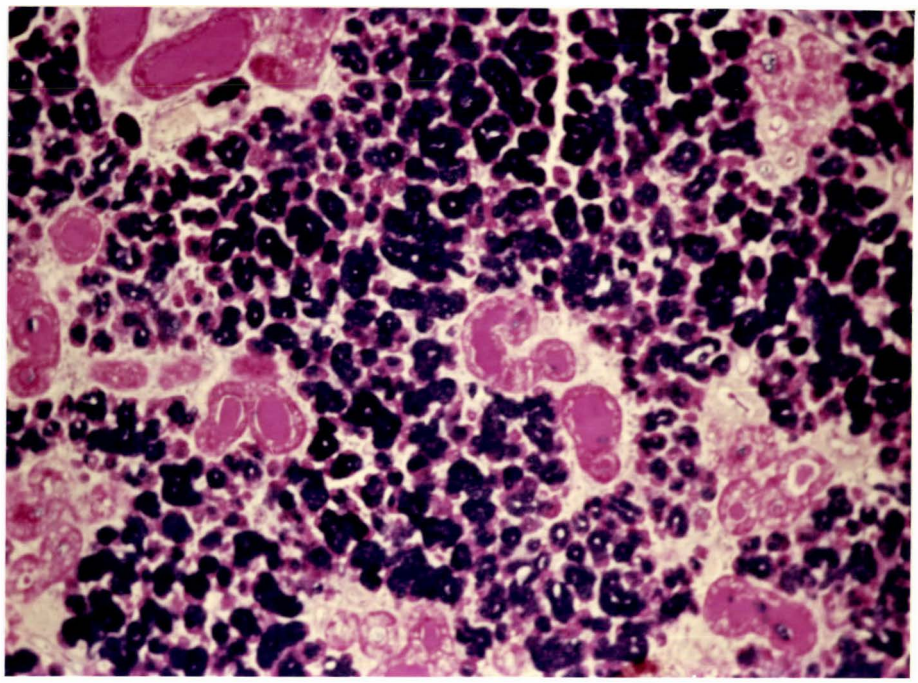
Note the secretion in the ductal lumina.

A. *Right gland*, after infusion of isoprenaline. This caused a sparse but very viscous secretion (0.37 g in 2h).

B. *Left gland*, after 2h of isoprenaline infusion during which time a background secretion was maintained by stimulation of left chorda lingual nerve. The total saliva secreted by this gland was 58.5 g in 2h.

(Paraffin sections; 10% formal saline fixative; AB/PAS/H staining).

A



B

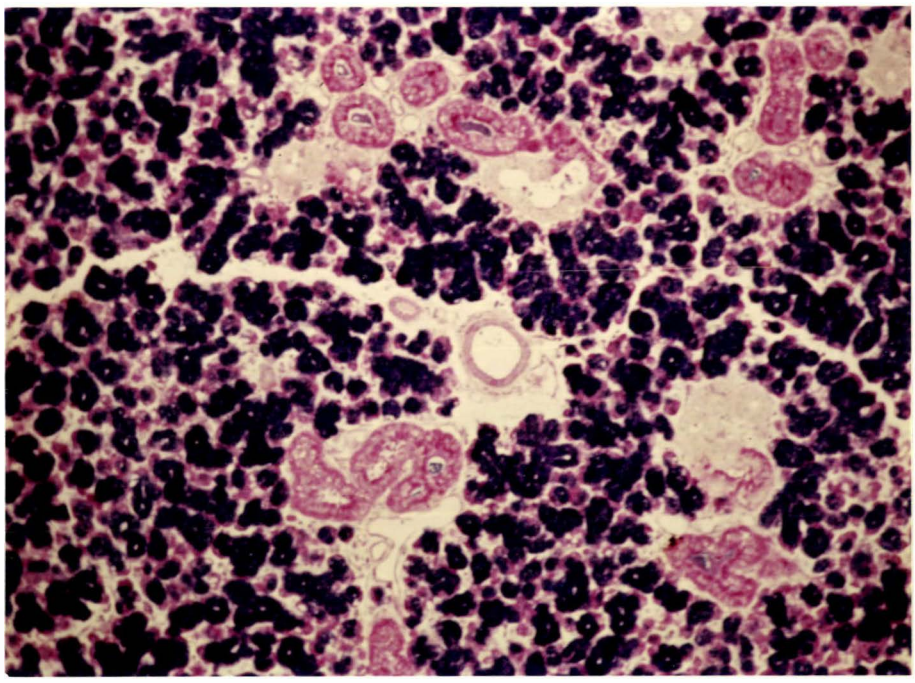


Figure 4.20: Electron micrographs obtained from the same sheep as in Figure 4.19.

(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A. Demilune cells of the right mandibular gland after infusion of isoprenaline for 2h. *Note* the presence of fused granules and also the large vacuoles (V) containing discharged granules. x10,500.

A

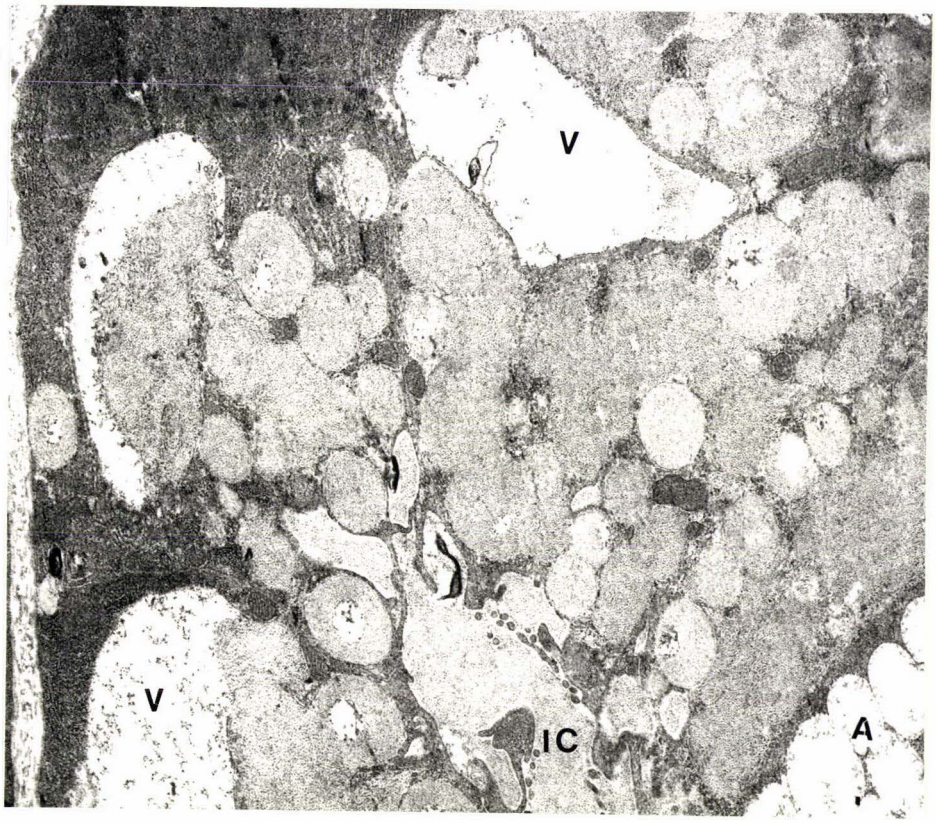


Figure 4.20:

B. A secretory endpiece of the left mandibular gland after the infusion of isoprenaline during stimulation of the left chorda lingual nerve. This shows the marked degranulation that occurred in the demilune (D), large vacuole (V) and the conspicuous intercellular canaliculi (IC), and dilated RER in the acinar cell (A). x7,900.

B

2h ISOPREN
0.3µg Kg⁻¹ min⁻¹
and
CHORDA
STIMULATION
A
RER
D
IC
V
IC
D

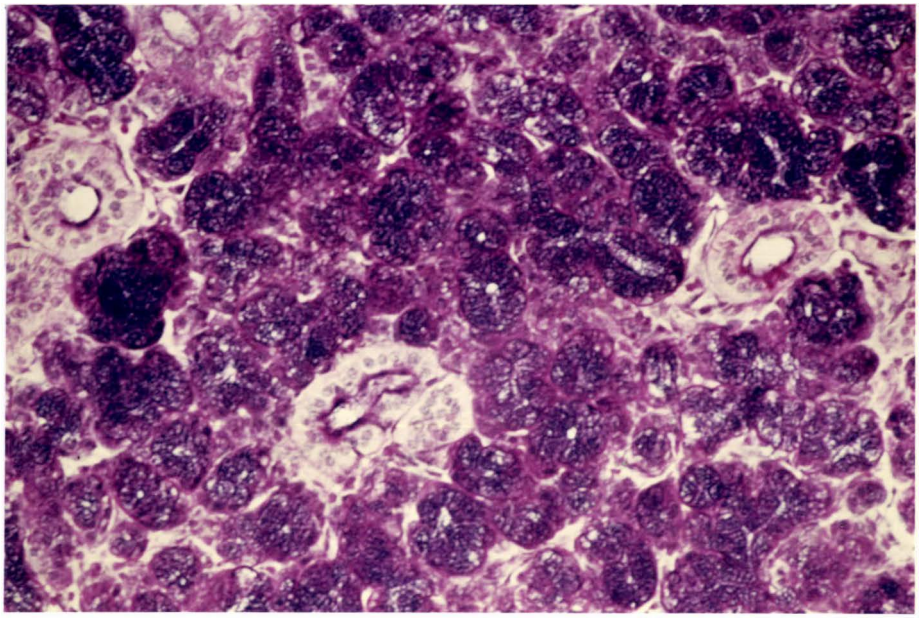
Figure 4.21: Light micrographs showing the effects of intravenous infusion of pilocarpine (5.8 µg kg^{-1} body weight min^{-1}) on the mandibular glands. x250.

A. *Right gland*. After 5h of pilocarpine infusion which caused a total secretion of 53.0 ml.

B. *Left gland*. After 5h of pilocarpine infusion plus 2h of left sympathetic nerve stimulation. (Same animal as A). A total of 49.0 ml of saliva was secreted.
Note the vacuolation in demilune regions and the damage in the basal regions of some striated duct cells (arrow).

(Paraffin sections; 10% formal saline fixative; AB/PAS/H staining).

A



B

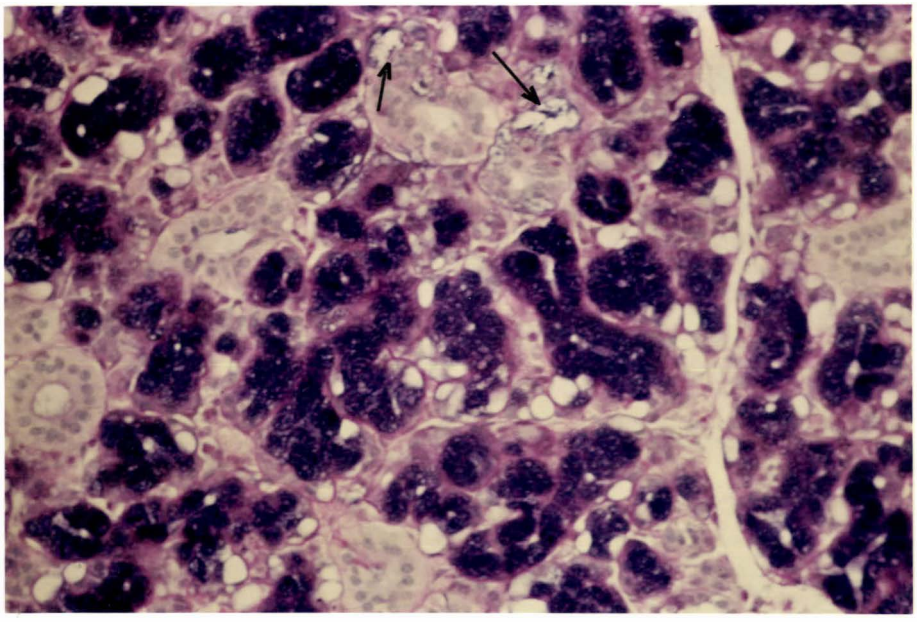


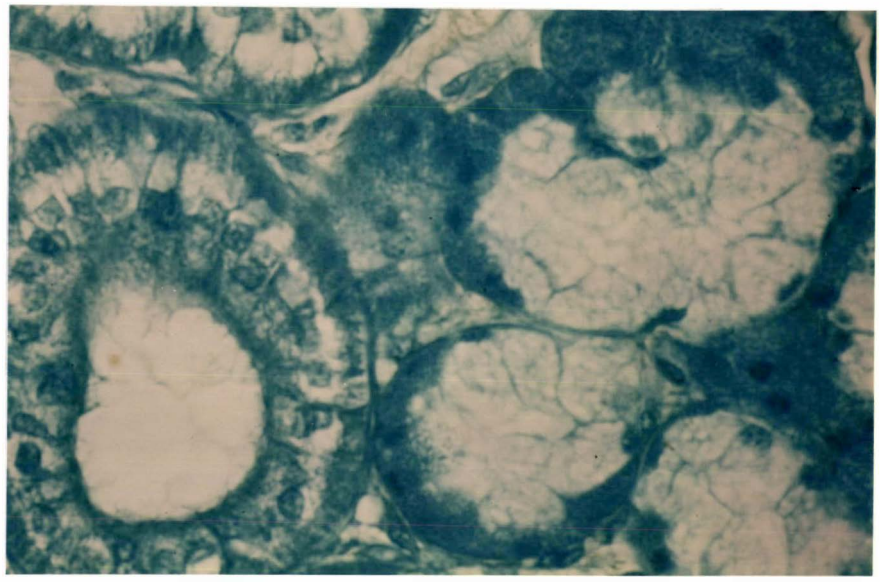
Figure 4.22: Light micrographs of a resting mandibular gland. Immunocytochemical localization of soluble salivary proteins from sympathetically evoked saliva is indicated by the presence of intense reaction products in demilune granules and some striated duct cells. (Paraffin sections fixed in Bouin's fluid; counterstained light green).

A. Control section, treated with rabbit non-immune immunoglobulin. x620.

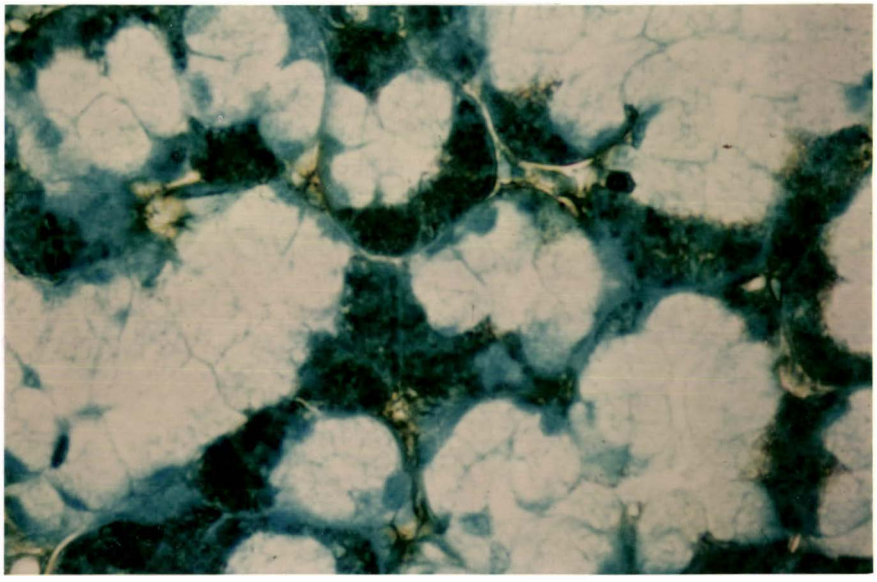
B. Section treated with rabbit immunoglobulin against sheep antigen I (1:2,000 dilution). x620.

C. Higher magnification of a striated duct (same section as in B). x1,250.

A



B



C

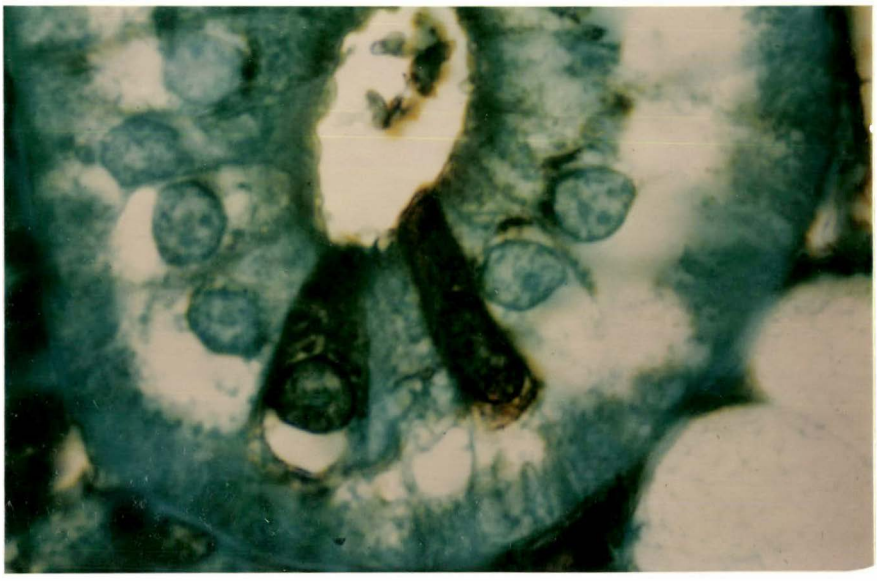


Figure 4.23: Immunocytochemical localization of insoluble salivary proteins from sympathetically evoked saliva. (Same method as Figure 4.22).

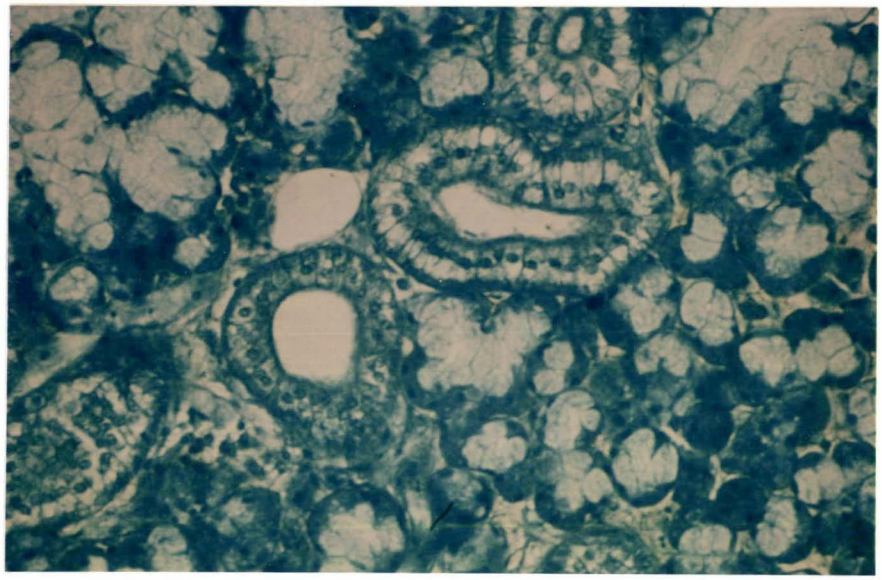
A. Control section, treated with rabbit non-immune immunoglobulin. x250.

B. Section treated with rabbit immunoglobulin against sheep antigen II (1:400 dilution). x250.

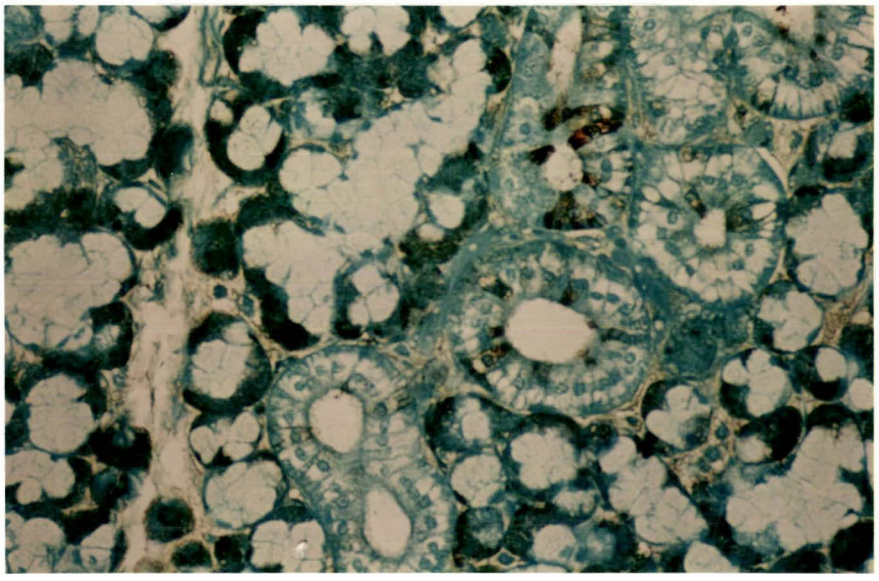
C. Higher magnification of B. x620.

Note the presence of reaction products at similar sites to those of the soluble major proteins.

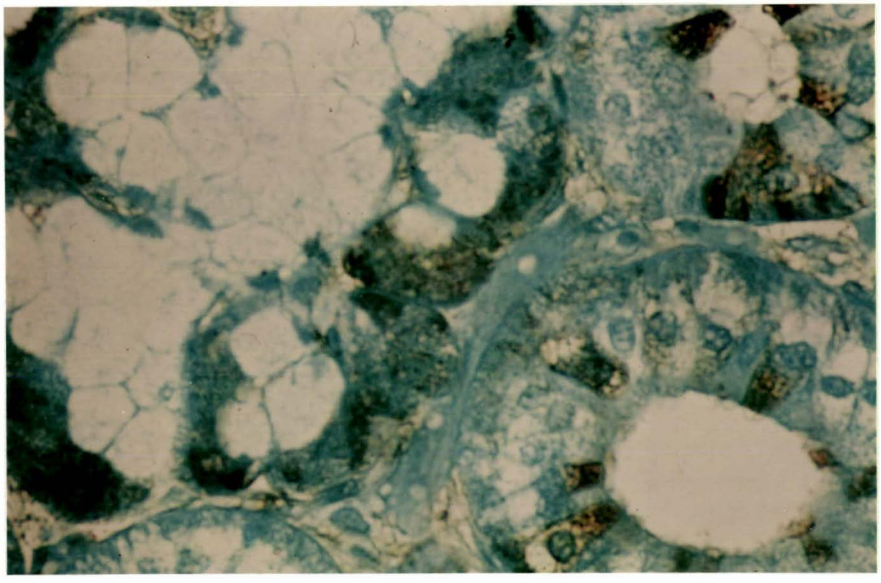
A



B



C



B. DISCUSSION

1. The Physiological Effects of Autonomic Nerve Stimulation

a. Mandibular Flow

In the present experiments, as in those of others who have worked with this gland (Kay, 1958*a*; Phillipson and Reid, 1958; Kay and Phillipson, 1959; Phillipson and Mangan, 1959) the flow of mandibular saliva in anaesthetized animals was normally slight or absent.

The production ofacopious secretion of clear saliva from the mandibular gland in response to parasympathetic stimulation also confirms earlier work. Phillipson and Mangan (1959) were able to stimulate a secretion such as is seen in Figure 4.2B in anaesthetized sheep by intravenous injection of carbachol while Patterson and Titchen (1977) achieved a similar effect with infusions of low doses of pilocarpine. Electrical stimulation of the chorda lingual (submaxillary) nerve (Kay, 1960) also results in a vigorous secretion from the ipsilateral mandibular gland which is atropine sensitive (Figure 4.2A).

This cholinergically stimulated secretion differs both in its volume of production and its physical properties from that obtained in response to sympathetic or sympathomimetic stimulation. Patterson and Titchen (1977) reported that electrical stimulation of the cervical sympathetic trunk and isoprenaline caused secretion and this was confirmed in the current study (Figure 4.3). In their experiments on anaesthetized calves Phillipson and Mangan (1959) observed the secretion of "a small quantity of extremely viscous and cloudy" mandibular saliva during inflation of the rumen while Kay and Phillipson (1959) produced a slow mandibular flow in anaesthetized sheep by distension of the lower cervical and thoracic oesophagus. It seems possible that these responses obtained by Phillipson and his co-workers arise from reflex activation of the sympathetic nerves to the mandibular gland. The susceptibility of this viscous and

cloudy secretion to the administration of propranolol (Figure 4.4: Patterson and Titchen, 1977) is consistent with it being due to activation of β-adrenoceptors in this gland.

The initial increase of flow evoked by sympathetic nerve stimulation on a background of parasympathetic activity (Figure 4.6) can be argued to have arisen through contraction of myoepithelial cells - an effect seen so clearly with the sheep parotid, in which a compensatory pause always follows a transient increase of flow (Coats *et al.*, 1956). In many salivary glands, including the sheep parotid this effect is mediated *via* the α-adrenergic innervation (Emmelin *et al.*, 1969*a*; Emmelin, Ohlin and Thulin, 1969*b*; Patterson and Titchen, 1975). The anatomical arrangement of myoepithelial cells (see Chapter III) with their cell processes embracing secretory endpieces and intercalated ducts, equips them to expel preformed saliva upon contraction. Both the light microscopic studies of the biogenic-amine and AChE activities, and electron microscopy revealed adrenergic, as well as cholinergic axons in close proximity to these cells.

The possibility of the myoepithelial cells also having a cholinergic innervation cannot be ruled out. In many experiments involving stimulation of the chorda lingual nerve (e.g. Figure 4.2A) there was an initial rapid increase of flow followed by a relatively steady but slightly lower flow. The possibility of the initial acceleration being due in part to a parasympathetically induced contraction of myoepithelial cells must also be considered as it has been demonstrated in both the cat's (Emmelin *et al.*, 1974) and the dog's (Emmelin, Gjörstrup and Thesleff, 1977*b*) mandibular glands.

An alternative interpretation of the initial acceleration of flow when sympathetic nerve stimulation was superimposed on a background of chorda lingual or pilocarpine stimulation is that it reflects a synergistic action of these two divisions of the autonomic nervous system. Such a synergism has recently been reported in other species (Emmelin and Gjörstrup, 1975; Emmelin and Gjörstrup, 1976*b*; Gjörstrup,

1977) and related to the phenomenon of augmented secretion described by Langley (1889).

The diminution of mandibular flow during continued sympathetic nerve stimulation (Figures 4.4; 4.6) probably resulted from many influences. One factor which appeared important was the vasoconstriction that has been widely reported to accompany sympathetic nerve stimulation (Langley, 1889; Emmelin 1967; 1972; Emmelin and Gjörstrup, 1976b; Gjörstrup, 1979). Evidence to support this contention arose from the partial recovery of flow following the administration of the α-adrenoceptor antagonist phentolamine (Figures 4.3D; 4.6B) and from the greater secretory responses in experiments in which intermittent stimulation of the cervical sympathetic trunk was employed (Figure 4.3C).

Another factor thought to contribute to the reduced flow was the viscosity of the saliva secreted under sympathetic stimulation. Patterson and Titchen (1977) remarked that following sympathetic nerve trunk or sympathomimetic stimulation the saliva changed from a clear viscid secretion to a stringy opalescent one. In the present study the reduction of flow from the cannula usually occurred at the time when fine white particles first became visible and intensified with the cloudiness of the secretion. This reduction was no doubt exaggerated by the reduced luminal diameter and increased resistance to flow produced by the extra length that the cannulae necessarily added to the duct system. The fact that flow progressively recovered after cessation of sympathetic stimulation, when there was a background of parasympathetically evoked flow (Figure 4.6A) and that this recovery correlated closely with reduced cloudiness and viscosity of the secretion (Figure 4.5) supports the interpretation that the low flow is due to increased viscosity.

b. Protein Secretion

In the present study (Tables 4.1 and 4.2) and that of Patterson and Titchen (1977) stimulation of the cervical sympathetic trunk and the administration of isoprenaline increased the protein concentration of mandibular saliva.

These increases in protein secretion were blocked by the β-adrenergic antagonist propranolol and thus may be concluded to be mediated by β-adrenoreceptors (Patterson and Titchen, 1977).

However, it cannot be argued that all proteins in mandibular saliva are secreted in response to β-adrenoreceptor activation because in none of the present experiments was the protein concentration reduced below 0.66 mg ml^{-1} by a β-blockade. It is of interest in this respect that Babara, Putney and Sherman (1976) found, using slices of rat parotid tissue, that amylase secretion could be stimulated *via* α-adrenoreceptors. Further, Gjörstrup (1979) has emphasized that sympathetic stimulation at frequencies subthreshold for fluid secretion (below 1 Hz for rabbit parotid glands), and in the presence of parasympathetic activity, strongly stimulated amylase secretion. Whether the low levels of protein secretion always present with cholinergic stimulation in the present experiments reflect a purely cholinergic influence or the effects of low levels of sympathetic activity or circulating catecholamines (perhaps *via* α-adrenoreceptor activation) added to cholinergic stimulation is open for conjecture. The fact that the low volumes of saliva occasionally secreted by resting glands in anaesthetized animals invariably had higher protein concentrations than secretion from their parasympathetically stimulated counterparts is evidence of a background of adrenergic stimulation. However, low protein concentrations (0.47 mg ml^{-1}) in cholinergically stimulated saliva were obtained even in the presence of both α- and β-adrenergic antagonists. This seems to support the involvement of parasympathetic activity in some protein secretion (Schneyer, 1974). Recently, Garrett and Kidd (1977) have reported that parasympathetic nerve stimulation caused a secretion of acid phosphatase from the acinar cells of cat's mandibular glands.

In the experiments reported here the protein concentration of pilocarpine stimulated saliva was always higher in secretion obtained from the normal gland than in that from

the contralateral gland after the cranial cervical ganglion on that side had been removed. This action of pilocarpine in activating postganglionic sympathetic fibres through ganglionic stimulation is well recognized (Schneyer and Hall, 1965; 1966; Goodman and Gilman, 1975).

Dische, Kahn, Rothschild, Danilchenko, Licking and Wang (1970) have reported markedly different glycoproteins are secreted in sympathetically and parasympathetically stimulated saliva. Their work was based on ultracentrifugal studies and analyses of carbohydrates. The only information available, however, on the protein composition of mandibular saliva in ruminants appears to be that of Phillipson and Mangan (1959) who reported that the secretion produced by stimulation of sympathetic nerves in the calf was rich in mucoprotein and had protein nitrogen levels up to six times greater than carbachol stimulated saliva.

The present preliminary electrophoretic studies on polyacrylamide gel revealed that at least two major soluble protein components were secreted during stimulation of the sympathetic innervation (Figure 4.7D) and that these were susceptible to the actions of propranolol. This sympathetically stimulated secretion also contained insoluble protein components (Figure 4.7E) similarly sensitive to β-adrenergic blockade. Neither the soluble nor the insoluble protein bands were present when a solely cholinergic stimulus to secretion was employed in the presence of both α- and β-antangonists.

Qualitative differences between the protein constituents of parasympathetically and sympathetically stimulated saliva have also been reported from studies on the cat's mandibular gland in which techniques of vertical disc electrophoresis were used (Kahn, Mandel, Licking, Wasserman and Morea, 1969). While they establish the secretion of distinctive protein fractions with stimulation of different divisions of the autonomic nervous system, electrophoretic studies of the type undertaken in the current experiments provide little detailed information on the proteins present. In particular the methods employed had a limited degree of resolution and the

consistent identification of minor protein bands was difficult. A more quantitative approach to this is desirable and represents an area of investigation that could be profitably developed.

2. Morphological Changes Induced by Stimulation of Autonomic Nerves

Stimulation of either the parasympathetic or sympathetic nerves to the mandibular gland, in addition to causing the differing physiological responses described, also induced cytological changes in the secretory cells. The differences in macroscopic appearance of the resting and stimulated glands, regardless of which division of the autonomic nervous system had been stimulated, seemed to relate to the presence or loss of secretory granules. As was pointed out by Langley (1898) and, emphasized more recently by Garrett and Thulin (1975), glands with densely packed secretory granules have a whitish opaque appearance grossly whereas those depleted of secretory granules become pinkish and more translucent.

a. Effects of Parasympathetic Stimulation

The acinar cells, in response to the parasympathetic nerve stimulation, showed marked exocytosis at the fine structural level (Figure 4.9) and this, together with the presence of purple-red to purple-blue materials in the ductal lumina in AB/PAS stained paraffin sections (Figure 4.8) strongly suggests that glycoproteins are secreted from the acinar cells under these conditions. This is similar to Kennerson's (1979) finding in the rat that parasympathetic stimulation evoked secretion of glycoproteins from the mucous cells of the sublingual glands. Although no measurement of the glycoprotein content in the saliva collected during various forms of autonomic nerve stimulation was made in the present study, Patterson and Titchen (1977) reported that the sheep mandibular saliva secreted in response to infusion of pilocarpine contains a considerable amount of free and bound neuraminic acids but has a low protein content.

The presence of dilated RER, small (presumably newly formed) secretory granules and well developed Golgi complexes (Figure 4.9) in acinar cells is taken to suggest that these cells are hyperactive, and engaged in synthetic activity accompanying exocytosis. A similar morphological appearance has been noted in the mucous acini at early stages of the secretory cycle both in other ruminants (Shackleford and Wilborn, 1969*b*, 1970*a*) and in humans (Testa-Riva, 1977). It is now well established that polypeptides are synthesized at the ribosomes of RER, pass into the cisternae, are transported to the Golgi apparatus, end up in the form of secretory granules, and are then stored prior to release at the cell surface (see reviews by Palade, 1975; Jamieson and Palade, 1977). Bogart (1977), using electron microscopic autoradiographic analysis, revealed that the uptake and intracellular transport of H^3-leucine for the synthesis of glycoprotein in the rat mandibular gland slices involves the same pathway as that in protein synthesis in the pancreas and other serous glands. Golgi complexes are believed to be the sites for the step by step addition of carbohydrate residues to the polypeptide core and also for the terminal glycosylation of glycoproteins (Leblond and Bennett, 1977). Thus, the morphological changes observed in acinar cells probably reflect the early stages of the synthesis of glycoprotein destined to migrate to secretory granules. Oschman, Wall and Gupta (1974) suggest that solute transport through the RER and Golgi complexes could create a stream of osmotic flow which facilitates transport of secretory (glyco) proteins and may also be involved in the condensation process. However, as Garrett, Thulin, and Kidd (1978) stressed, it is not known whether the dilation of the RER is an essential part of the resynthetic phase in the cells or just another relatively unimportant variation in cell activity.

The secretion evoked by both parasympathetic nerve and parasympathomimetic stimulation is characteristically copious and of relatively low protein content. Although the site of

primary fluid secretion in salivary glands is generally considered to be the secretory cells (Schneyer, *et al.*, 1972; Young and van Lennep, 1979), the fact that the secretory endpieces in the sheep mandibular gland consist of two different cell types, makes it difficult to decide the source of the fluid secretion. Unfortunately, there is no well-established anatomical entity which can be used as a general criterion for identifying the dynamic changes in fluid and solute transport. This deficiency may have been remedied with the recent report of Møllgard and Rostgaard (1978) who describe, with the aid of freeze fracture techniques and thick section electron microscopy, the presence of a transcellular system of tubulo-cisternal endoplasmic reticulum (TER) which appears to connect the apical and basolateral surfaces in some sodium transporting epithelia. They propose that the TER may be an entity concerned with transcellular transport of sodium in production of an isotonic fluid.

So far, the morphological evidence dealing with fluid and solute transport has been described mainly in relation to changes in the intercellular route (*via* lateral intercellular spaces and the tight junctions) where a standing gradient flow system could be established (see Oschman *et al.*, 1974). Distension of lateral intercellular spaces after fluid transport has been observed in epithelia, e.g. rabbit gall-bladder, reptile renal tubules and insect rectum (Oschman *et al.*, 1974), as well as between adjacent acinar cells in the rat mandibular gland 30 minutes after intraperitoneal injection of isoprenaline (20 mg kg^{-1}; Bogart, 1975). Blomfield *et al.* (1980) have also reported that fluid secretion evoked *in vitro* by acetylcholine caused distension of intercellular spaces between acinar cells and expanded their basolateral membranes. However, in the rat parotid gland, Garrett and Thulin (1975) were unable to identify changes in the morphology of the acinar cells in response to parasympathetic nerve stimulation despite the copious secretion of a fluid low in amylase. In the present study several intercellular changes appeared to correlate with the

fluid secretion induced by parasympathetic nerve stimulation. These included the presence of dilated basolateral and lateral intercellular spaces between demilune cells and between adjacent demilune and acinar cells, expanded basolateral canaliculi, and wide intercellular canaliculi among demilunes. (The distension of basolateral canaliculi is likely to reflect an enhanced fluid transport from blood into the secretory endpieces). In contrast, no changes in lateral intercellular spaces between adjacent acinar cells were observed. Thus, although not established conclusively, the involvement of both acini and demilunes in fluid secretion appears likely.

The phenomenon of exocytosis by mucous cells in salivary glands has been demonstrated in many species (Tandler, Denning, Mandel and Kutscher, 1969; Kim, Nasjleti and Ham, 1972; Tandler and Poulsen, 1976; Testa-Riva, 1977) although with two different modes of secretion. The present study has shown that in the sheep mandibular gland, the discharge of secretory granules from the mucous acini follows a typical exocytotic pattern. As is evident in Figure 4.9A, the content of the secretory granules was liberated, *via* the gaps in the fused granule membrane and luminal membrane, into the central acinar lumen.

A detailed study of mucous cell secretion has been described by Tandler and Poulsen (1976) in the cat mandibular gland. They revealed that a 5-layered contact is formed when the limiting membrane of mucous granule fuses with the luminal plasma membrane and subsequently converted to a 3-layered membrane by avulsion of the plasmalemma. Subsequent attenuation and rupture of this membrane barrier permits the contents of the mucous granules to flow into the lumen. The avulsed plasmalemmae possibly appear in the lumen as membrane ghosts such as were seen in Figure 4.9A where these membrane ghosts are present together with secretory materials and dense bodies.

The mode of mucous cell secretion in sheep mandibular glands thus differs from that described in the rat sublingual

gland (Kim *et al.*, 1972) and the human submandibular (Testa-Riva, 1977) and labial glands (Tandler *et al.*, 1969) where secretory granules with intact membranes are discharged from the acinar cells along with membrane fragments.

b. Effects of Sympathetic Stimulation

The discharge of secretory granules from the demilunes in response to sympathetic nerve stimulation resembled that described in the rat parotid acinar cells (Amsterdam, Ohad and Schramm 1969; Simson, 1969) and the rat mandibular acinar cells (Bogart, 1975), after the administration of isoprenaline. There was similar scalloping of intercellular canaliculi perhaps formed by granule membranes remaining with the luminal plasma membranes following exocytosis. However, the invaginations of intercellular canaliculi observed after sympathetic nerve stimulation in the present study seemed to be less conspicuous than those seen in sheep (present study) and reported in the rat after the infusion of isoprenaline. It is not possible to meaningfully compare the degrees of exocytosis because of differences in the species, types and extent of stimulation. In the present study stimulation of the cervical sympathetic trunk would have excited both α and β-adrenoreceptors, but only a β-adrenergic agonist was given in the experiments with rats.

The irregularly fused granules with electron-pale contents frequently observed within the demilunes after the stimulation of sympathetic nerves (Figure 4.12AB) or the infusion of isoprenaline (Figure 4.20A) may be secretory granules that have already liberated their secretory content (including the characteristic substructures) by chain exocytosis (Young and van Lennep 1978). This possibility is supported by the occasional finding of fused rows of secretory granules in connection with the intercellular canaliculus.

The extrusion of demilune granules, induced either by sympathetic nerve stimulation or by infusion of the β-adrenergic agonist isoprenaline, occurred at the time high protein concentrations were detected in the saliva. This correlation

of morphological and physiological events in the present study, together with Patterson and Titchen's (1977) findings, suggests that sympathetic nerve stimulation induces the secretion of proteins and glycoproteins *via* the secretory granules of demilunes.

The immunocytochemical findings in the present study confirm that the major soluble salivary proteins and those of the salivary sediment secreted under the influence of the sympathetic innervation are localized predominantly in the demilunes of resting glands (Figures 4.22; 4.23) with little or none present in the acinar cells.

The occasional presence in lumina of cytoplasmic ghosts and cellular organelles (probably from the damaged demilune cells) after sympathetic nerve stimulation is taken to indicate an ability of secretory cells to excrete their unwanted waste materials directly into the saliva. A similar excretion of redundant cytoplasmic structures has been reported in other species following sympathetic stimulation (Garrett *et al.*, 1977).

The presence of the granular content of demilunes in an enlarged basolateral canaliculus after sympathetic nerve stimulation, in otherwise normal secretory endpieces (Figure 4.12C) suggests that extrusion of secretory products might have occurred through ruptures in the basolateral plasma membrane. Several factors may be involved in the rupture of basolateral plasma membranes. Firstly, as argued by Simson (1972), adrenergic stimulation through its effects on membrane permeability occasionally results in foci of cell membrane disruption. Secondly, both the secretion of highly viscous saliva and contractions of myoepithelial cells during the sympathetic nerve stimulation may result in a great increase in the intraglandular pressure, probably ranging from the rupture of basolateral membranes in normal demilune cells (Figure 4.12C) to severe intracellular damage (Figure 4.13). The occasional presence of an intact acinar granule in the basolateral canaliculus (Figure 4.12C) may also be accounted for by rupture of the basolateral plasma membrane.

More general changes in demilune cells after sympathetic nerve stimulation (Figure 4.13) can also be related to the cell damage reported by Simson (1972) in the acinar cells of the rat's mandibular and parotid glands after injection of gross amounts of isoprenaline (5 mg, ip). His own observations suggested that marked cytoplasmic disruption was produced by high doses of isoprenaline but that very few of the altered cells were irreversibly damaged (Simson, 1972). It seems likely that rupture of secretory cells is a relatively normal event in the gland in response to sympathetic stimulation.

Another ultrastructural feature found in demilune cells was the presence of large cytoplasmic vacuoles (Figures 4.12B; 4.20; 4.21B). Vacuolation in the demilunes after adrenergic stimulation was reported by Rawlinson (1933) in the cat mandibular gland, and it is also present in acinar cells of rat mandibular and parotid glands (Tapp, 1968; Batzri, Amsterdam, Selinger, Ohad and Schramm, 1971). Schramm and Selinger (1974) have further shown that vacuole formation can be induced in rat acinar cells by activation of either α-adrenergic or cholinergic receptors and have argued that it is related to a rapid release of potassium ions. In the present study the presence of these vacuoles was not uniform: they were observed only occasionally after sympathetic stimulation alone (Figures 4.12B; 4.20A) but more frequently after both the sympathetic and parasympathetic nervous systems were involved e.g. in the demilunes after 2h sympathetic nerve stimulation during a 5h infusion of pilocarpine (Figure 4.21B). These findings in many ways resemble those of Garrett *et al.* (1978) who proposed that the presence of vacuolation depends on the pre-existing metabolic state of the acinar cells at the time of stimulation. These authors found that variable sized vacuoles occurred extensively and consistently in the parasympathetically stimulated acini that had been degranulated previously (either by performing bilateral superior cervical ganglion-ectomy, or by isoprenaline treatment) but that parasympathetic nerve stimulation alone did not produce vacuolation.

Vacuolation may be part of the secretory process (Tapp and Trowell, 1967; Fleming, Teitelman and Sturgess, 1980). It has been suggested to arise because of a temporary imbalance between the uptake and output of water by the cells (Tapp and Trowell, 1967; Tapp, 1968, 1969). Perhaps concurrent stimulation of both types of autonomic nerves has a synergistic action which enhances degranulation of demilunes, alters the metabolic state of the cells and enhances the movement of water and salts across the cell membrane. The vacuoles may then develop by segregation of fluid from the oedematous cytoplasm as Tapp (1969) proposed. On the other hand, Fleming *et al.* (1980) argued from a study of dissociated acini of rat mandibular glands treated with isoprenaline or carbachol that large vacuoles may result from the fusion of secretory granules as a part of the process of exocytosis. This seems to be consistent with the changes depicted in Figure 4.20A.

Based on the fine structure of the secretory endpieces in the sheep mandibular gland, it appeared in the present study that the changes induced in the demilunes after dual nerve stimulation were more marked than those observed after sympathetic nerve stimulation alone. The presence of abundant ribosomes, mitochondria and dilated Golgi membranes after 2 hours of the combined nerve stimulation (Figure 4.18A,B) is compatible with the involvement of demilunes in the early stage of protein synthesis. Further structural evidence of such an involvement in protein synthesis - the formation of condensing vacuoles and newly formed granules, was observed after 3 hours of sympathetic nerve stimulation added to a background of parasympathetic secretion (Figure 4.18C-E). The presence at the basal region of these cells of whorl-like structures composed of concentric arrays of RER was believed by Scott and Pease (1964) to be closely related to increased glandular activity. Whorl-like structures have been found in rat's acinar cells under conditions of hyperactivity, i.e. after refeeding, and pilocarpine injection, but are not present under normal conditions, or conditions of hypoactivity, such as

accompany starvation or atropine administration (Scott and Pease, 1964). They suggested that these structures resembled "ergastoplasmic Nebenkern" seen with light microscopy and resulted from the apposition of ergastoplasmic lamellae.

In the present study the size of the intercellular canaliculi in glands receiving stimulation of both the sympathetic and parasympathetic systems appeared to be reduced when compared with glands subjected to sympathetic stimulation alone, and this reduction was accompanied by the appearance of small smooth vesicles at luminal regions and adjacent to the dilated Golgi complexes (Figure 4.18C-E). These observations may be accounted for by the retrieval of membranes from the luminal surface which Amsterdam *et al*. (1969) postulated to occur. Such retrieved membranes, it is argued, may be eventually utilized by the Golgi system (Amsterdam *et al*. 1969; Herzog and Farquhar, 1977). No attempt at systematic observations on the secretory cycles of membranes was made in the present study.

The general depletion of PAS-positive materials in striated duct cells after sympathetic stimulation probably reflects a contribution of these cells to secretion of neutral glycoproteins. Although no related cellular structures were identified at electron microscopic level (as discussed in chapter III), immunocytochemical investigations indicated that the striated duct cells did contain proteins which were produced in sympathetically evoked saliva (Figures 4.22; 4.23).

Many of the morphological changes arising in striated duct cells during sympathetic stimulation can be regarded as being a consequence of the physical properties of the secretion that is produced. This saliva is typically thick and viscous. Damage to striated duct cells has been reported in the dog's mandibular gland (Emmelin *et al*., 1977*a*) under conditions of increased intraluminal pressure and a high resistance to flow. The changes noted in the present study (Figures 4.11; 4.16) closely resemble the ballooning disruption of striated duct cells (in which fluid was accumulated intercellularly) and

the intracellular vesiculation described by Emmelin *et al.* (1977*a*). Alcian Blue and PAS-staining (Figure 4.11) demonstrated the presence of both AB and PAS positive materials, perhaps secretion, in the disrupted cells as well as in the ductal lumina. The multivesicles present in electron microscopic studies (Figure 4.16B) are interpreted to arise from the breakdown of basal plasma membrane folds and the large vacuoles with double or multi-membrane boundaries (Figure 4.16A) to be the greatly swollen basal processes of neighbouring striated duct cells. Such intracellular and intercellular disruptions are probably exacerbated by the contraction of myoepithelial cells that apparently occurs during sympathetic stimulation. The contraction of these cells, embracing as they do the secretory endpieces and intercalated ducts (see Figures 3.4A; 3.11A), would be expected to place the striated duct under additional pressure and might additionally explain why the disruption with sympathetic stimulation is confined mainly to the striated duct cells. Secretory endpiece cells, like those of the intercalated ducts, would be protected by their basket cells.

An argument along similar lines can be advanced to account for the less frequent disruption of striated duct cells that occurs if sympathetic stimulation is added to a background of chorda lingual nerve activity. Under these conditions it would be expected that a lesser resistance to flow would be associated with the less viscous saliva produced. It is pertinent to note that administration of the β-adrenergic agonist isoprenaline alone did not result in damage to striated duct cells and neither was there damage in the single experiment in which sympathetic nerve stimulation was performed after administration of the α-adrenergic antagonist phentolamine. This lack of intercellular disruption in circumstances where it would otherwise have been predicted provides additional support for the idea that myoepithelial cells function *via* an α-adrenergic innervation. In this latter experiment the lumina of intercalated ducts were widely dilated with secretion as were the basolateral

canaliculi of demilunes, and damage to demilune cells appeared unusually frequent and severe. This suggests that in the absence of myoepithelial contraction the flow of viscous saliva evoked by sympathetic nerve stimulation was greatly reduced, although its continuing secretion caused marked increases in the pressure in endpieces and induced cell rupture within these.

CHAPTER V

OBSERVATIONS IN CONSCIOUS SHEEP: EFFECTS OF FEEDING

A. RESULTS

The results obtained in this section of investigation are divided into two parts. The first part is concerned with physiological responses of the sheep mandibular glands to feeding and the second with the morphological changes that resulted.

1. Physiological Effects

Measurements of salivary flow, salivary protein concentration and protein output were studied in seven animals with chronic, unilateral, cannulations of a mandibular duct. (Hereafter the gland whose duct was cannulated is referred to as the 'cannulated gland' and the contralateral one as the 'uncannulated gland'). Six of the sheep showed no structural differences between the left cannulated and the right uncannulated glands when these were subsequently examined in stained paraffin sections. The remaining sheep developed a partial occlusion of the cannula which resulted in reduced mandibular flow and some structural changes in the gland (described later).

The mean weight of the cannulated glands from the six animals as measured at the end of experiments was 11.19 $\pm$ 1.15 g wet weight ($\bar{X} \pm$ SE) which did not differ significantly ($p < 0.01$) from the contralateral uncannulated ones (12.17 $\pm$ 1.00 g). The difference in gland weight between individual right and left glands was 0.99 $\pm$ 0.26 g.

a. Mandibular Flow

(i) Resting Secretion

During the 20 hours when food was not available, a variable amount of mandibular saliva was secreted (0.6-53.1 g

in 18h, n=52). The average resting flow was 0.95 ± 0.09 g h^{-1} ($\bar{X}$ ± SE) which contributed some 7.6 ± 1.4% of that secreted during eating. No mandibular secretion was observed during rumination. Marked mandibular flow occurred, however, when the animals drank water and during activities, such as licking of their lips, and foot chewing or licking of the metabolism crates (Figure 5.1).

(ii) Mandibular Flow in Anticipation of Feeding

The arrival of the usual attendant responsible for feeding, the sound of his voice and of food being prepared, or the sight of sheep in adjacent crates feeding, all evoked mandibular secretion.

Ten experiments were undertaken on four sheep to study psychic influences on mandibular secretion. When the sheep were teased they showed behavioural changes - stretching out and attempting to reach the food, occasional licking and nibbling of the crates, and foot stamping (in one sheep). This was accompanied in all of the animals by the flow of mandibular secretion. Such behaviour disappeared after several unsuccessful attempts at obtaining food even though the teasing procedure continued. Figures 5.2A and 5.3A show that mandibular secretion appeared as soon as the food was given to other sheep and includes the brief augmentation of flow that usually occurred when teasing was performed in front of the subject under observation. Typically, secretory responses to teasing became weaker if the experiment was repeated on succeeding days.

To determine whether the secretion in response to teasing involved cholinergic stimulation, atropine was given intravenously to the four sheep 10-15 minutes prior to the normal feeding time in the same dosage (0.1 mg kg^{-1}) that produced a complete blockade of mandibular flow evoked by electrical stimulation of the chorda lingual nerve in acute experiments. After atropine, all sheep showed signs of drying of the

mouth - this included lip licking and slight dysphagia. One of the animals in addition nibbled at its crate and feet and this was accompanied by a slow flow of mandibular secretion which lasted about 15 minutes (Figure 5.3C). In the three others there was no mandibular flow (Figures 5.2B; 5.3B) - as was the case before atropine administration.

In two of the animals secretory responses to teasing remained after pretreatment with atropine although they were weaker than those obtained in the absence of atropine (Figures 5.2B; 5.3C). Such secretion was absent after treatment with both atropine (0.1 mg kg^{-1}) and propranolol (1.0 mg kg^{-1}; Figure 5.2C). In the remaining two sheep, teasing responses were abolished by atropine alone (Figure 5.3B).

(iii) Mandibular Flow during Feeding

Immediately fresh food was given a substantial mandibular flow commenced (Figures 5.2; 5.3) and it ceased almost as soon as eating stopped. The flow rate during eating was up to 2.85 g min^{-1} while the total mandibular secretion during the 4 hours food was available was 256.90 $\pm$ 13.47 g ($\bar{X} \pm$ SE, n=52).

The patterns of mandibular flow during eating were examined in the six sheep and are presented in Figures 5.4-5.6. The flow rate was always highest at the initial 10 or 20 minutes of feeding (1.90 $\pm$ 0.14 and 1.70 $\pm$ 0.10 g min^{-1}; $\bar{X} \pm$ SE, n=18) and then gradually declined as feeding continued, so that after the first and second hours of feeding flows were 1.26 $\pm$ 0.11 and 0.89 $\pm$ 0.09 g min^{-1}, respectively. Typically the rate of eating also decreased steadily, e.g. 468.6 $\pm$ 22.9 g , 220.7 $\pm$ 15.7 g and 280.5 $\pm$ 20.6 g for the first, the second, and the last two hours, respectively (n=32).

After the first 30 minutes of eating the rates of mandibular flow showed much greater variability. By this time, interruptions of eating often occurred as the animals were more sensitive to environmental disturbances that arose,

for example, when someone entered the animal room, conversed, and to noises outside the room. These pauses in eating resulted in pauses in secretion. Also at this time the eating behaviour of the individual animals changed, perhaps as they became more replete. Some sheep, after eating almost continuously for 30-90 minutes tended to stop from time to time while others only paused after most of the food was consumed.

Further, variations in flow during eating were present on different days even in individual animals and a brief period of rumination was occasionally interspersed late in the feeding periods. Rumination did not stimulate mandibular secretion.

The rate of mandibular secretion during feeding was not affected by propranolol (Figure 5.4), either with respect to average flow or with respect to the shape of the response in time (see Appendix 5), but in contrast, the flow rate was reduced significantly after atropine had been injected. All of the sheep, however, had substantial secretory responses to feeding after the intravenous administration of atropine at 0.1 mg kg^{-1}.

b. Protein Secretion

(i) Resting Secretion

The protein concentration of mandibular saliva collected during periods when food was not available varied from 0.67-3.56 mg min^{-1} (n=45) with a mean concentration of 1.72 $\pm$ 0.13 mg ml^{-1}.

(ii) In Anticipation of Feeding

Saliva with a higher protein concentration was secreted in response to teasing (2.73 $\pm$ 0.20 mg ml^{-1}, n=8).

(iii) During Feeding

Saliva produced during feeding had its highest protein concentrations as soon as the eating began (1.65 $\pm$ 0.06 mg ml^{-1}, n=18), remained high with a slight diminution during the first hour (1.55 $\pm$ 0.06 mg ml^{-1}) and fell thereafter,

e.g. 1.37 mg ml^{-1} at 60 minutes of eating (Figure 5.4).

The mean changes in protein concentration, mandibular flow and protein output are presented in Figures 5.4-5.6, while variations of protein concentrations among individual animals are shown in Figure 5.5 and Figure 5.6. In three of the animals, a maximal concentration of proteins was secreted within the first 2-8 minutes of feeding and then declined gradually, although the levels remained relatively high during the first hour of eating (Figure 5.5). In other sheep (Figure 5.6), the saliva produced during eating did not show any peak protein concentration but had a variable range of high protein levels throughout the first and second hours of the feeding period.

Fluctuations in protein concentrations were small during the first 30 minutes of feeding but became more extensive as eating continued. These variations were associated temporally with the fluctuation of mandibular flow.

(iv) Effects of the β-Blocker Propranolol on Protein Secretion during Feeding

Propranolol in a dosage of 1.0 mg kg^{-1} (iv) caused a partial reduction of the protein secretion in saliva during feeding, without significantly changing the flow rates (Figure 5.4). A reduction of protein output, ranging from 24.6% - 70.7%, was observed in six sheep in the first 20 minutes of eating. This supports the suggestion that the secretion of salivary proteins is mediated at least in part *via* a β-adrenergic mechanism.

(v) Effects of Atropine on Feeding Secretion

Feeding after the administration of atropine (0.1 mg kg^{-1}) resulted in a marked decrease in mandibular flow with reciprocal changes in its protein concentration (e.g. an increase up to 5.36 mg ml^{-1}). The protein output after atropine was affected by the salivary flow rate. Taking the first 20 minutes of eating, only in animals in which flow was reduced by more than 50% did the protein output fall. The greatest

reduction (to 45% of control value) was in the animal where flow rate fell to 16% of the control value after atropine.

(vi) Gel Electrophoresis of Feeding Saliva

A preliminary electrophoretic study of salivary protein obtained at different stages of the feeding period was undertaken. Results are shown in Figure 5.7. All saliva obtained during the feeding period contained a major protein band but the intensity of its staining decreased as eating continued. The most intense staining of the protein band occurred in saliva collected within the first 10 minutes of feeding. This band in feeding saliva corresponded to the soluble protein band X of sympathetically evoked saliva in acute experiments. The staining of this band was markedly reduced in saliva from animals given propranolol 10-15 minutes before feeding commenced (Figure 5.7B).

2. Morphological Changes Induced by Feeding

The morphological changes induced by feeding were examined in 15 animals. In these animals the average daily food eaten was 1015.5 ± 30.4 g. The rates of food intake were 507.5 ± 27.2; 219.2 ± 18.3; and 304.5 ± 35.1 g for the first, second, and the final two hours of feeding, respectively. The morphological changes in the different cell populations of the glands are described below.

a. Acinar Cells

(i) Light Microscopy (Figure 5.8A-F)

Eating for 1 hour and 4 hours resulted in a progressive decrease of the preformed granules in acini examined at the light microscopic level. As eating continued the secretory granules were found predominantly at the apical regions near the widened acinar lumina. Although it was not examined systematically, a reduction of cell size was apparent most obviously after eating for 4 hours. Within 1-2 hours postprandially secretory granules had re-accumulated in the acinar cells, but not in the concentrations noted in the resting glands.

(ii) Electron Microscopy (Figure 5.9)

Ultrastructurally, the discharge of granular content of acini was obvious as were signs of hyperactivity associated with synthesis. These were most marked after eating for 4 hours, when degranulation was so extensive that only the apical regions of the cells contained large fused granules and other regions were occupied by highly dilated RER and what were interpreted to be newly formed granules. Mitochondria became prominent, while Golgi complexes appeared as narrow cisternae. After eating ceased, particularly within 2 hours postprandially, secretory granules re-accumulated in the cytoplasm of acinar cells and the dilated RER, mitochondria and Golgi complexes became less prominent.

b. Demilunes

(i) Light Microscopy

No obvious changes in demilunes were detected from paraffin sections, although they were observed in 0.5 µm toluidine blue stained sections (Figure 5.8). In general the density of staining of demilune granules in secretory endpieces after eating was always variable, however, after eating for 1 hour, the majority of demilunes contained few dark-staining granules and this contrasted with their appearance after eating for 4 hours when a higher proportion of dark-staining granules had reappeared. At 1-2 hours postprandially, both dark and pale staining granules were present in the cells.

(ii) Electron Microscopy (Figure 5.10)

Tissues examined with the electron microscope showed that the variation in the degree of degranulation in the demilunes during eating was considerable, and that the most marked changes were always present in animals that had been eating for 1 hour. After eating for 1 hour fused granules, which appeared pale and homogeneous and lacked their typical substructure, were usually evident (Figure 5.10A). These

fused granules seemed to connect with the widened intercellular canaliculi. Golgi complexes with multiple cisternae were also present.

In contrast, after eating for 4 hours, most demilune cells showed little alteration compared with the resting gland although it was always possible to find some cells in the same gland which displayed similar changes to those described after eating for 1 hour. (Figure 5.10C,D).

Changes to demilune granules after eating for 1 hour were much reduced if the β-blocker, propranolol (1.2 mg kg^{-1}, iv) was given prior to feeding (Figure 5.10A,B). This drug had no detectable effects on the acini.

Within 1-2 hours postprandially (Figure 5.10E-H) the apical regions of demilunes contained granules of varying sizes (0.4-2.0 μm diameter). They usually showed a bipartite structure with uniformly dense and pale regions. Only a minority of granules displayed the tripartite features typical of the resting gland. One to two hours postprandially, the substructure of tripartite granules appeared as an aggregation of finely fibrillar structures and did not show any membrane-like boundaries, (Figure 5.10 E-H). Golgi complexes, typically located at the perinuclear regions and close to the RER stacks at the cell base, remained as swollen cisternae with small vesicles at their periphery.

c. Striated Ducts

(i) Light Microscopy

No obvious structural changes in striated duct cells were detected after eating either for 1 hour or 4 hours, except for the presence of secretory products (PAS-positive and/or, AB-positive materials) in the ductal lumina and a loss of PAS-staining from these cells.

(ii) Electron Microscopy

The fine structure of the epithelial cells of striated ducts, however, showed numerous empty vesicles throughout

the cytoplasm of cells (types I, II and III) and less frequently in their basal regions. RER cisternae in the perinuclear regions were dilated and appeared as empty vesicles, especially after 4 hours of eating.

Although not systematically examined, lysosomes (primary) seemed to be present more frequently in the cytoplasm after eating for 1 hour (Figure 5.11A) and small dense bodies adjacent to the apical surface of striated duct cells (Figure 5.11B) were prominant and appeared to be extruded into the lumen. No changes in the striated duct cells were detected 2 hours postprandially.

3. Observations on the Animal with a Partial Occluded Mandibular Duct

As previously mentioned, one of the seven sheep (sheep No. 1) with chronically placed mandibular duct cannula developed a reduced salivary flow due to a partial occlusion of the cannula. This was confirmed when the sheep was sacrificed 30 days after the cannulation was performed. At this time the cannulated gland weighed 11.13 g, while the uncannulated one was 9.25 g.

a. Physiological Observations

During eating, the flow from this sheep's cannula was very slow and the total secretion collected 10 days after cannulation was 78.58 g in 4 hour feeding period. The mean daily feeding collected from days 20 to 29 after the cannulation was 88.28 $\pm$ 7.02 g (range: 57.03-120.00 g, n=10), compared with a mean value of 251.04 $\pm$ 9.7 g (n=18) for the other six sheep. Thus the flow from this sheep was only 30-40% of the others over a period of almost three weeks. This reduction in flow was independent of food intake, as the total food intake in sheep No. 1 was consistent (555-670 g) throughout the experimental period.

Samples of mandibular saliva were collected at various intervals of feeding period on the 20th and 21st days after cannulation, and the mandibular flow, salivary protein

concentration and protein output estimated. The results showed a marked reduction of both protein concentration and output throughout the feeding period (Figure 5.12).

b. Morphological Observations

After perfusion fixation with half-strength Karnovsky's fixative, the uncannulated gland had a pinkish appearance while the cannulated gland appeared dark.

At the light microscopic level (Figure 5.13), the cannulated gland showed morphological changes in both the secretory endpieces and ductal system. The size of secretory endpieces, both the demilunes and acini, was reduced compared with the uncannulated gland. The lumina of central acinar cells, intercalated and striated duct cells were greatly dilated.

At the fine structural level, only slight changes were observed in secretory endpieces (Figure 5.14A). The secretory granules of the demilune cells seemed to have lost their characteristic appearance. There were conspicuous intercellular canaliculi and wide intercellular spaces between the myoepithelial cells and the underlying demilunes.

The most striking changes appeared in the ductal system (Figure 5.14B,C). The intercalated duct had wide intercellular spaces both between adjacent intercalated duct cells and between the myoepithelial and underlying cells. The striated duct cells exhibited greatly dilated basolateral intercellular spaces - a feature similar to that seen after the stimulation of sympathetic nerves (see Figure 5.16A). Despite these changes in the cells of ducts and endpieces, their basal membranes remained intact.

Figure 5.1: Record of mandibular salivary flow from the chronically placed unilateral cannula of sheep No. 5, during drinking (a); licking and chewing foot (b).

The uppermost trace indicates the mandibular flow (drops per 30 sec), the middle trace is of a one-minute time marker and the lowermost trace is the event marker.

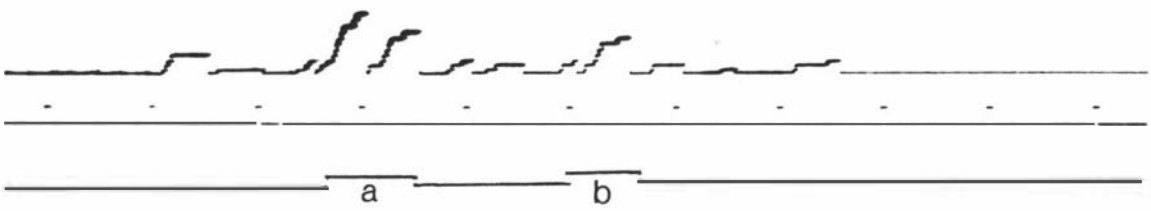

a
b

Figure 5.2: Mandibular salivary flow during teasing (c to f) and at the beginning of feeding (f onward) in sheep No. 5.

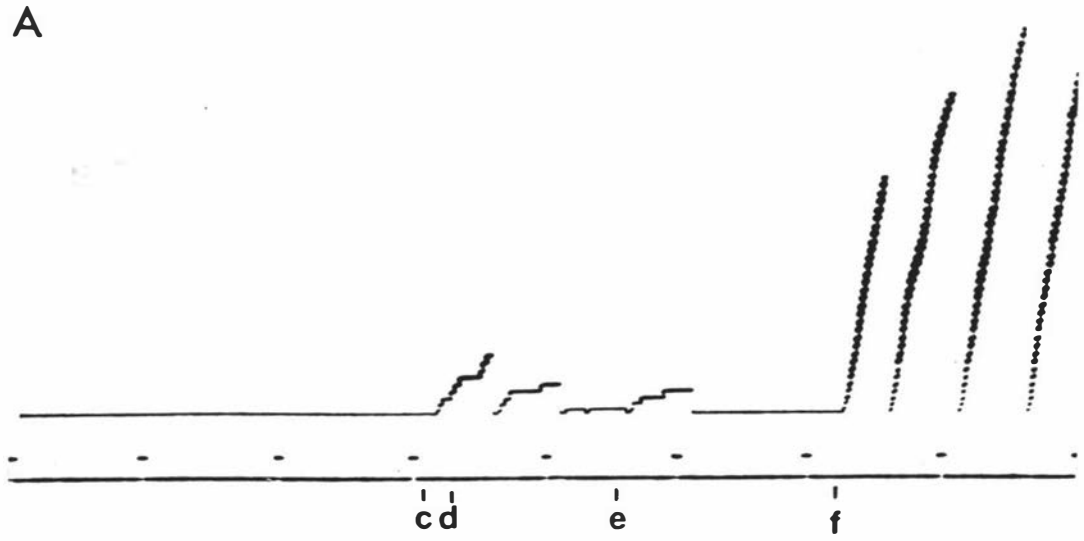
A. The normal conditions.

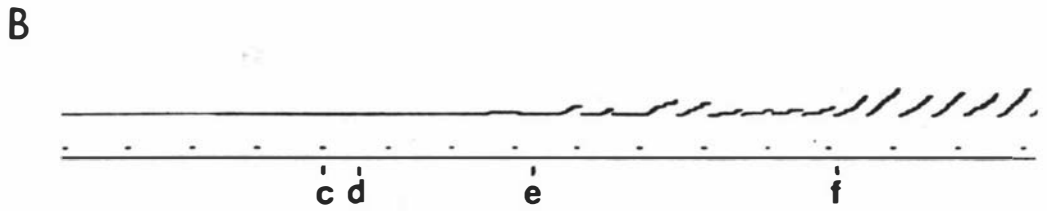
c - onset of feeding two other sheep
d - onset of teasing the subject with food
e - giving the food to the third sheep
f - onset of feeding the subject

B. Injection of atropine (0.1 mg kg^{-1}, iv) 10 minutes prior to teasing. *Note* the reduction in secretory responses to both teasing and feeding.

C. Injection of both atropine (0.1 mg kg^{-1}, iv) and propranolol (1.0 mg kg^{-1}, iv) 10 minutes prior to teasing. Showing the absence of a secretory response to teasing.

In records of A and B, the upper trace was from a chronically placed unilateral cannula and the lower trace one-minute marker, but in C they have been reversed.


A
c d
e
f


B
c d
e
f

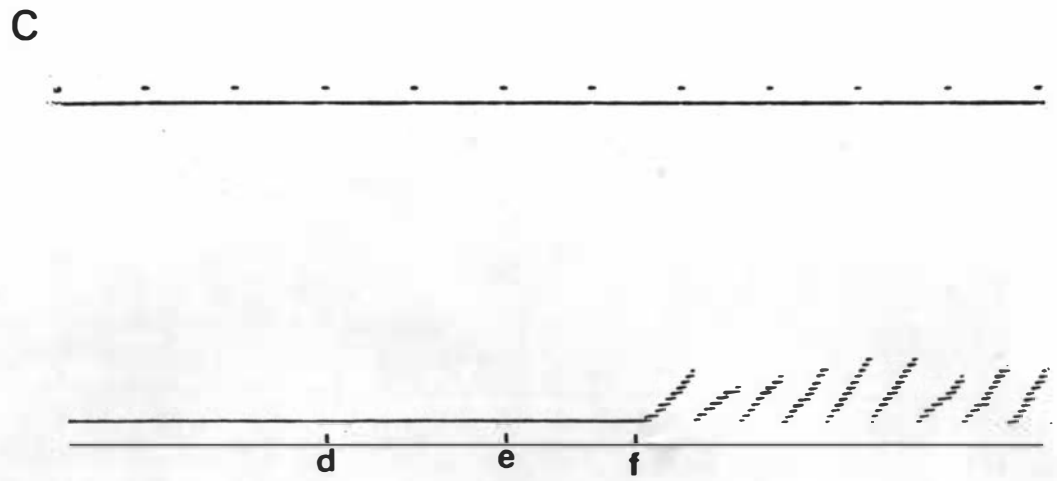

C
d
e
f

Figure 5.3: Mandibular salivary flow during teasing (c to f) and at the beginning of feeding (f onward) in sheep No. 4 and No. 6. (Similar records to Figure 5.2).

A. *Sheep No. 4.* The normal conditions.

B. *Sheep No. 4.* After injection of atropine (0.1 mg kg^{-1} body weight, iv) 10 minutes before teasing began. Showing a minimal secretory response to teasing.

C. *Sheep No. 6.* In this animal the injection of atropine (0.1 mg kg^{-1} body weight, iv) was followed after 4 minutes by a marked mandibular flow. The secretory response to teasing, however, was less than was recorded without atropine treatment.

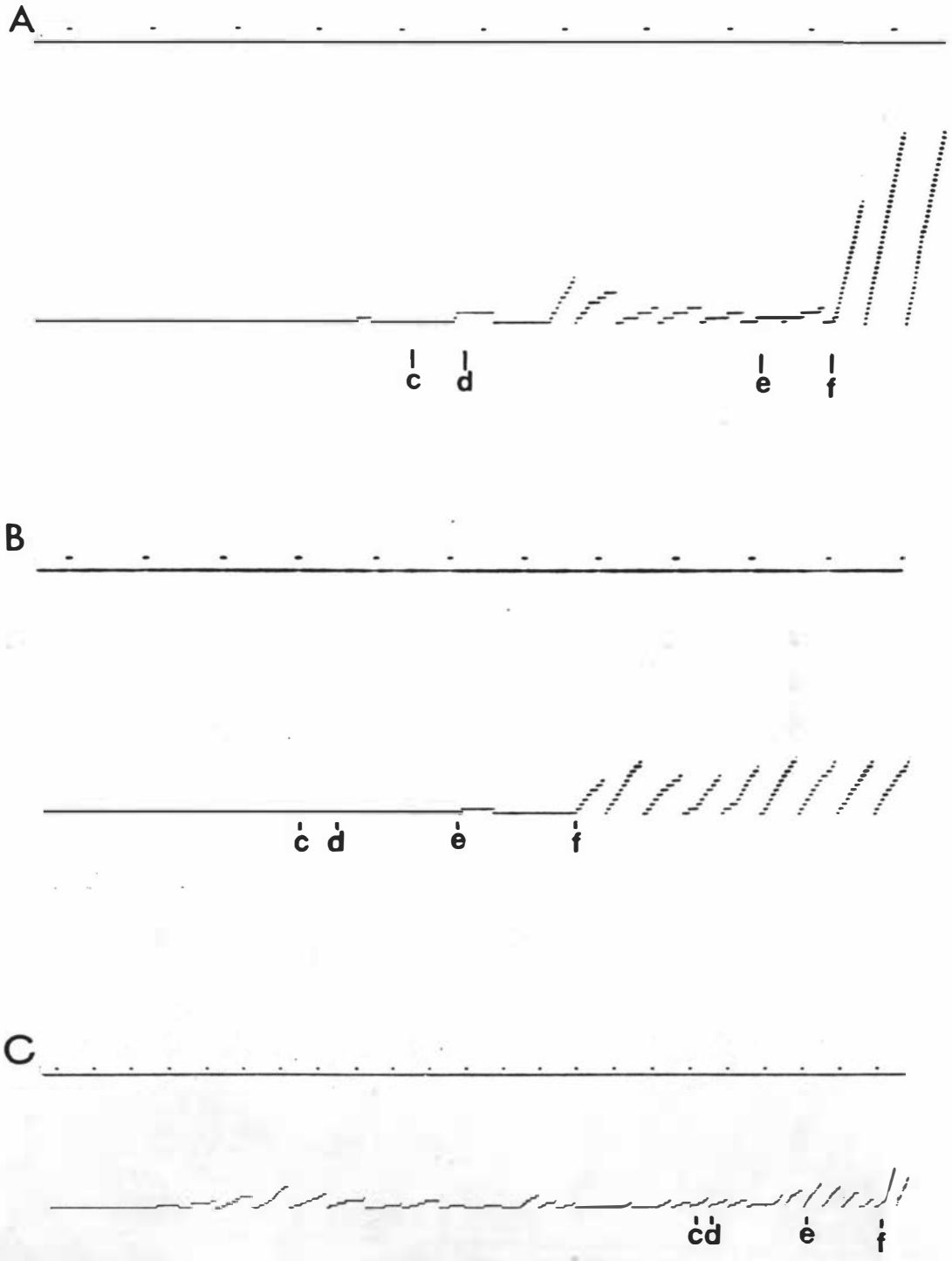

A
c
d
e
f
B
c
d
e
f
C
cd
e
f

Figure 5.4: Changes in mandibular salivary flow (g min^{-1}), protein concentration (mg ml^{-1}) and protein output (mg min^{-1}) in 6 sheep during feeding. The mean values (Ŧ; $\bar{X} \pm$ SE, n=18) were obtained from 3 successive experiments on each sheep.

Propranolol (1.0 mg kg^{-1}) was given intravenously 15 minutes prior to feeding, and the mean values (Ŧ; $\bar{X} \pm$ SE, n=6) were plotted against the duration of eating.

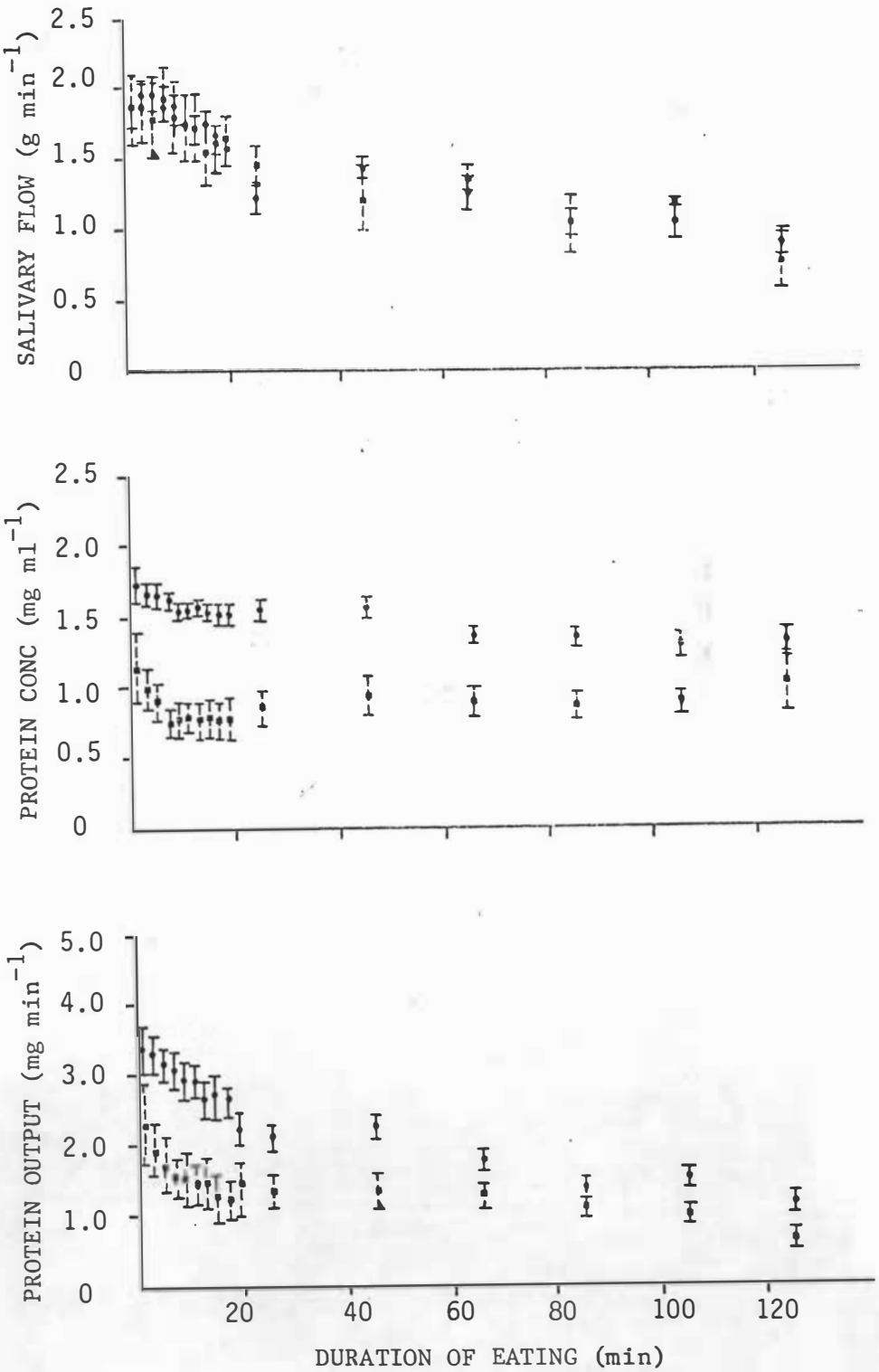

SALIVARY FLOW (g min^{-1})
2.5
2.0
1.5
1.0
0.5
0
PROTEIN CONC (mg ml^{-1})
2.5
2.0
1.5
1.0
0.5
0
PROTEIN OUTPUT (mg min^{-1})
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
120
DURATION OF EATING (min)

Figure 5.5: Changes in mandibular salivary flow ($g\ min^{-1}$), protein concentration ($mg\ ml^{-1}$) and protein output ($mg\ min^{-1}$) in sheep No. 2 during feeding.
The mean values (I; $\bar{X} \pm SE$, n=6) were obtained from 6 successive experiments. Propranolol was given intravenously 15 minutes prior to feeding (■).

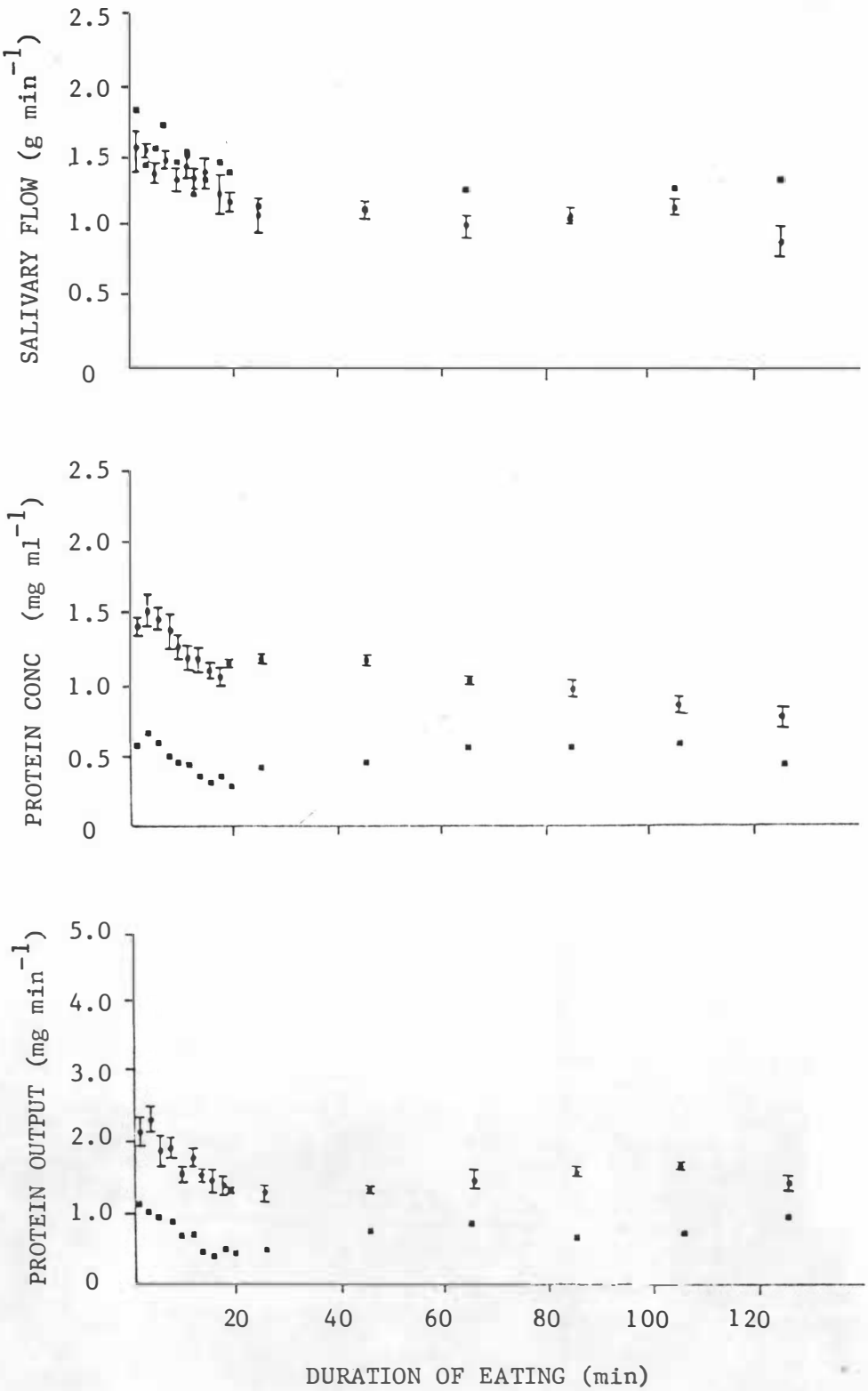

SALIVARY FLOW (g min^{-1})
2.5
2.0
1.5
1.0
0.5
0
PROTEIN CONC (mg ml^{-1})
2.5
2.0
1.5
1.0
0.5
0
PROTEIN OUTPUT (mg min^{-1})
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
120
DURATION OF EATING (min)

Figure 5.6: Observations on Sheep No. 6 under the same conditions as Sheep No. 2 in Figure 5.5. (Sheep No. 6 usually ate very quickly).

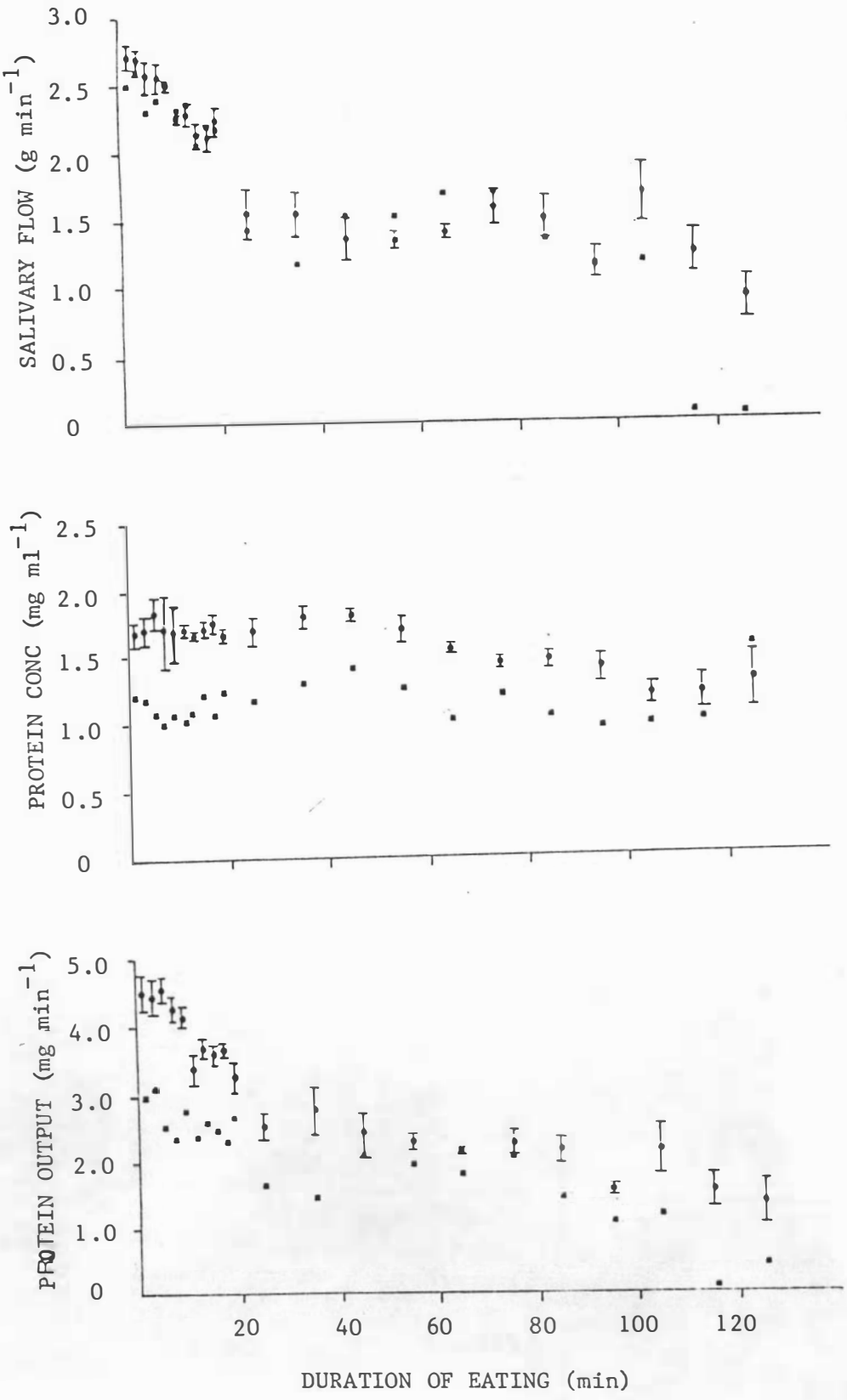

SALIVARY FLOW (g min^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0
PROTEIN CONC (mg ml^{-1})
2.5
2.0
1.5
1.0
0.5
0
PROTEIN OUTPUT (mg min^{-1})
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
120
DURATION OF EATING (min)

Figure 5.7: Electrophoretic pattern of mandibular saliva collected during feeding (from sheep No. 2).

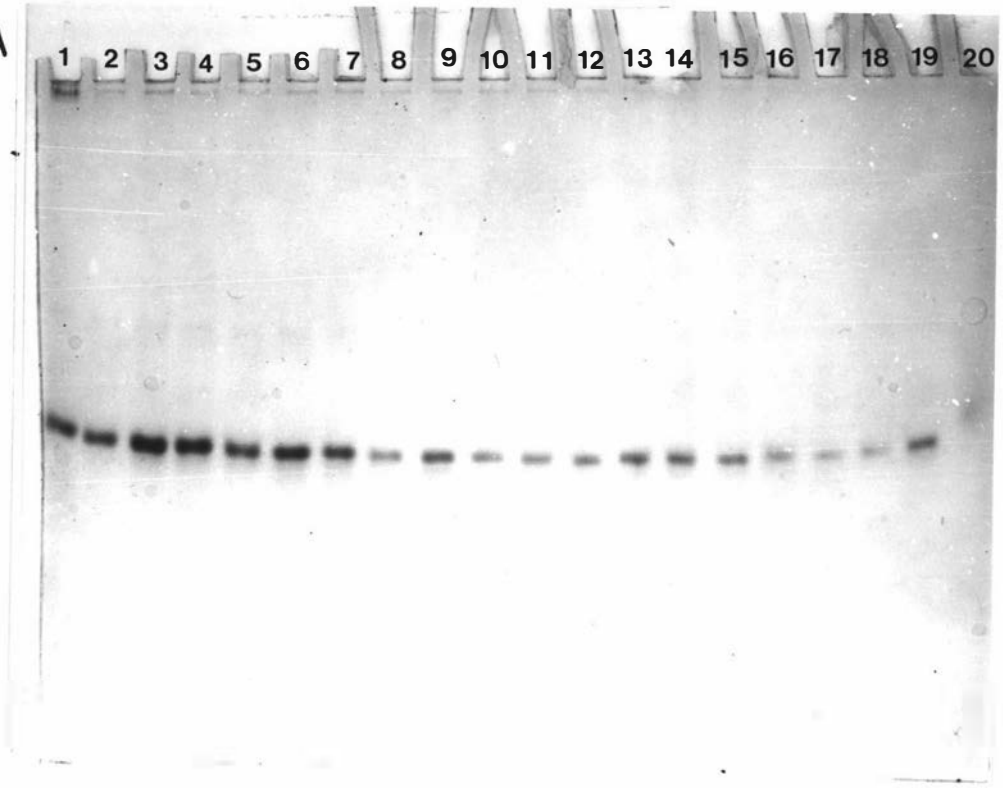
A. *Note* the decrease in intensity of the major protein band as feeding continued.

B. *Note* the reduction of the intensity of the major protein band in saliva collected from the same sheep after the treatment with propranolol (1.0 mg kg^{-1}) 10 min prior to feeding.

Saliva samples from left to right:

A. (3.0 µl saliva per pocket)			B. (6.0 µl saliva per pocket)		
Pocket No.	Feeding Time (min)	Protein Conc ($mg\ ml^{-1}$)	Pocket No.	Feeding Time (min)	Protein Conc ($mg\ ml^{-1}$)
(1)	Dead space saliva	1.52	(1)	Same sample as A(3)	1.92
(2)	0-2	1.62	(2)	Dead space saliva	0.86
(3)	2-4	1.92	(3)	0-2	0.31
(4)	4-6	1.82	(4)	2-4	0.46
(5)	6-8	1.71	(5)	4-6	0.49
(6)	8-10	1.52	(6)	6-8	0.45
(7)	10-12	1.34	(7)	8-10	0.46
(8)	12-14	1.09	(8)	10-12	0.41
(9)	14-16	1.26	(9)	12-14	0.42
(10)	16-18	1.07	(10)	14-16	0.40
(11)	18-20	1.13			
(12)	20-30	1.20			
(13)	40-50	1.32			
(14)	60-70	1.09			
(15)	80-90	1.19			
(16)	120-130	0.75			
(17)	140-150	0.72			
(18)	160-170	0.67			
(19)	180-190	0.93			
(20)	-	-			

A



B

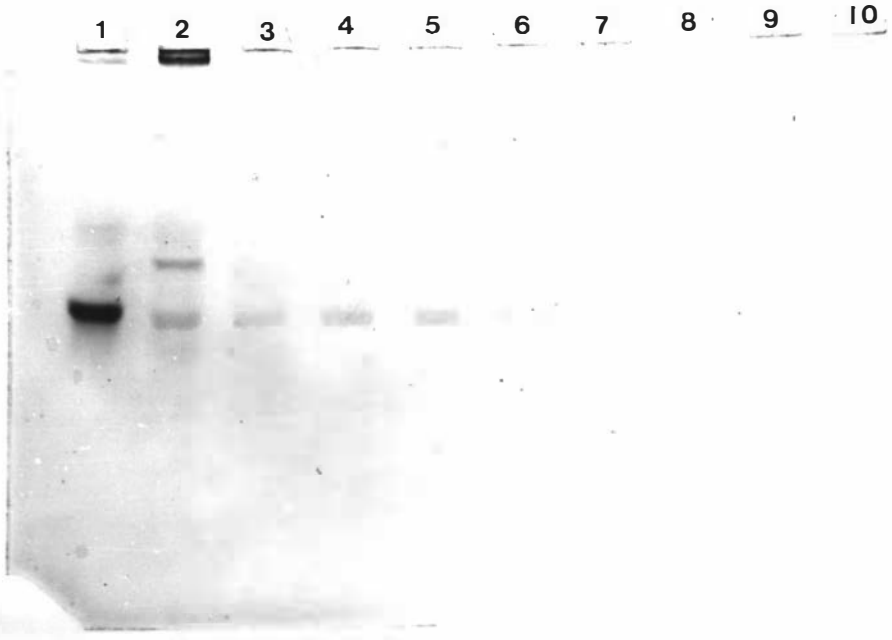


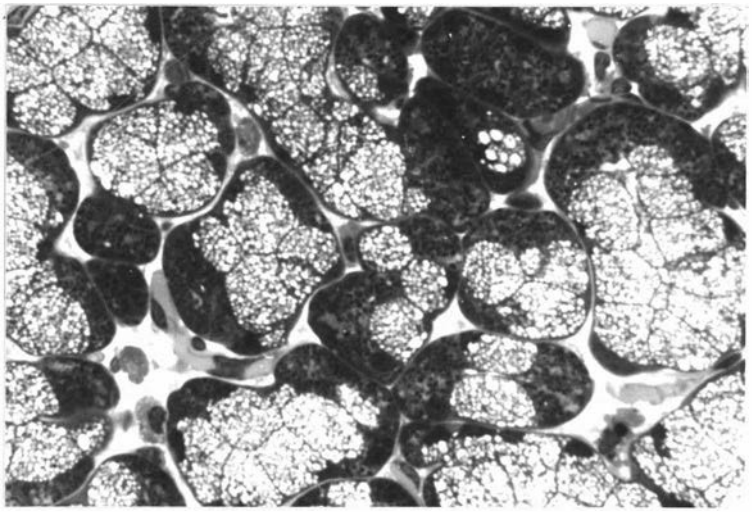
Figure 5.8: Light micrographs of mandibular glands after different periods of eating. (0.5 µm Araldite sections, toluidine blue staining). x500.

A. Resting gland (after food had been withheld for 20 hours).

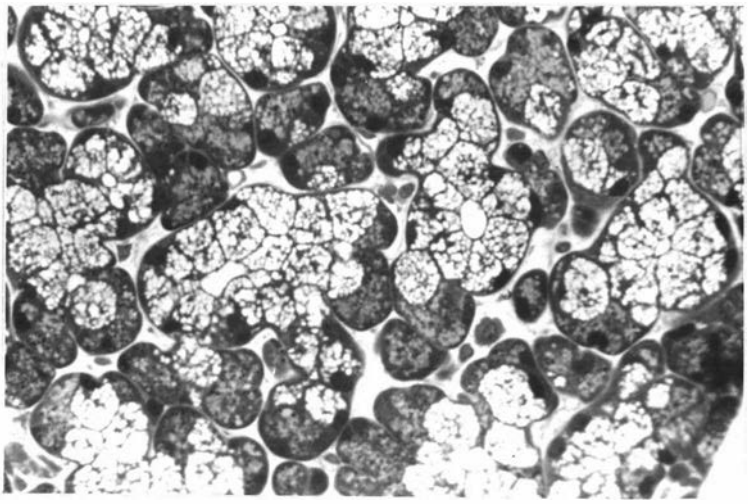
B. After eating for one hour.

C. After eating for one hour with injection of propranolol 15 minutes before feeding.

A



B



C

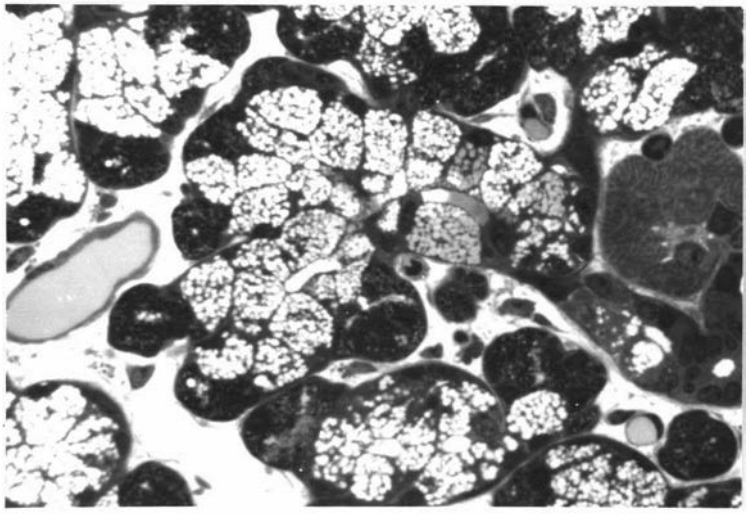


Figure 5.8:

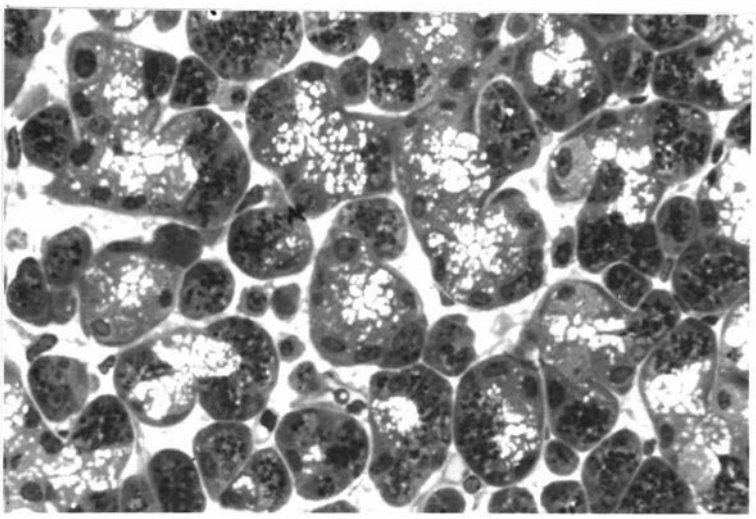
D. After eating for four hours.

E. One hour postprandially.

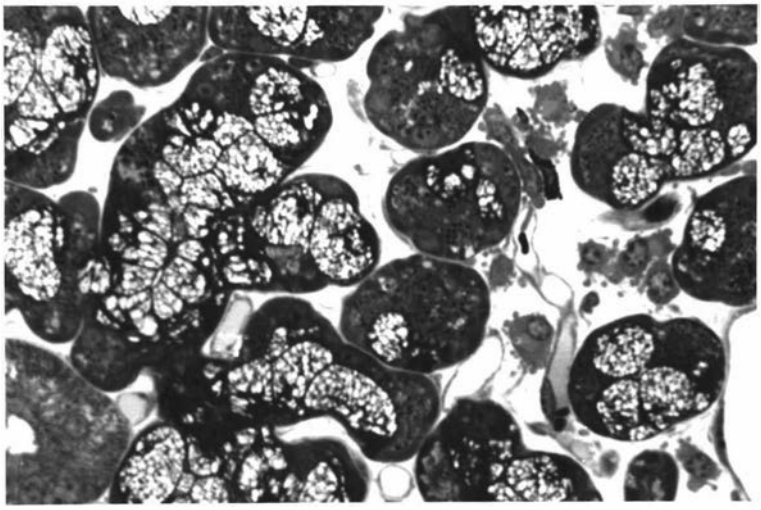
F. Two hours postprandially.

(See text for details of changes).

D



E



F

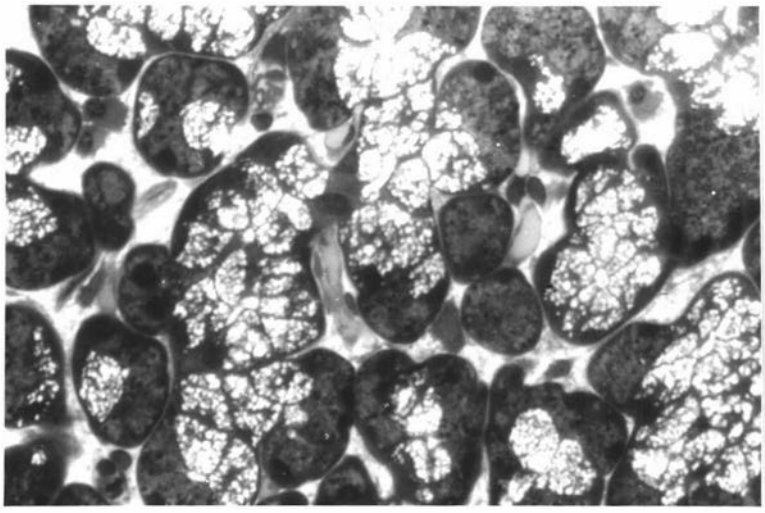
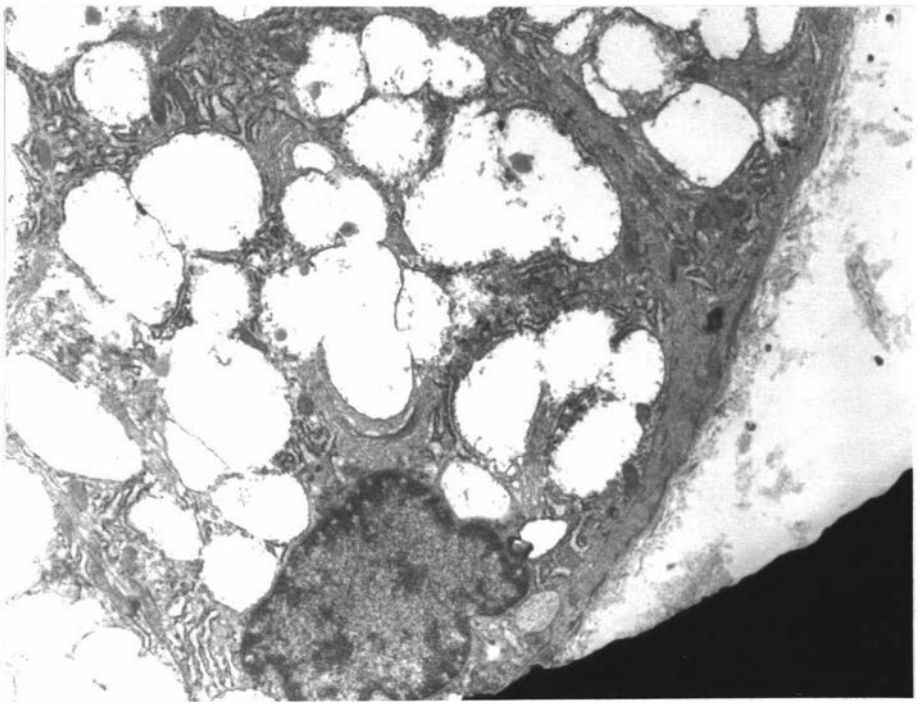


Figure 5.9: Electron micrographs of mucous acinar cells of mandibular glands after different periods of eating. Araldite sections. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A. After eating for one hour. x10,500.

B. After eating for four hours. x5,500.

A



B

Figure 5.9:

C. Two hours postprandially.
This shows the reaccumulation of secretory granules in the mucous acinar cells.
x10,500.

C

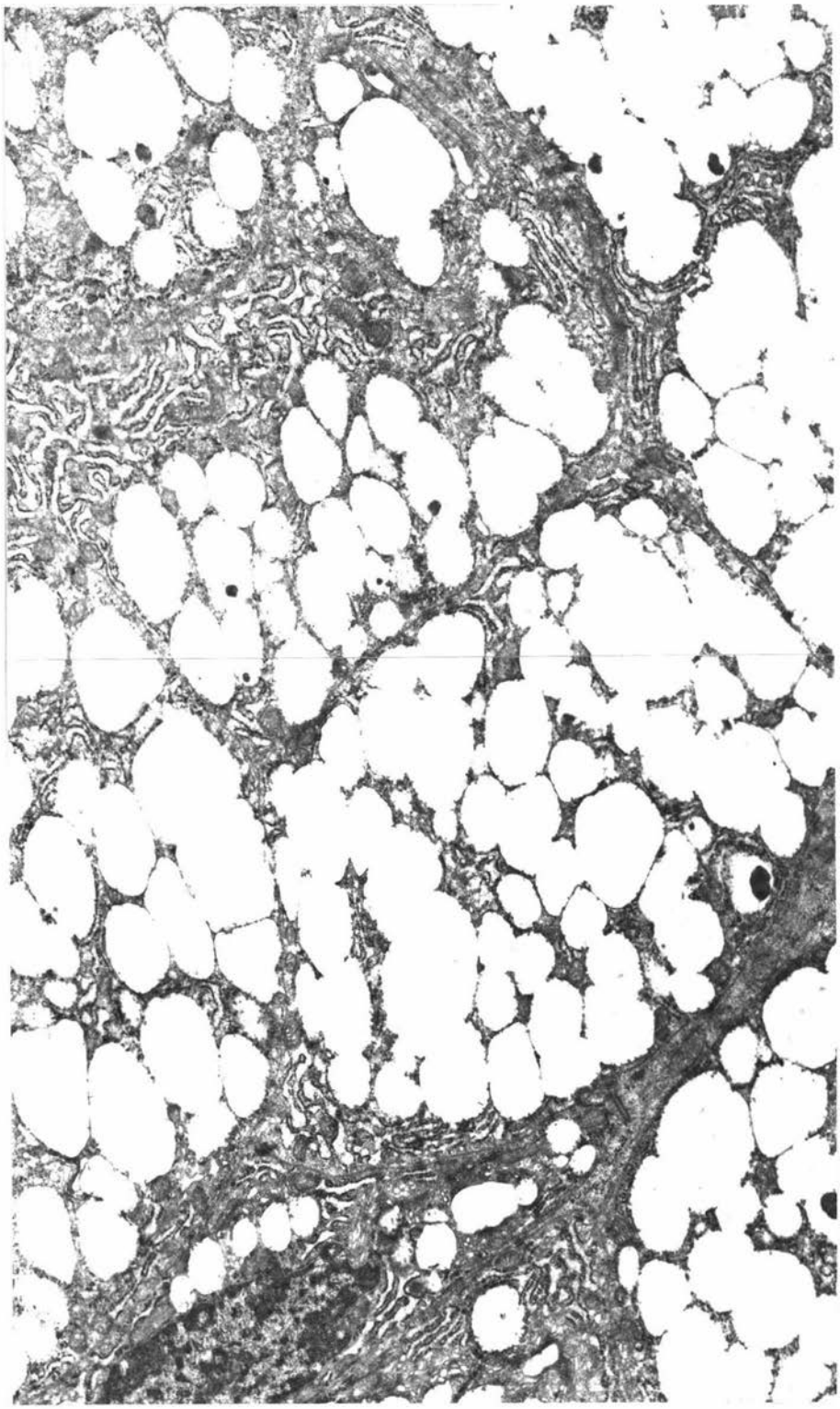


Figure 5.10: Electron micrographs of demilune cells after different periods of eating. Araldite sections. (Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A. After eating for one hour with prior injection of normal saline. *Note* fused granules (F).

B. After eating for one hour with prior treatment of propranolol (1.2 mg kg^{-1} body weight).

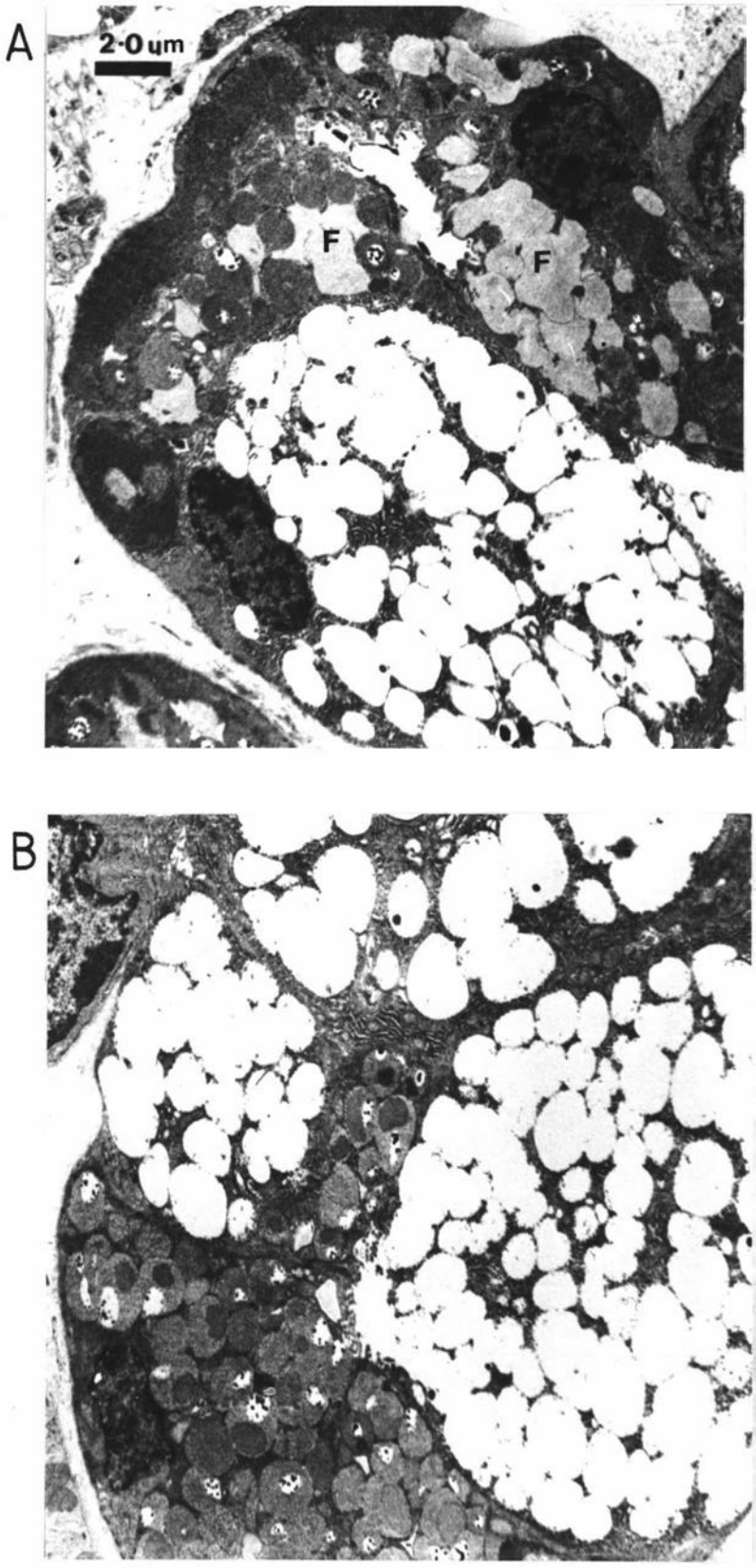
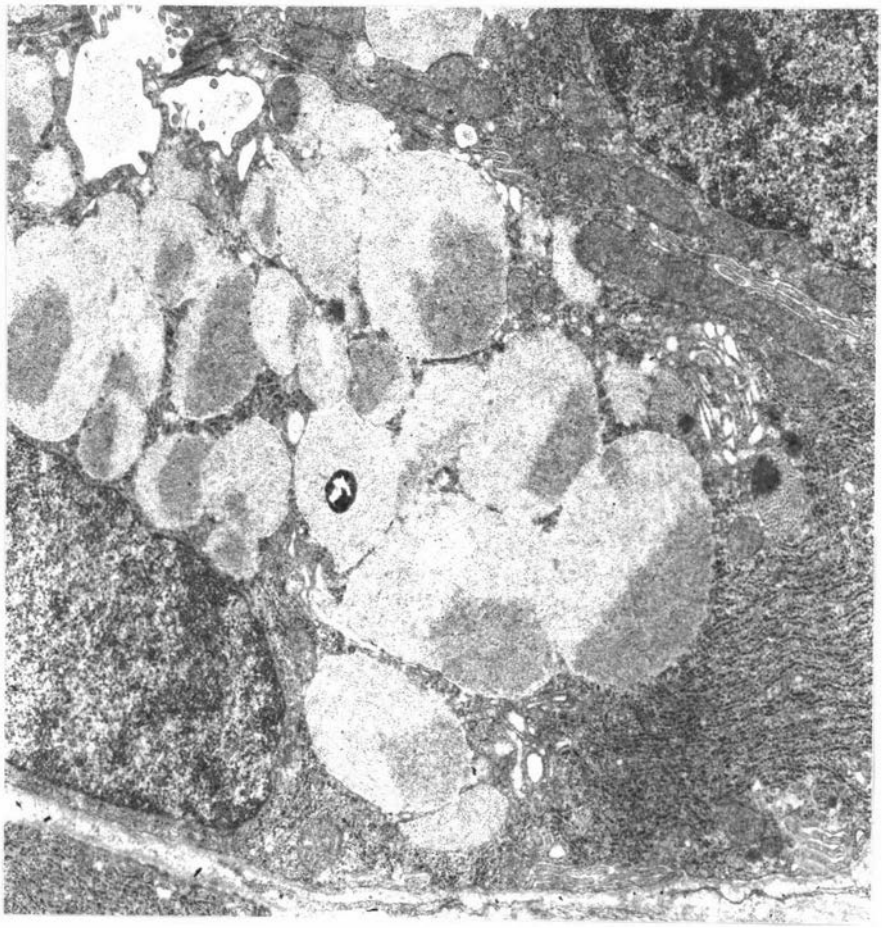

A
2·0 µm
F
F
B

Figure 5.10:

C&D After eating for four hours, showing variable changes in the demilune cells of two secretory endpieces of the same gland.
C. x13,500; D. x10,500

C



D

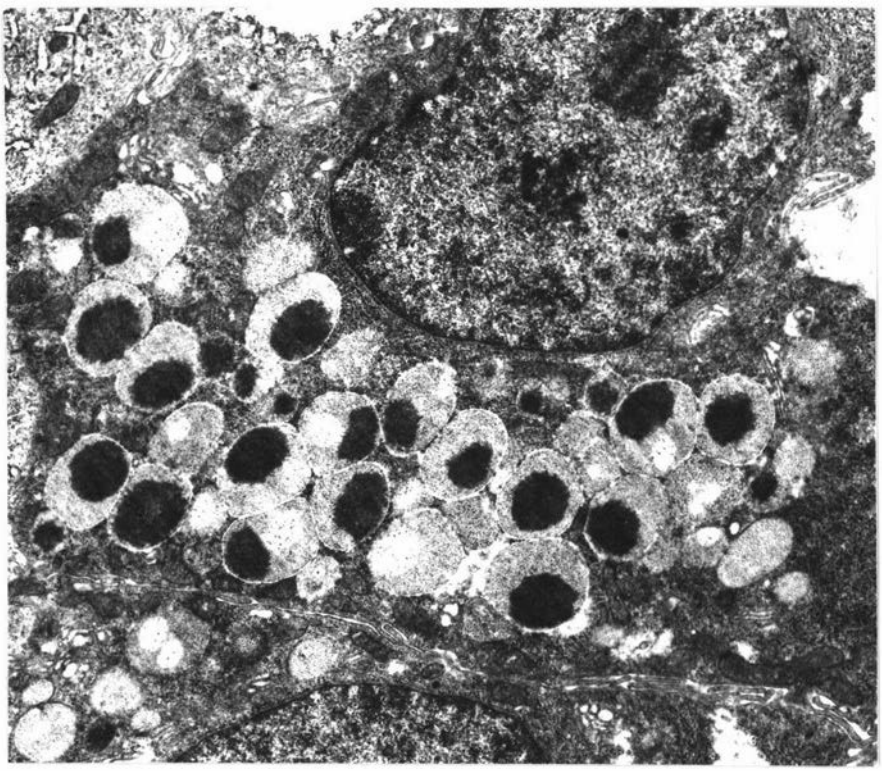
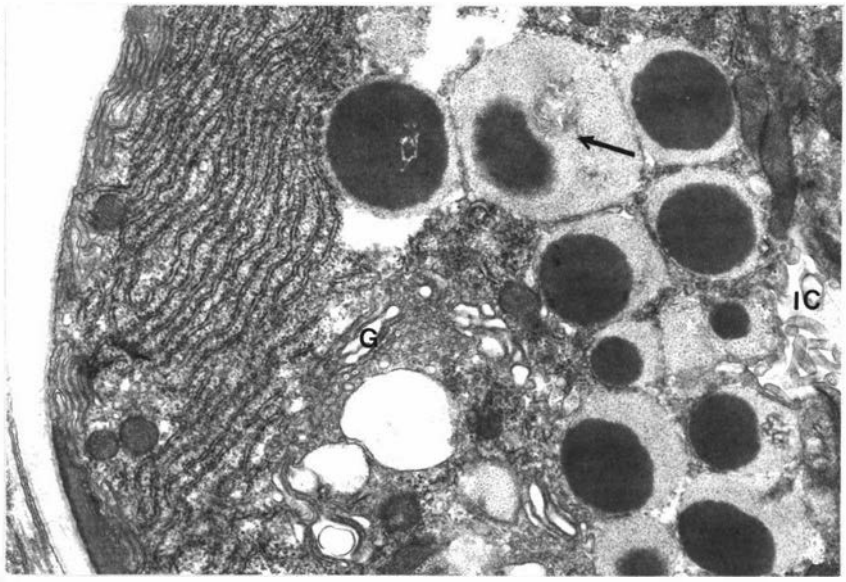


Figure 5.10:

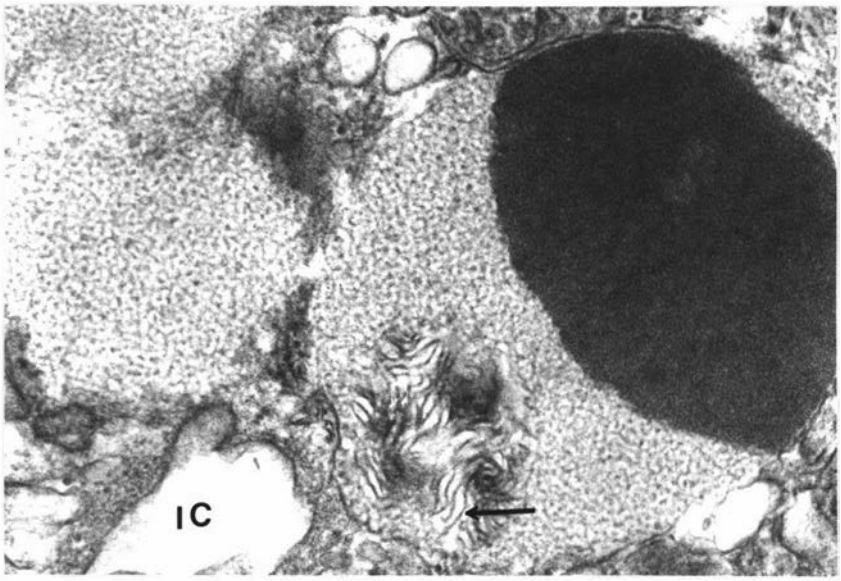
E. One hour postprandially.
Note the structure of secretory granules in the demilune (arrow). x13,500.

F&G Higher magnification of secretory granules of demilune at one hour postprandially. x46,000.

E



F



G

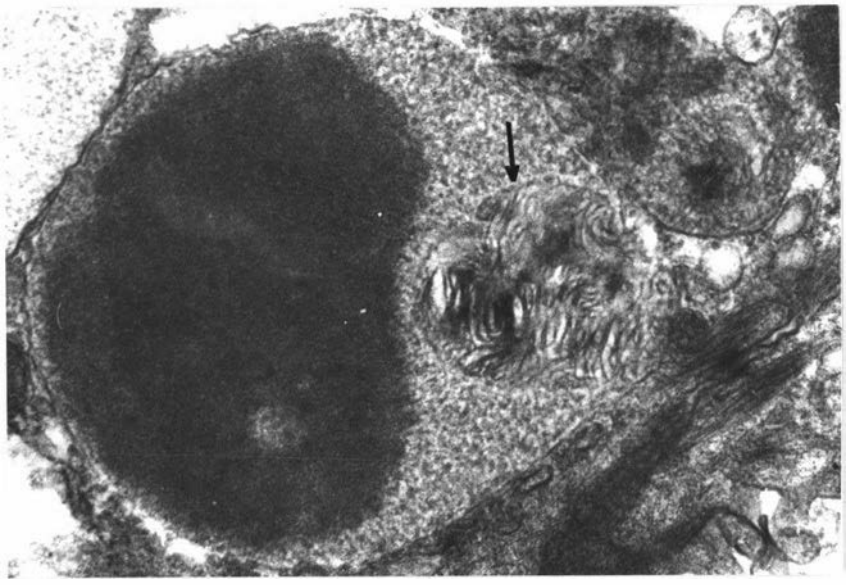


Figure 5.10:

H. Two hours postprandially, showing the reappearance of secretory granules (arrow) in demilune cells. x26,200.

H

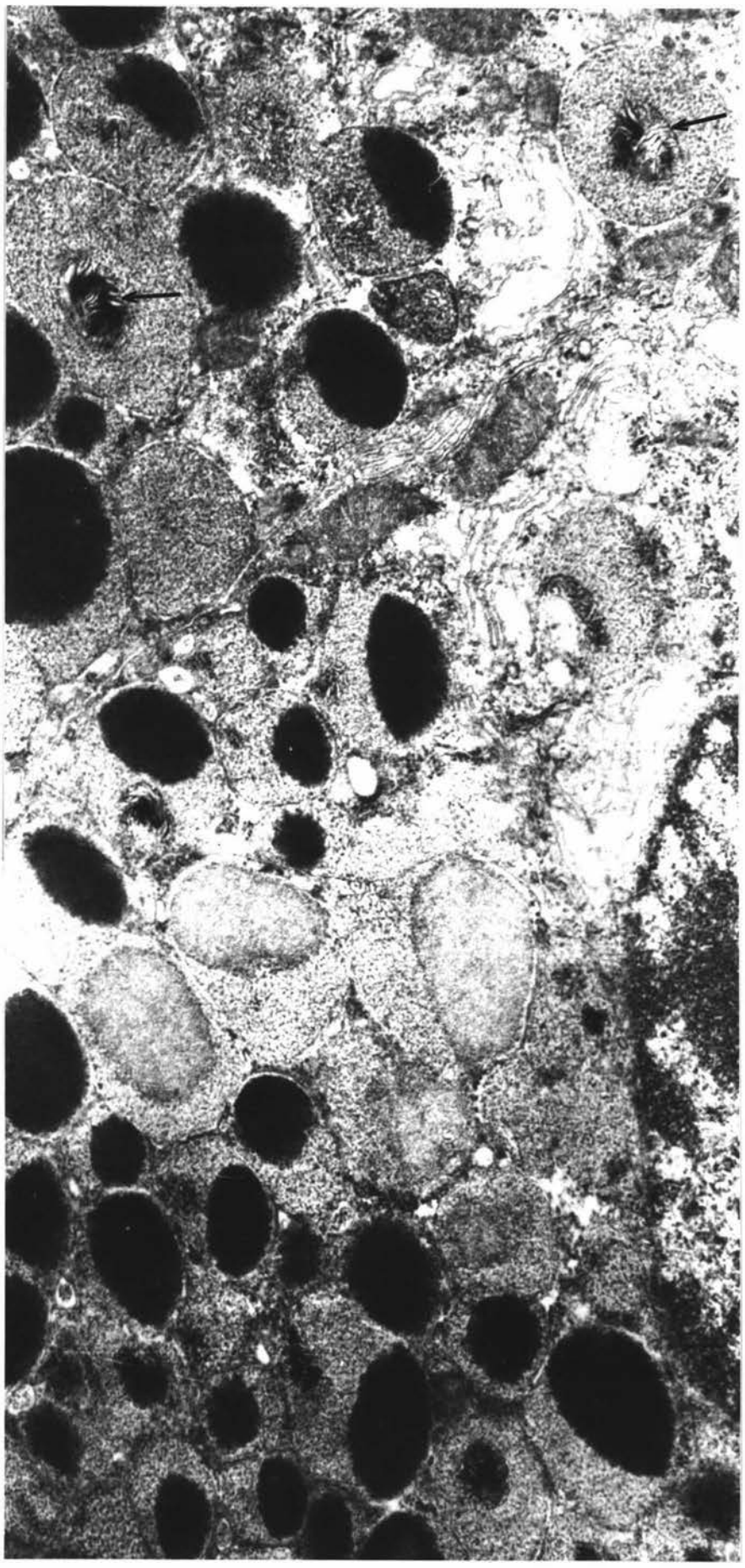
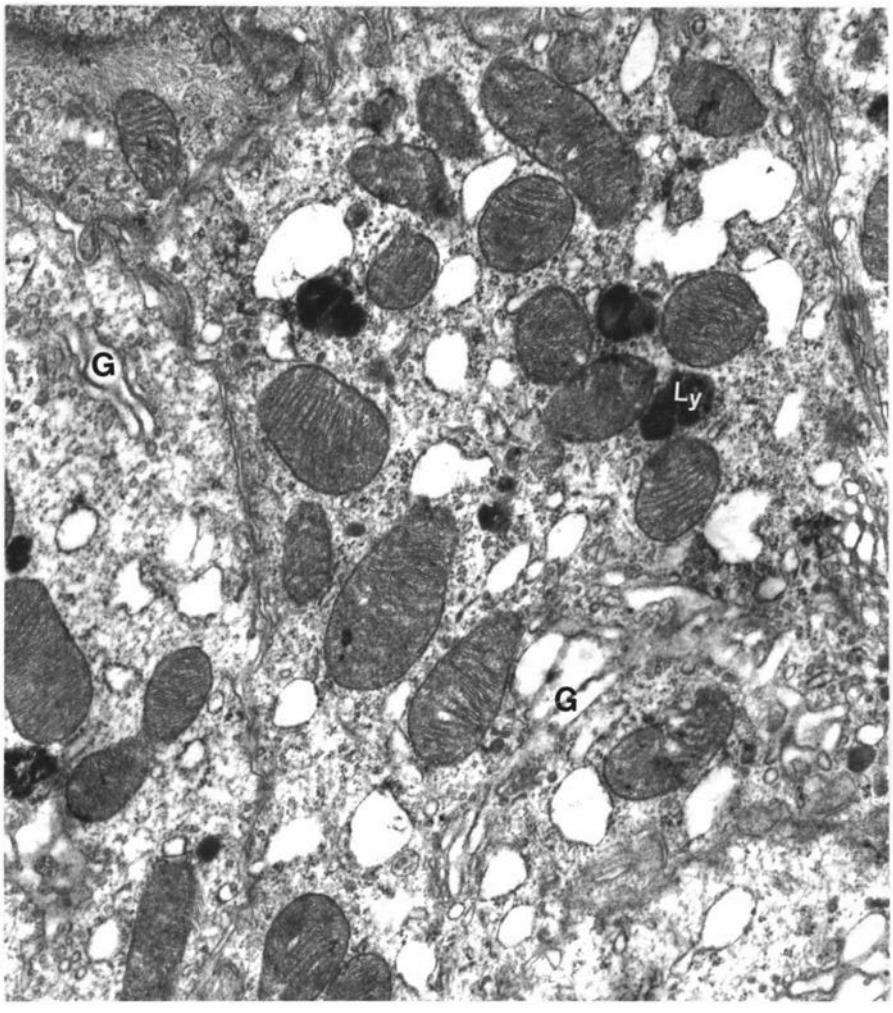


Figure 5.11: Electron micrographs of striated duct cells after eating for one hour.
(Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation; *en bloc* staining).

A. A portion of a type I striated duct cell. Numerous empty vesicles, dilated Golgi complexes (G), and lysosomes (Ly) are present. x17,500.

B. The apical region of a type I striated duct cell, showing the small dense bodies and part of a type III cell. x22,500.

A



B

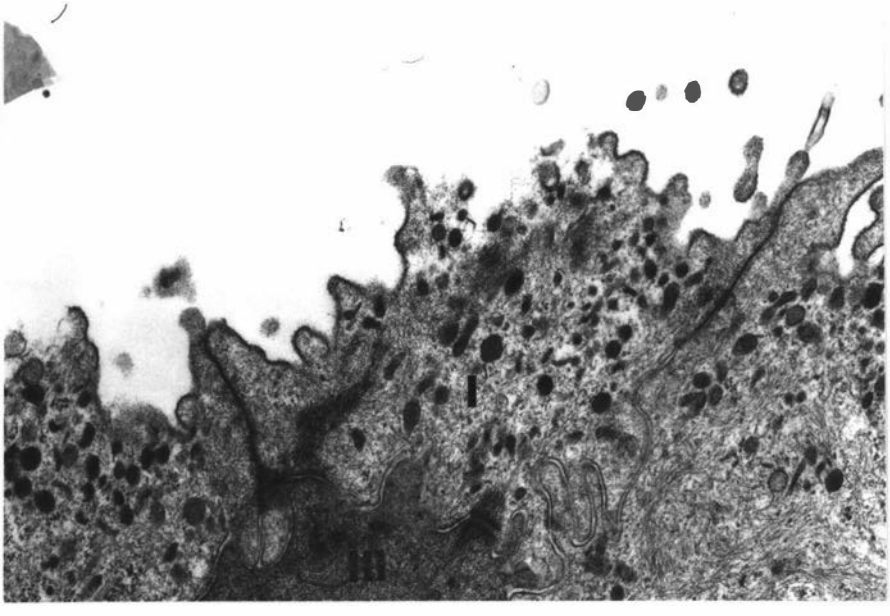


Figure 5.12: Changes in the mandibular salivary flow ($g\ min^{-1}$), protein concentration ($mg\ ml^{-1}$) and protein output ($mg\ min^{-1}$) during feeding in sheep No. 1 whose cannulated duct developed a partial occlusion. (Saliva samples were collected on the 20th (•) and 21st (▲) days after cannulation).

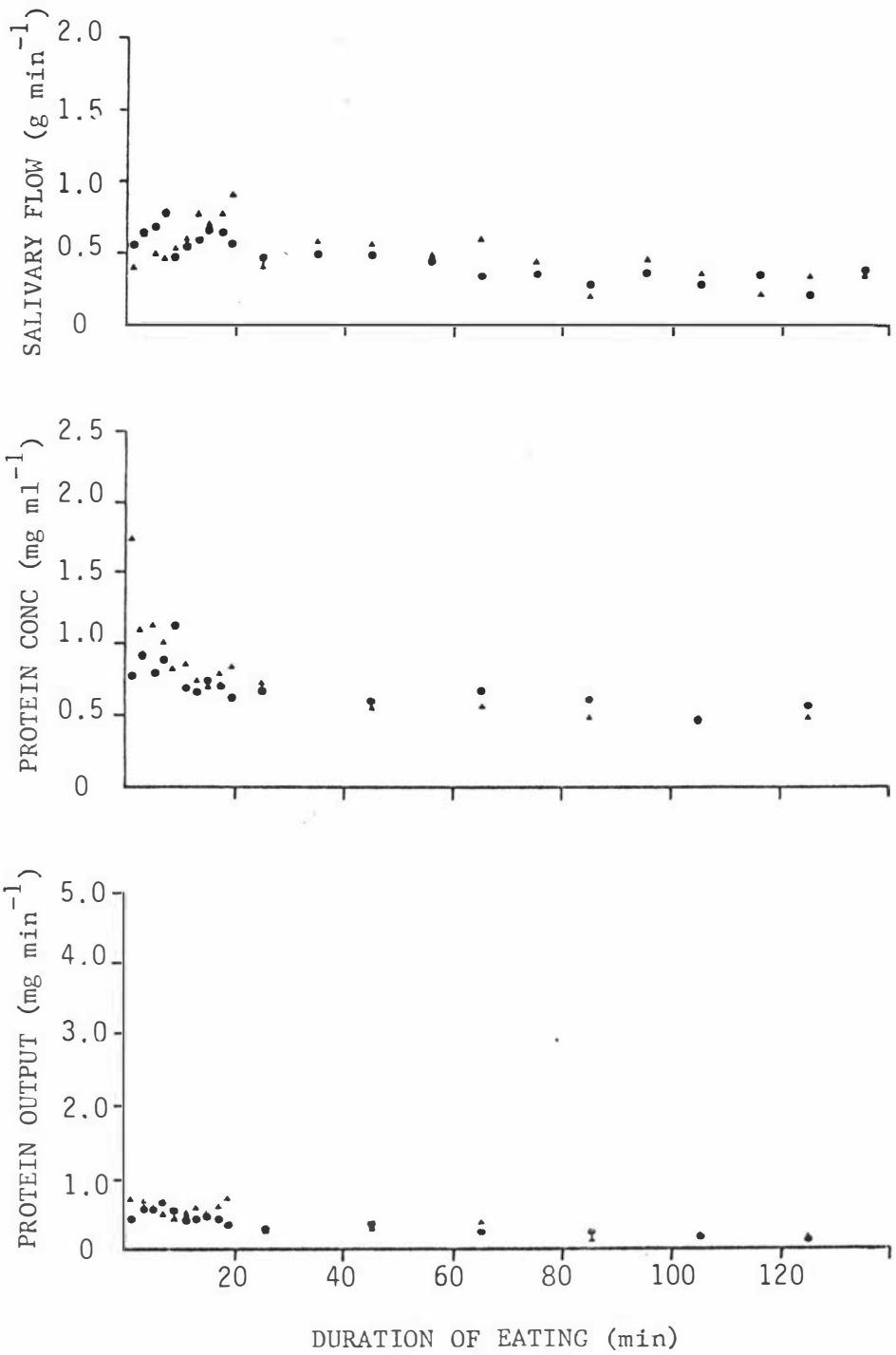

SALIVARY FLOW (g min^{-1})
2.0
1.5
1.0
0.5
0
PROTEIN CONC (mg ml^{-1})
2.5
2.0
1.5
1.0
0.5
0
PROTEIN OUTPUT (mg min^{-1})
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
120
DURATION OF EATING (min)

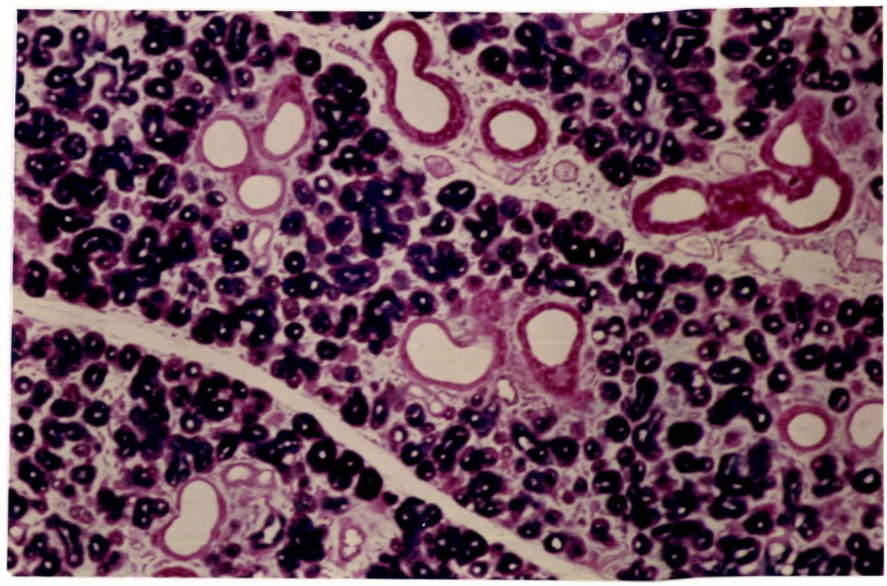
Figure 5.13: Light micrographs of the mandibular glands of sheep No. 1 (food withheld for 20h). Comparison between the left cannulated gland which had a partial obstruction of its mandibular duct cannula for 30 days and the right uncannulated gland.

A&B *Left cannulated gland.* A. x100; B. x250. Marked distension of both the ductal and acinar lumina is evident as is a reduction in the size of secretory endpieces.

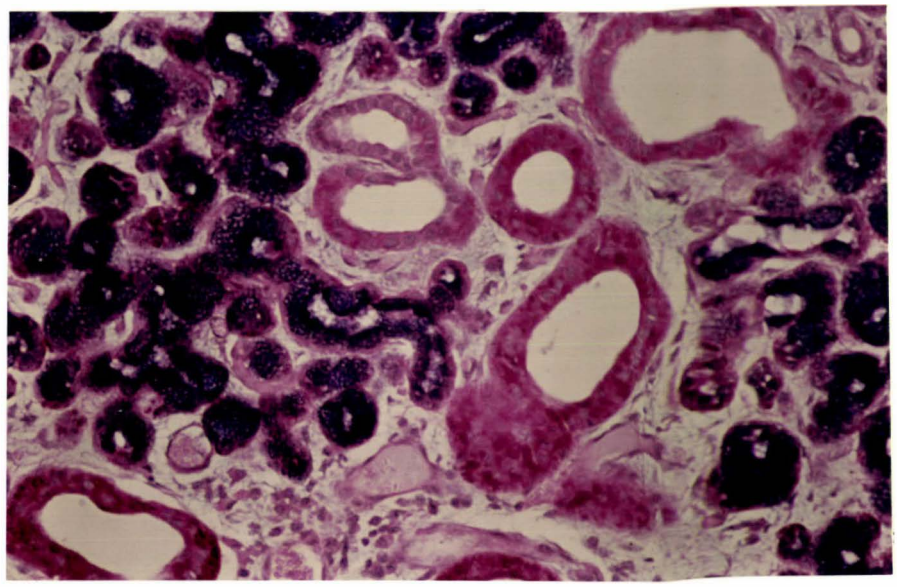
(Karnovsky's fixed paraffin sections, AB/PAS/H staining).

C. *Right uncannulated gland.* x250. The ductal and acinar lumina are typical of other resting glands.

A



B



C

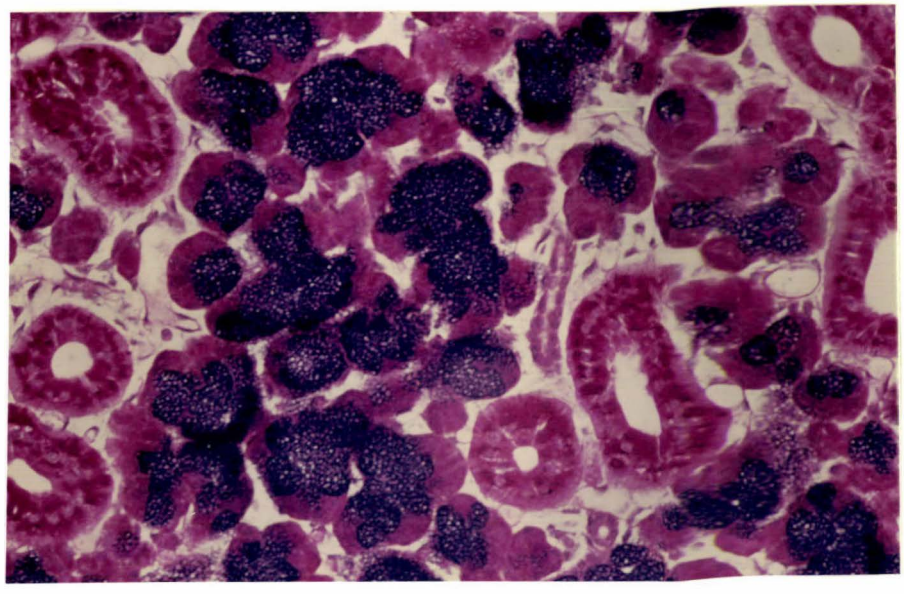


Figure 5.14: Electron micrographs of the left cannulated gland of sheep No. 1. (Same animal as in Figure 5.13).

(Epon-Araldite sections. Perfusion fixation: half-strength Karnovsky's fixative; OsO_4 postfixation).

A. A portion of a secretory endpiece. x10,500.

(A - acinar cells, d - demilune,
IC - intercellular canaliculus,
MEC - myoepithelial cell process).

A

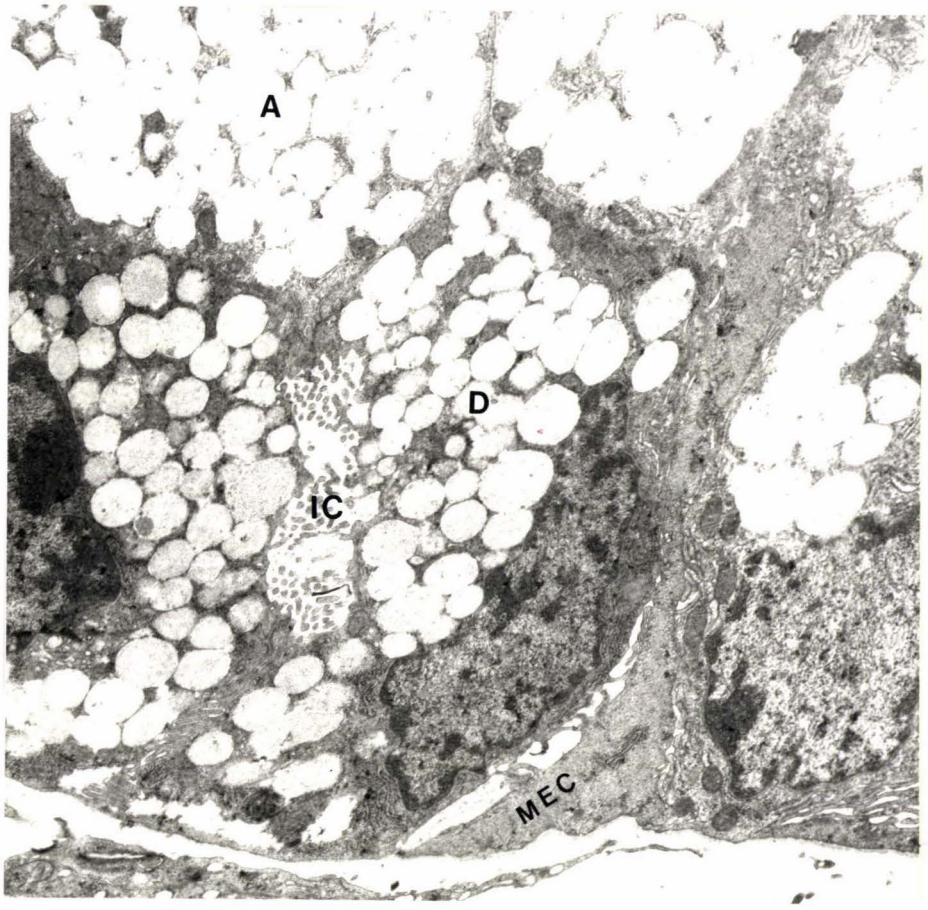
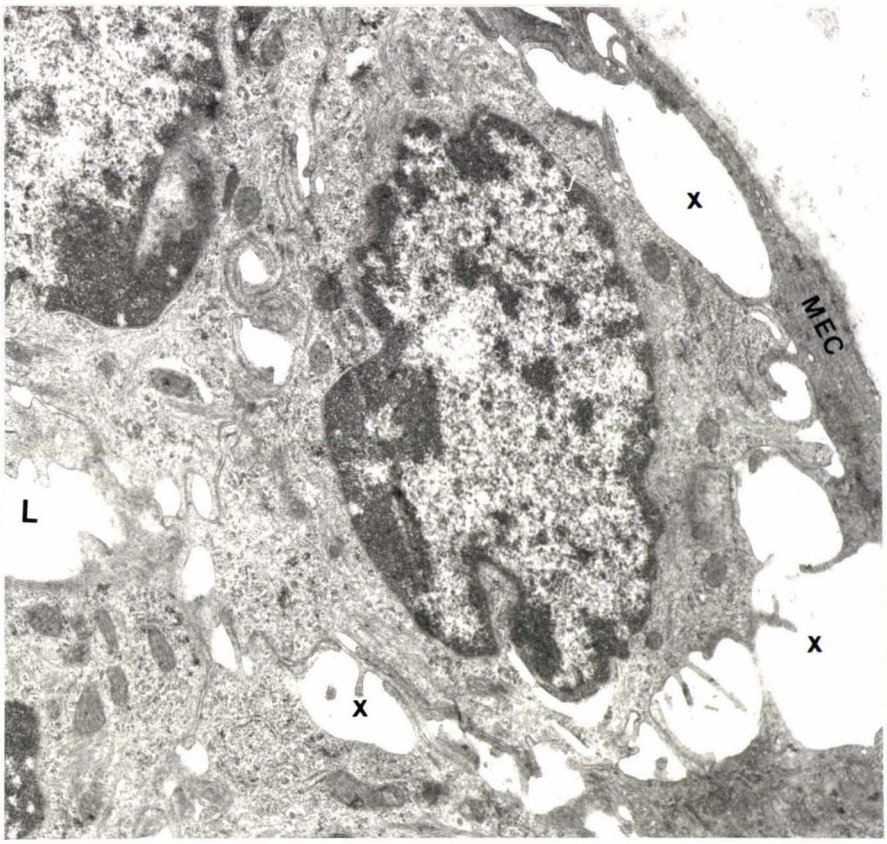


Figure 5.14:

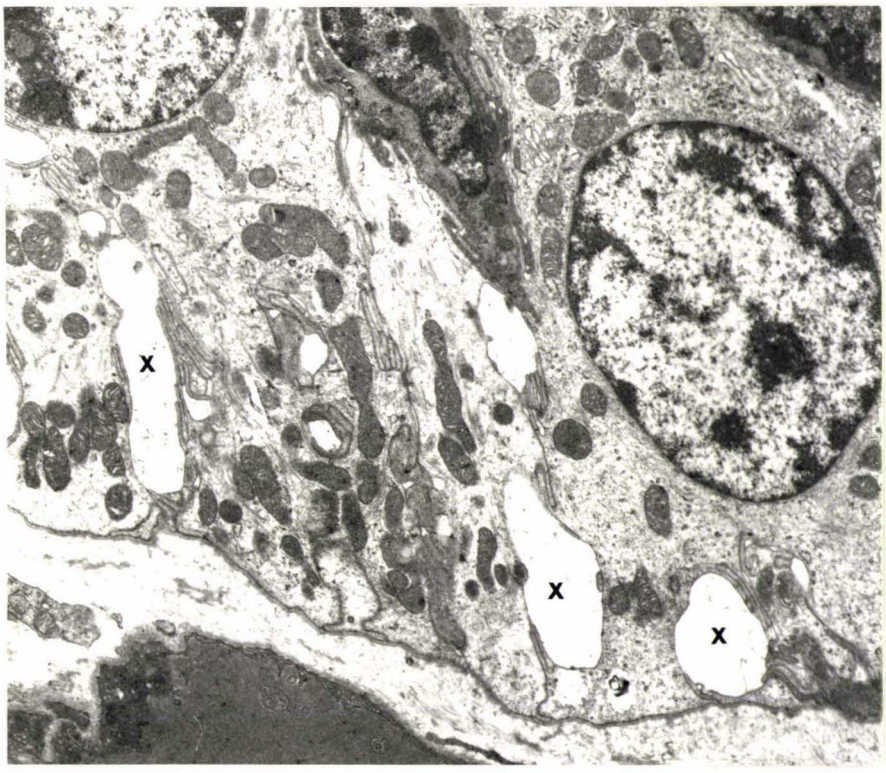
B. Intercalated duct cells.
Note the expanded intercellular spaces between adjacent duct cells (**x**), and between myoepithelial cell processes and underlying cells. (L - ductal lumen). x13,500.

C. The basal region of striated duct cells, showing the dilation of intercellular basal folds (**x**). x8,100.

B



C



B. DISCUSSION

1. Physiological Effects Induced by Feeding

a. Mandibular Flow

The absence of a marked mandibular secretion from the cannulated glands during rumination or in the intervals between meals is in accord with the reports of others (Scheunert and Trautmann, 1921; Ash and Kay, 1959; Kay 1960; Carr *et al.*, 1977) who have worked with this gland. In the present study the term 'resting' has been used, as by Langley (1898), to describe glands not wittingly stimulated to secrete and the term 'active' for glands which have been secreting in response to intentional stimuli. Some inherent difficulty in definition seems unavoidable. The resting mandibular flow in this context is, therefore, the secretion obtained when the animals did not have food available. Recently, Emmelin and Gjörstrup (1975, 1976*b*) have suggested that in the awake state, there is normally a slow resting flow elicited by impulses in the parasympathetic nerve pathways. Such resting secretion is believed to be evoked partly by reflex stimulation of the mouth and throat during tongue movements, swallowing and other buccal activities (Babkin, 1950).

Sheep, with their continuous secretion of parotid saliva, are noted for a continued high rate of swallowing, but in addition to the buccal stimulation noted above, a contribution to resting secretion may also arise from stimulation of the rumino-reticulum during movements of digesta and from rumino-reticular contractions. This suggestion is based on the work of Ash and Kay (1959) who reported that, either tactile stimulation of the anterior wall of reticulum, or stretching of the oesophagus, cardia or reticulo-omasal orifice in conscious sheep caused a moderate secretion of mandibular saliva.

Two types of gastric mechanoreceptors have been identified electrophysiologically in ruminants - tension

receptors, functionally in-series with the muscle fibres (Iggo, 1955; Leek 1969) and tactile receptors in the epithelium (Leek, 1972). However, although afferent impulses arising from gastric mechanoreceptors have been shown to affect reflexly the secretion of parotid saliva, gastric movements and rumination (Titchen 1958; Ash and Kay, 1959), there is little direct evidence of such impulses influencing the reflex secretion of mandibular saliva (Kay, 1958*a*).

A marked mandibular flow occurs in association with drinking (Figure 5.1) which, it is interesting to note, was reported in ruminants nearly a century ago by Ellenberger and Höfmeister (1887). Water receptors occur in the tongues of many mammals (Wolf and Monnier, 1975) although not apparently in sheep (Iggo and Leek, 1967). None the less, both cold receptors and mechanoreceptors with thermal sensitivity are present in the sheep's tongue (Iggo and Leek, 1967) and these would be expected to have been excited during drinking in the present experiments where the ambient temperature was about 10^{o}C. The activation of these may have reflexly contributed to mandibular secretion. This secretion can also be argued to be associated with buccal and oesophageal stimulation delivered by the swallowed water (Ash and Kay, 1959). In contrast to this response of the mandibular gland, both Wilson (1963) and Carr (1974) observed a reduction in parotid secretion during drinking together with an increase in monophasic contractions of the reticulum (Carr, 1974).

A flow of mandibular secretion was also present when animals licked the metabolic crates in which they were kept and chewed at their feet and coat. This is interpreted to involve both buccal and central stimulation. The organization of the medullary centres regulating mandibular secretion thus appears to differ from those controlling the parotid gland as reductions of parotid flow were noted by Carr (1974) when the animals behaved similarly. An enhanced resting mandibular flow was also observed in one atropinized animal (Figure 5.3C) which showed signs of restlessness. This is

suggestive of involvement of the sympathetic nervous system with psychically stimulated secretion - the other animals (Figures 5.2B and 5.3B) which remained calm showed no flow after atropine injection.

Evidence from other sources suggests that a general central nervous system arousal is associated with the expectation of eating when food is present in front of the animal at the usual feeding time. Electroencephalographic records taken immediately before the usual time of feeding often show a desynchronization and an increase in the frequency of cortical discharges (see Wyrwicka, 1967). Psychic effects on salivary secretion have been reported previously in ruminants (Scheunert and Trautmann, 1921; Blokh, 1939; Denton, 1957*b*; Carr, 1974). In the present study, teasing the sheep with food usually caused an increase in mandibular secretion over a period of several minutes (Figures 5.2A; 5.3A) as was the case in Scheunert and Trautmann's (1921) experiments in the sheep and in Blokh's (1939) with the bull. This response to teasing appears typical of the conditioned salivary reflexes described by Pavlov (1910) and which involve visual, auditory, and olfactory sensations. It seems probable that not all the mandibular flow under these conditions truly reflects an increased secretion by the gland. The abrupt rise in mandibular secretion evident in Figure 5.2 is interpreted to arise from contraction of myoepithelial cells in the gland and thus to be an expulsion of saliva.

Psychic stimulation at least of mandibular salivary secretion appears, from the current results, to be associated with efferent impulses in both the parasympathetic and sympathetic pathways. This suggestion arises from the fact that the secretion induced by teasing was completely blocked by atropine in two of four animals (Figure 5.3B) while the other atropinized animals showed a diminished response which was completely annulled if both atropine and propranolol were given (Figure 5.3C). The higher concentration of protein secreted in the saliva during teasing gives added

support to the idea of greater sympathetic activity during teasing as does the work of Blair-West and Brook (1969) who reported an increased heart rate in sheep when teased with food.

Evidence indicating the involvement of parasympathetic efferent activity in secretory responses induced by teasing has been provided by Denton (1957*b*) for the parotid secretion in a sheep in which the sympathetic innervation had been extirpated. The rapid rises in the volume and the output of acid and pepsin from fundic pouches of the abomasum in response to teasing in sheep have also been suggested to be mediated parasympathetically (McLeay and Titchen, 1970).

It is interesting that rumination, although causing a pronounced secretion from the parotid gland (Carr, 1974; Brightling *et al.*, 1977), has no effect on the mandibular. It seems that the buccal stimulation of remastication is not an effective stimulus to mandibular flow. This may be due to the failure of the moist regurgitated food to stimulate mandibular secretion as suggested by Hungate (1966) or it may reflect a central inhibition *via* the salivary centres in the medulla oblongata. Electroencephalographic records during rumination showed a quiescent (synchronized) pattern which differed markedly from that recorded immediately at the feeding time (Wyrwicka, 1967).

The present observations confirm that a rapid and sustained mandibular flow in sheep is only elicited in response to feeding, and that flow almost ceases when eating stops. This secretion during eating doubtless arises as a reflex event involving afferent impulses from receptors in the lips, mouth, tongue and pharynx (Babkin, 1950), and perhaps additionally a minor contribution from stimulation of the forestomach and oesophagus (Phillipson and Mangan, 1959; Ash and Kay, 1959; Kay, 1960). The profuse secretion initially present during feeding cannot, however, be regarded as only resulting from stimulation of the mouth and gut, as psychic stimulation at the time of feeding appears also

to be involved. Blair-West and Brook (1969) recorded rises in both heart rate and blood pressure soon after feeding commenced. These changes seem to be temporally related to the highest mandibular flows which were present in the present study.

The slower flow rates observed as eating continued are thus argued to be due in part to a reduction in psychic stimulation but no doubt an important factor is also a reduction in appetite and a consequently slower rate of eating. Pauses in secretion are present with each pause of eating. It seems that the progressive slowing of secretion can be accounted for by the reduced afferent stimulation that occurs as eating becomes less avid, and that there is no strong evidence for the intervention of inhibitory stimuli. However, although both the rates of mandibular secretion and the amounts of food consumed gradually declined as eating continued the decrease in flow rate does not appear to be in direct proportion to the amount of food being eaten. Recently, Carr *et al.* (1977) have reported that the hourly mandibular secretion in the first 3 hours of eating in sheep with once daily feeding (1200 g lucerne chaff) was 0.20 $\pm$ 0.02, 0.28 $\pm$ 0.02, and 0.58 $\pm$ 0.10 g saliva per g food eaten, respectively. The increased mandibular secretion per g food eaten in the later stages of feeding may help compensate for the marked reduction in parotid secretion that occurs at this time (Carr and Titchen, 1978).

Experiments with autonomic blocking agents indicate that the reflex changes in flow induced by feeding are mainly parasympathetic and that the β-adrenergic innervation has a relatively minor effect on the volume of saliva produced. The fact that the same dose of atropine that was found to block mandibular secretion evoked by parasympathetic stimulation completely (see Chapter IV) caused only a partial blockade during feeding, suggests that grossly increased amounts of neurotransmitters are released in response to the natural stimulus of feeding compared with electrical stimulation of parasympathetic nerves. It may be,

however, that as in the parotid glands of rabbits (Alm and Ekström, 1976), dogs (Ekström and Holmberg, 1972) and cats (Ekström and Emmelin, 1974), the chorda lingual nerve is not the only parasympathetic route to the gland, or that the frequency of electrical stimulation of nerves was much below that present naturally. Further studies on secretory responses after denervation will help to clarify this matter.

b. Protein Secretion

The experiments on anaesthetized sheep (see Chapter IV) indicate that β-adrenergic stimulation has a pronounced effect on protein secretion by the mandibular gland and also causes a characteristically meagre flow. The reverse effects appear to be mediated *via* cholinergic stimulation. Variations in the levels of salivary protein concentration can thus be argued to reflect the nature of autonomic nerve activation of the gland. The variable protein concentration of the resting secretion in conscious animals found both within sheep on different days and between sheep can be accounted for in this way.

The higher protein concentration in saliva obtained in the prefeeding period when the animals were teased with food appears to involve sympathetic activation arising from psychic stimulation. There may be a more general activation of the sympathetic nervous system at this time as has already been described (Blair-West and Brook, 1969).

The present observations on salivary responses to feeding show that there is often a higher level of salivary protein secretion in the first 30-60 minutes of feeding than later in the eating period (Figure 5.4). Although, in some sheep, the peak protein concentrations occurred during the first 2-8 minutes of eating (Figure 5.5), in others no peak was observed (Figure 5.6). These initial higher protein concentrations are interpreted to arise from sympathetic activation associated with a state of excitement in hungry animals. A number of effects associated with increased sympathetic activity in the initial stages of

eating have been reported in the calf, lamb and sheep (Blair-West and Brook, 1969; Bloom, Edwards, Hardy, Malinowska and Silver, 1975). Bloom, Edwards and Vaughan (1973) demonstrated a rise in plasma glucocorticoid due to sympathetic activation while Blair-West and Brook (1969) showed that reflex responses to rapid eating include an abrupt rise in arterial blood pressure within 5 minutes of starting to eat and also a gradual elevation in heart rate. The blood pressure in their sheep returned to normal levels over 30 minutes of eating whereas heart rate after reaching a peak rate, gradually declined although persisting at higher than prefeeding levels for up to 3 hours. These workers also recorded a rise in plasma renin concentrations which reached a peak within 30 minutes of starting to eat and then gradually fell to near normal levels 3 hours after feeding started. They proposed that the increased release of renin (due to a sudden hypovolemia caused by the rapid salivary secretion) during feeding depended upon increased sympathetic activity. In addition, the stimulating action of angiotensin II on the sympathoadrenal system has also been recently reviewed by Fitzsimons (1980). On the other hand, animals that ate their food intermittently over 12-24h showed no regular changes in the plasma renin concentrations (Blair-West and Brook, 1969).

It therefore seems likely that the lower level of protein secretion after eating has been in progress for 30-60 minutes is due to a reduction of sympathetic activity as the initial excitement at receiving food abates. Other factors may also be important. The decline in protein secretion is possibly in part due to a depletion of preformed secretory proteins - in one acute experiment, the repeated intravenous injection of single doses of isoprenaline (2-4 $\mu g\ kg^{-1}$) during a background parasympathetic stimulation resulted in a progressive decline in protein secretion (see Chapter IV).

The involvement of a β-adrenergic innervation in protein secretion during the early eating period was confirmed by

observations on the effects of administering propranolol prior to feeding. This resulted in a marked reduction (ranging from 24.6% to 70.7%) in salivary protein secretion during the initial feeding period.

Preliminary electrophoretic studies showed that the saliva secreted early in the eating period contained an intensely staining protein band (Figure 5.8A) and that the intensity of this usually decreased as eating continued. This protein band corresponds to the major soluble protein band of sympathetically evoked saliva and its intensity was also diminished following propranolol injection (Figure 5.8B). This suggested role of sympathetic innervation in causing protein secretion is further supported by the experiments involving partial blockade with atropine (0.1 mg kg^{-1}) in which in the first 20 minutes of eating higher protein concentrations were present than in the control experiments.

The variations in the protein concentration of mandibular saliva secreted later in the feeding periods that were often observed both within and between sheep on different days could arise through variations in chewing activity. Gjörstrup (1980*b*) has recently shown that during feeding sympathetic secreto-motor nerves can be activated *via* both taste stimulation and chewing, while the parasympathetic activity is mainly influenced by chewing. Chewing has been suggested as a major stimulus to the secretion of salivary amylase from the parotid gland of the rat (Schneyer, 1974) and the rabbit (Gjörstrup, 1980*a*) and in man chewing dental wax also causes secretion of amylase (Newbrun, 1962). Four types of mechanoreceptors with different response characteristics have been found in the sheep's tongue apart from the taste receptors (Iggo and Leek, 1967). It is possible that differences in the magnitude of the afferent discharge from mechanoreceptors stimulated by chewing contributed to variations in the reflex secretion of protein in the present study through sympathetic pathways.

2. Morphological Changes Induced by Eating

In the present study the morphological features characterizing the secretory cycle associated with feeding were investigated in the mandibular glands of sheep. These cellular changes which underly glandular activity appeared mainly as changes in the secretory granules of secretory endpieces, i.e. discharge-synthesis-storage, as was recognized by Heidenhain (1868). Less pronounced changes were observed in the cells of striated ducts.

a. The Effects of Eating

(i) Changes in Secretory Endpieces

Eating in sheep is a potent secretory stimulus effectively causing exocytosis in both the acinar cells and demilunes of their mandibular glands and a reduction in the size of secretory endpieces. The structure of acinar cells apparent during eating (Figures 5.8B-D; 5.9A,B) indicates that the cells were hyperactive. Specifically, there were signs of synthetic activity occurring concurrently with exocytosis (Figure 5.9A,B), and these were more marked with increased duration of eating. The inference of hyperactivity is based on the apparent reorganization of intracellular organelles which included the appearance of small newly formed granules, abundant dilated RER and mitochondria in regions which, in resting glands, were notable for their content of closely packed mature secretory granules. Morphological changes in acinar cells induced by feeding resemble, in more extreme form, those seen after stimulation of the chorda lingual nerve in the anaesthetized sheep, and thus strongly suggest the involvement of parasympathetic activity throughout eating.

Changes in the demilunes, on the other hand, were usually most marked early in the feeding period and were somewhat less after eating for 4h (Figures 5.8; 5.10A,C). The presence of fused granules with a loss of their typical substructures is similar to the appearance after sympathetic

nerve stimulation (see Chapter IV). This finding is most readily explained by assuming the sympathetic innervation is particularly active in the early stages of the eating period. This contention, of sympathetic involvement in the secretion of granules from the demilunes, is strengthened by the absence of gross morphological changes when the β-adrenergic blocking agent propranolol was given before eating began (Figures 5.8C; 5.10B). These morphological changes in both acinar cells and demilune under natural reflex stimulation are taken to imply that both divisions of autonomic nervous system are normally involved in regulating secretion from this gland. Further, the nature of the morphological changes in the presence of a β-blocker is evidence that changes in the acinar cells do not depend upon the sympathetic innervation.

A common finding was considerable variation in the degree of degranulation between cells of individual secretory endpieces and of adjacent lobules during eating. This was more obvious in the demilune cells which showed variation of secretory granule fusion and discharge after eating for 1h and, particularly after eating for 4h, reappearance of darkly stained granules to varying degrees. Variable changes in different cells of the same gland have been reported recently in the parotid acinar cells of rats fed on hard chow (Harrop and Garrett, 1974). Garrett and his co-workers (Harrop and Garrett, 1974; Garrett *et al.*, 1977) have proposed that these variations probably indicate an asynchrony in the traffic of nerve impulses to the gland. Gjörstrup's (1980*b*) observation in the rabbit that chewing and taste stimuli evoked different afferent discharges and contributed to asynchronous firing in different efferent pathways during naturally induced reflex secretion is particularly relevant. A similar explanation may be applied to the sheep gland where it was apparent from direct observation that the nature of chewing differed throughout extended periods of eating.

(ii) Changes in Striated Ducts

Babkin (1950) commented that "whenever the salivary gland passes from a state of resting to a state of activity, the cells of striated ducts change their appearance". This proposition was influenced by the work of Rawlinson (1935) who established that morphological changes occurred in the striated duct cells of cat mandibular glands after the stimulation of parasympathetic or sympathetic nerves, following an intravenous injection of adrenalin, and after chronic denervation. With the limited histological techniques available at that time, he (Rawlinson, 1935) described the occurrence of ragged edges of luminal membranes and the presence of unstained clear areas in the cells of striated ducts both after moderate (1-2h) stimulation of the chorda tympani nerve and 2 weeks after section of this nerve. He also reported a lack of uniformity in the striated duct cells after sympathetic stimulation and interpreted these changes as evidence of secretory activity. Recently, Garrett and Kidd (1975) have reinvestigated this and confirmed that changes do occur in the fine structure of striated duct cells under similar treatments.

The present study revealed that eating caused a depletion of PAS-positive materials (presumably glycogen and neutral glycoproteins) in the cells of striated ducts as was also the case with sympathetic nerve stimulation (see Figure 4.11). This depletion of neutral glycoproteins suggests a secretory role in the striated ducts and thus agrees with results described by Garrett and co-workers (Garrett and Kidd, 1975; Garrett *et al.*, 1977) in the cat mandibular gland. They reported a depletion of neutral glycoprotein (which they identified to be kallikrein) in the apical regions of striated duct cells after sympathetic nerve stimulation (Garrett and Kidd, 1975) and during ductal ligation (Harrison and Garrett, 1976). Loss of glycogen was also observed after both sympathetic and parasympathetic nerve stimulation (Garrett and Kidd, 1975). Examination of the fine structure of striated duct cells in the sheep

glands provided morphological evidence supporting the prevailing view on the secretory role of striated duct cells. This arises from the apparent discharge of small dense bodies from the apical regions of striated duct cells into the duct lumen during eating (Figure 5.11B). These dense bodies were normally present in apical regions in the resting state.

Another obvious change in the fine structure of striated duct cells in the sheep mandibular gland following eating was the appearance of numerous empty vesicles, dilated fragments of RER and lysosomes (Figure 5.11A). The presence of intracellular vesiculation has also been mentioned in the striated duct cells of the cat mandibular gland after the stimulation of its sympathetic (Emmelin *et al.*, 1977*a*) and parasympathetic innervation (Garrett and Kidd, 1975) though without comment. The significance of these vesicles is not clear. Those bounded by membranes have been speculated to represent secretory granules that have lost their secretory material (Tandler, 1978). This explanation seems inappropriate for the present study since no secretory granules or other cellular structures were identified in the resting glands that relate to the empty vesicles seen after eating at electron microscopic level. This was despite the fact that depletion of PAS-positive materials (both glycogen and neutral glycoprotein) was associated with eating. Such PAS-positive materials seem likely to have been stored throughout the cytoplasm without packaging into secretory granules.

The presence of intracellular vesiculation may have some connection with the ductal reabsorption that is known to occur during saliva formation and which is believed to occur in the striated and excretory ducts (Young and van Lennep, 1979). Because the salivation induced by eating was more profuse than with nerve or pharmacological stimulation and because changes in the electrolyte content in the final saliva are closely related to changes in the flow rates (Kay, 1960) which in turn reflect the salivary osmolarity, it is possible that dilation and vesiculation of various membrane-bounded compartments, e.g. RER, Golgi

complexes, are due to changes in the state of hydration in the cytoplasm (Cheville, 1976). The striated duct cells after a period of eating (Figure 5.11) seem to exhibit features characteristic of cell swelling as indicated by highly dilated RER. Cheville (1976) mentioned that cell swelling is often a subtle change and is best seen in the rapidly metabolizing parenchymal cells of liver, heart, and kidney. The appearance of the active striated duct cells after reflex stimulation also bears similarities to those described by Cheville (1976) in the early stages of cell degeneration. Changes in the striated duct cells it appears, however, are reversible.

b. Changes after Eating (Postprandially)

Reaccumulation of secretory granules occurred within the secretory endpieces when eating ceased. The fine structure of these cells clearly showed that they were in an active phase of synthesis and storage (Figures 5.9C,D; 5.10E-H). It is fascinating that the secretory granules in the demilunes, although present in large numbers at 1h and 2h postprandially, exhibited substructures quite different from the mature granules seen in the resting gland (Figure 5.10E-H). They contained both bipartite granules with electron dense and pale characteristics and tripartite granules. The latter consisted of a small component with aggregated fibrillar structure but lacked the membrane-like boundary (Figure 5.10H) typical of the granules in the resting state. This difference in the appearance of these granules probably reflects different stages of maturation of secretory granules. The structure present in Figure 5.10E-H perhaps represents granules during the process of assembly. Different rates of formation of different components appears to take place within the secretory granules.

The fine structure of striated duct cells showed no differences in appearance between the postprandial and resting states indicating a rapid recovery of these cells

after cessation of natural stimulation. This seems reasonable because there was no obvious cell damage that could be observed in response to feeding. Unlike the situation with sympathetic or combined nerve stimulation, feeding did not induce changes in the basal regions of the striated duct cells.

3. The Effects of Cannulation

Chronic mandibular duct cannulations were only associated with definite structural changes in the gland of one sheep (sheep No. 1). The other six sheep showed no histological differences between their cannulated and uncannulated glands and they exhibited similar and consistent secretory responses to feeding.

The markedly reduced mandibular flow in sheep No. 1 during eating (Figure 5.12) was related to a partial constriction of its duct at the point of cannulation. The morphological changes of this gland after 30 days of the cannulation can be accounted for by the combined effects of increased resistance to flow and retention of secretion. These included a marked increase in the diameter of all ductal and acinar lumina (Figure 5.13) and dilation of intercellular spaces in ducts (Figure 5.14B,C) and of intercellular canaliculi in demilunes (Figure 5.14A).

Studies on the backdiffusion of materials from the ducts of resting mandibular glands in anaesthetized dogs (Parsons and Garrett 1977; Emmelin *et al.*, 1977*a*) have shown that there is movement from the ductal lumina into the interstices of the gland. After ductal injection of horseradish peroxidase solution, Parsons and Garrett (1977) found that movement of this enzyme occurred principally *via* the intercellular junctions between acinar cells. Nevertheless, the distribution of this marker in the gland was usually irregular and diffuse cytoplasmic uptake of horseradish peroxidase by some striated duct cells was also evident. It is speculated that, in the presence of high intraluminal pressure, backdiffusion of fluids from the ductal lumina into the interstices probably occurred by similar routes in the sheep mandibular gland.

Although fluid was also accumulated in the intercellular spaces between duct cells, it was not possible to be certain about the precise sites of fluid leakage.

The development of oedema of the interstitial and interacinar spaces as a result of ductal obstruction was described by Garrett (1966*b*) in the cat mandibular glands. He found that the oedema occurred in the first two days after ductal ligation at a time when the acinar tissues and nerve fibres remained unchanged. The morphological changes in the sheep gland after 30 days of partial ductal obstruction thus resemble the early stages of complete ductal occlusion. The retention of fluid in the gland probably accounts for the greater weight of this gland compared with its uncannulated counterpart.

The dilation of basolateral intercellular spaces in striated duct cells appears to be similar to the ballooning distension demonstrated by Emmelin *et al*. (1977*a*), although to a lesser degree and apparently without damage to these cells.

The changes in the secretory endpieces in the present study were relatively minor. Only slight reductions in cells size were noted and the secretory granules which packed in the demilune cells did not show their characteristic appearance (Figure 5.14). Further, no glandular atrophy was detected in contrast with the effects of complete ductal obstruction reported in other species, where a progressive atrophy of secretory endpieces was observed (Garrett, 1966*b*; Tamarin, 1967; Harrison, 1972). However, the slight changes in the demilunes may account for the secretion of saliva of lower protein concentration during eating (Figure 5.12).

Changes in the secretory cells, if marked, would be expected to cause an even greater reduction in secretion as Emmelin *et al*.,(1974) showed in glands in which the main salivary duct had been ligated for 12 to 81 days. More severe changes due to complete obstruction have been reported recently in the bovine mandibular gland (Birtles,

1981). In this gland there was evidence of duct and end-piece damage and also a reduction in the gland weight after a cannula had been completely blocked for 9 days.

CHAPTER VI

GENERAL DISCUSSION & CONCLUSIONS

The overall objective of the present study has been to explain the normal activity in a ruminant salivary gland. The present study confirmed (e.g. Figure 5.2) the earlier observations of Scheunert and Trautmann (1921) and Kay (1960) that in sheep the mandibular gland only secretes markedly during eating, and the major thrust of the present experiments was, therefore, directed towards explaining the changes observed during feeding. The feeding studies were facilitated by the use of chronically cannulated mandibular salivary ducts for the collection of saliva. In none of the cannulated animals was there evidence of disordered eating or digestion as a result of the secretion of one mandibular gland failing to enter the alimentary canal: all of the animals retained a normal appetite and body weight and, with the one exception in which the cannula was partially blocked, had normal glands at the completion of the experiments after some six weeks of study. Additionally, the cannulated and uncannulated glands of each animal were of comparable weight, and showed no gross differences from the glands of other, uncannulated sheep. The mandibular secretion obtained in chronic experiments was, therefore, assumed to have come from normal glands.

Details of the structure of the resting mandibular gland have already been considered in chapter III. These demonstrated that while considerable differences exist at the fine structural level between the glands of sheep and those of other ruminants, the general features of the gland were consistent with it serving a similar role in each of these different animals. This role appears mainly to be the production of mucus glycoproteins which serve as lubricants both for the food and digestive apparatus itself.

It seems that the mucous acinar cells are a major source of the mucus glycoproteins. This view is based upon histochemical staining and on electron microscopic investigations of acinar cells which demonstrated the presence of electron lucent granules (Figure 3.8) characteristic of those containing glycoproteins (Gallagher *et al.*, 1969). In contrast, the seromucous demilunes contained secretory granules of varying electron density which typically displayed a tripartite structure (Figure 3.10). They appear to be the source of the secretory proteins, of unknown structure and function, which were demonstrated immunocytochemically in the present study (Figures 4.22; 4.23). Although no supporting evidence was obtained, the secretory materials in these demilunes probably include the acid phosphatase and non-specific esterase enzymes demonstrated histochemically by Chauncey and Quintarelli (1963) in both the demilunes and duct cells, but not acini, of ovine mandibular glands. These enzymes were also detected in the mandibular saliva (Chauncey *et al.*, 1961).

The striated duct epithelium of sheep had four cell types. The fine structure of the principal cell type (type-I) resembled that of the principal cells of other ruminants and of many non-ruminants - they were tall columnar cells with electron lucent cytoplasm and possessed well developed basal infoldings with associated mitochondria and apically contained small dense bodies (Figure 3.13). The other cell types were present on an occasional basis.

Myoepithelial cells were found within the basement membranes of both secretory endpieces and intercalated ducts (Figures 3.1C; 3.10A; 3.11), and were arranged so that their cell processes embraced both of these structures. They were seldom seen, however, in association with the striated ducts although alkaline phosphatase activity indicated they were present at the excretory ducts.

Nerves were seen to be closely associated with myoepithelial cells, secretory endpieces, intercalated ducts, striated ducts and blood vessels. Histochemically, both cholinergic (Figure 3.6B,C) and adrenergic (Figure 3.5B,C) nerve fibres were found densely arranged around the secretory endpieces, coursing alongside the intralobular duct system and with the

walls of blood vessels. This distribution was confirmed at the ultrastructural level (e.g. Figure 3,16) where nerve fibres were also found in close proximity to myoepithelial cells (Figure 3.16B).

The finding of axons containing a population of large granular vesicles in the same unmyelinated nerve bundles as axons containing small agranular and granular vesicles (Figure 3.16A) is interesting because the former axons show the general characteristics in their granule content, of the peptidergic nerves which have recently been described in other species (Kidd and Garrett, 1979). Comparative evidence from immunocytochemical studies on the cat's mandibular gland (Uddman, 1980), as well as electron microscopic evidence (Kidd and Garrett, 1979; Al-Gailani, Asking, Emmelin and Garrett, 1981) suggests that a correlation between vasoactive intestinal polypeptide (VIP) and large granular vesicles exists. It is of interest that stimulation of the chorda lingual nerve in both the cat and sheep is reported to cause an atropine resistant vasodilation in the mandibular gland (Beilenson *et al.*, 1968). Detailed studies using VIP-immunocytochemical techniques are obviously required in the sheep and are a fascinating area for future study.

Morphologically, eating had profound effects on both acini and demilunes in that the acinar cells showed extensive degranulation and concurrent synthesis which was proportional to the duration of eating (Figure 5.9), while in demilunes, in contrast, changes were not proportional to the duration of eating but were greatest in its early phases (Figure 5.10). In addition, eating caused a depletion of PAS-positive materials from the striated duct cells, but at the fine structural level only slight changes were present - a discrepancy which has not been adequately accounted for. Discharge of small dense bodies from the apical regions of the striated duct cells was also noted with eating, thus supporting the possibility of striated duct cells contributing to the final secretion as in many other species (see Young and van Lennep, 1978).

The experiments involving direct stimulation of autonomic nerves (chapter IV) provided evidence that in the sheep mandibular gland, parasympathetic and sympathetic nerves exert their major roles on different types of secretory cells. Thus, parasympathetic stimulation mainly affected the acinar cells, whereas the sympathetic nerves affected the demilunes and striated ducts - findings which support the general concepts developed from observations on the cat's mandibular gland by Rawlinson (1933, 1935) and more recently by Garrett and Kidd (1977). These workers found, in a gland which also contains two types of secretory cells, that sympathetic nerve stimulation causes a reduction in demilune size and is mainly responsible for the secretion of peroxidase and its synthesis in the demilunes. Parasympathetic stimulation causes extensive degranulation of the acini with secretion of acid phosphatase (Garrett and Kidd, 1977). However, it remains a possibility that this action on either demilune or acinar cells is not exclusive since in the experiments of Garrett and Kidd (1977), and in the present study, a low level of protein was found in cholinergically evoked saliva even after injection of both α and β-adrenergic antagonists.

From a comparison of the morphological changes noted after autonomic nerve stimulation in acute experiments with those present after eating, it is plausible to suggest that natural stimulation of the gland involves both divisions of the autonomic nervous system. The parasympathetic innervation probably plays an important role throughout the eating period whereas sympathetic activity is only obvious under particular conditions. Although synthesis and reaccumulation of secretory granules were in evidence after eating, this study gave no information about the roles of autonomic nerves in the reappearance of secretory granules after either eating or autonomic nerve stimulation.

Turning now to the role of autonomic nerves in changing the flow and composition of saliva, it appears that the predominant effect of sympathetic activity was to stimulate

protein secretion whereas the main contribution to the volume of saliva secreted arose from parasympathetic activity.

In the acute experiments, the saliva secreted in response to stimulation of the chorda lingual nerve or injection of carbachol, appeared more quickly, flowed more copiously and contained lower levels of protein than that obtained during stimulation of the cervical sympathetic trunk. Simultaneous stimulation of both types of nerves resulted in a secretion which, although of higher protein content, was never more voluminous than that obtained by stimulation of parasympathetic nerves alone. Since mucus glycoprotein makes an important contribution to the viscosity of saliva (see Herp *et al.*, 1979), its secretion from the gland may require large amounts of fluid to wash it through the ductal system, and it is appropriate, therefore, that both fluid and mucus glycoprotein secretion should be mediated *via* the same control mechanism.

That the effect of sympathetic nerves on secretion of salivary proteins is mainly *via* β-adrenoceptors can be inferred from the fact that in the present study, and that of Patterson and Titchen (1977), saliva obtained during stimulation of a cervical sympathetic trunk or after administration of isoprenaline had the highest protein concentrations. Further, propranolol but not the α-adrenergic antagonist, phentolamine, blocked the increases in flow and protein secretion produced by sympathetic stimulation (Figures 4.3; 4.4). The evidence from the present experiments involving electrical or pharmacological stimulation was supported by electrophoretic studies which established the presence of particular protein components (Figure 4.7) in saliva secreted during sympathetic or sympathomimetic stimulation, but showed them to be virtually absent in parasympathetically evoked saliva.

The actions of parasympathetic and sympathetic nerves in the sheep thus conform to a pattern that is general for salivary glands in many species: "a principal role of the adrenergic innervation is to cause secretion of gland proteins, with elaboration of water only a minor role; a primary role

of the cholinergic innervation is to cause elaboration of copious quantities of fluid" (Schneyer, 1974).

From the responses to stimulation of autonomic nerves in anaesthetized animals, it therefore seems reasonable to argue that the reflex secretion induced by eating is mediated *via* both parasympathetic and sympathetic pathways. Judged by the large quantities of saliva produced during the feeding period, the major contribution is from the parasympathetic innervation. Further evidence for this arose from the reductions in secretion during eating that occurred following injection of atropine. A reservation must be expressed, however, about the use of atropine in conscious animals: it seemed, at the same dose, to be less effective than in acute experiments yet the use of higher dose rates risks appreciable central nervous excitation (Goodman and Gilman, 1975) and, therefore, difficulties in interpretation of results.

The secretion of the higher levels of protein during the early stages of eating appeared to be mainly a sympathetic effect. Three pieces of evidence support this view. First, in conscious animals the injection of propranolol resulted in marked reductions in protein secretion during eating without causing associated changes in the salivary flow (Figures 5.4-5.6). Secondly, the gel electrophoretic pattern of saliva collected during the eating period contained a major protein band (Figure 5.7A) which corresponded to the soluble protein band X of sympathetically evoked saliva from acute experiments, and which propranolol treatment confirmed was under β-adrenergic control (Figure 5.7B). Thirdly, morphological studies on the mandibular glands taken from animals after eating for one hour, showed changes in the demilunes (fusion and discharge of their secretory granules) which could be prevented by the β-adrenergic blocker propranolol (Figure 5.10A,B).

Both physiological and morphological evidence obtained in the present study supports a role for myoepithelial cells, in the normal secretion of mandibular saliva in sheep, along

the lines reviewed by both Young and van Lennep (1977) and Emmelin and Garrett (1979). Contraction of myoepithelial cells will, as well as reducing the loss of fluid into tissues and providing support for the underlying parenchyma, speed the flow of preformed saliva by increasing the secretory pressure and shortening the ductal lumina (Young and van Lennep, 1977; Emmelin and Garrett, 1979). A rapid flow of secretion occurred in response to teasing (Figure 5.2), on first eating (Figure 5.2), and at the onset of sympathetic nerve stimulation when imposed on a parasympathetic background (Figure 4.6). It is likely that myoepithelial cell contraction is especially important in assisting the secretion of viscous saliva from this gland as compared with, for example, the watery secretion of the parotid gland. The viscosity of the mandibular secretion is not uniform during feeding but tends to be greater at times of high protein concentration, such as in the early stages of eating, and teasing, when sympathetic activity is increased. It is speculated that this would also be a time of greater myoepithelial cell activity, since the present acute experiments suggested an association between myoepithelial cell and sympathetic activity.

Although the physiological investigations on mandibular flow in the present study gave support for a sympathetically induced myoepithelial cell contraction, it was not possible to eliminate a contribution from a parasympathetic innervation of these cells because of the prominence of a copious flow in response to cholinergic stimulation.

It is worth noting that the sympathetic nerve stimulation employed in the anaesthetized animals, while of an extent which gave clearcut morphological effects, appeared to exceed that which occurs naturally, in that eating appeared to cause neither cellular disruption of demilunes nor discharge of contents of secretory granules from acinar cells or demilunes into the basolateral canaliculi. That changes of this nature were present in acute experiments (Figures 4.12C; 4.13), even with a background of parasympathetic stimulation (Figure 4.18B), suggests more than one factor may have been

contributing to their appearance. As discussed earlier (chapter IV), some of the cellular disruption may have arisen from the effects of high levels of sympathetic transmitters on basolateral plasma membranes, but there may also have been a disruptive effect due to increased intraluminal pressure with intense myoepithelial contraction. Further, the highly viscous secretion that was produced in the acute experiments no doubt contributed to an increased pressure in the secretory endpieces and ductal lumina. With the natural stimulation afforded by eating there was always a voluminous secretion of fluid, and this would have diluted viscosity changes.

Sheep often inhabit environments in which they find it necessary, to eat and digest large quantities of dry, fibrous food. On a diet of this type a prime requirement of saliva is to facilitate chewing and swallowing. Once the initial problem of chewing and swallowing has been overcome the liquid nature of the gastric contents reduces the need for further secretion during, for example, the remastication of rumination. The results described in this thesis demonstrate that the mandibular glands of sheep are admirably equipped, structurally, to satisfy the demands of providing lubricant and that the mechanisms that stimulate them are so organized that secretion occurs at the time of greatest need, that is during eating.

Possible Future Studies

The results of the present study provide a background knowledge of autonomic nervous influences on the structure and function of the mandibular gland in sheep. However, this basic information needs expansion in many areas. In particular further systematic morphological studies using quantitative morphometric or stereological analysis of changes in the acinar cells, demilunes and striated ducts, and their cytoplasmic components, are now necessary to give a wider understanding of the detailed changes in these cells and of the kinetics of their secretory activity under varying conditions of stimulation.

In addition, in order to gain a better understanding of the autonomic nervous influences on the mandibular gland, more detailed ultrastructural examinations of the innervation pattern in the salivary gland are required. The electron microscopic methods employed (with Karnovsky's fixative and osmium tetroxide) in the present study were adequate only for a general survey of sites of innervation, and did not always allow precise definition of the types of nerves observed. The use of improved techniques for the preservation of small granular vesicles (such as glutaraldehyde-sodium chromate-osmium tetroxide-uranyl acetate [Tranzer and Richards, 1976]) is thus desirable. The techniques could be profitably performed under various conditions, e.g. on normal glands as well as after specific denervations and reinnervation and would logically be accompanied by parallel histochemical investigations of AChE and biogenic amine activity.

Another area for future research is the study of the nature and origin of the salivary proteins secreted during eating and nerve stimulation. This study would necessarily involve improved methods for separation and analysis of the proteins with the objective of isolating specific proteins and determining, perhaps by immunocytochemistry, their cellular origins. Such a study would provide for a better understanding of the functions of the salivary proteins produced, particularly during sympathetic stimulation. Clark and Reid (1974) have commented on the possibility of foam-stabilizing agents being present in saliva and contributing to the condition of bloat - a minor problem in sheep but one of considerable economic importance in cattle. Foam-stabilizing actions of salivary proteins are recognized (D.H. Carr, personal communication) and it is at least theoretically possible that differences in the susceptibility of animals to bloat involve unexplained differences in their sympathetic activation of protein secretion.

The suggestion by Bartley, Meyer and Fina (1975) that immunoglobulins of salivary origin may influence the

population of micro-organisms present in the rumino-reticulum adds to the need for improving knowledge of protein secretion. Secretory IgA is present in relatively high levels in the mandibular saliva of sheep (Cripps and Lascelles, 1976) but details concerning its secretion and function are lacking.

Finally, in future studies the use of chronically cannulated animals is likely to become increasingly important in investigations on the composition and control of mandibular secretion, especially since cannulations can be established without any apparent discomfort to the animals. Ideally, future developments would include re-entrant technique of cannulation so that objections to diversion of the saliva can be countered. As well as being useful in the protein studies referred to above, the influence of different experimental conditions, such as different diets, or different conditions of feeding, e.g. *ad libitum* feeding, would also seem important areas to examine and their study should lead to a better knowledge of the mandibular gland and the contribution it normally makes to digestion in sheep.

APPENDIX 1

THE UNLABELLED PEROXIDASE ANTI/PEROXIDASE (PAP) METHOD (After Taylor, 1978; Sternberger, 1979)

Procedure

1. Tissues are fixed in 10% phosphate-buffered formalin solution or Bouin's fixative, processed routinely, and embedded in paraffin.
2. Sections 4 µm thick are left in an oven at 56°C for 30 min. (Sections are deparaffinized immediately before staining).
3. The deparaffinized sections are treated with 0.3% H_2O_2 in methanol for 30 min to block endogeneous peroxidases (Burns, 1975).
4. Wash in buffered saline (Tris/phosphate buffered saline, 0.1M, pH 7.6) for 15 min.
5. Incubate for 10 min in normal swine serum (NSS, diluted 1:3 in Tris/phosphate buffer). The excess serum is drained, not washed off.
6. Apply the primary antibody produced in a rabbit (i.e. rabbit anti-sheep salivary protein) for 30 min in appropriate dilutions (i.e. 1:2,000). Always use NSS: buffer, 1:9, as diluents for antibodies and PAP reagent.
7. Wash in buffered saline and follow by a 10 min application of NSS (as in step 5).
8. Treat with swine anti/rabbit (Dakopatts A/S, Denmark) 1:20, 15 min.
9. Wash in buffered saline and apply NSS (as in step 5).
10. Incubate in PAP (Dakopatts A/S) 1:20 for 30 min and follow by a 15 min wash in buffered saline.

11. Incubate in 3-3', 4-4' diaminobenzidine tetrahydrochloride (DAB) solution (Sigma Chemicals Co., St. Louis) according to Graham and Karnovsky (1966) - preincubate sections in DAB solution in 0.05M Tris buffer, pH 7.6, 1 min for even staining and after addition of 0.02% H_2O_2 continue staining for about 3 min with intermittent agitation.

12. Wash the sections in water for 5 min.

13. Counterstain with either haematoxylin or 1% light green.

14. Dehydrate, clear and mount in DPX.

During the procedure in steps 5-10, sections are held in moist chambers (such as provided by a wet pad of filter paper in a Petri dish). The reagents used in immunostaining are applied as drops on top of the sections using one to three drops of solution depending on the size of the section. It is worth etching the area around each section with a glass pencil to minimize running of the solutions used for staining. Any isotonic buffer, pH 7.2 to 7.6, is suitable, except that for the enzyme reaction itself (step 11) 0.05M Tris buffer, pH 7.6 is required. Keep sections covered all the time except when actually applying reagents to avoid contamination, since momentary exposure of a section to highly diluted antiserum from another section during washing could result in staining (Sternberger, 1979).

APPENDIX 2

THE ETHANOL-CHLOROFORM PROCEDURE FOR PARAFFIN WAX PROCESSING

Process	Reagent	Time
Dehydration	70% ethanol	1 hour
	95% ethanol	1 hour
	100% ethanol	1 hour
	100% ethanol	1 hour
	100% ethanol	2 hours
Clearing	chloroform	1 hour
	xylene	1 hour
	xylene	1 hour
Impregnation	paraffin wax 56°C M.P.	2 hours
	paraffin wax 56°C M.P.	2 hours

After processing, tissue blocks were embedded in 56°C melting point paraffin wax.

APPENDIX 3

STAINING METHODS FOR PARAFFIN SECTIONS

A. PERIODIC ACID SCHIFF/HAEMATOXYLIN (PAS/H)

Method

1. Dewax and bring sections to water.
2. Oxidize in 1% periodic acid for 8 min.
3. Wash in running tapwater for 5 min.
4. Wash in at least 3 changes of distilled water.
5. React with Schiff's reagent for 10 min.
6. Wash in running tapwater for 10 min.
7. Stain in Mayer's haemalum for 5 min.
8. Rinse in tapwater.
9. Blue in Scott's tapwater for 2 min.
10. Rinse in tapwater.
11. Dehydrate, clear and mount in DPX.

To Prepare Schiff's Reagent

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

B. ALCIAN BLUE, pH 2.5 (AB)
(After Culling, 1974)

Method

1. Dewax and bring sections to water.
2. Stain in 0.3% Alcian Blue 8GX (Gurr), pH 2.5 for 10 min.
3. Rinse in tapwater.
4. Counterstain as desired (e.g. with H&E; option).
5. Dehydrate, clear and mount.

C. ALCIAN BLUE, pH 2.5/PERIODIC ACID SCHIFF/HAEMATOXYLIN (AB/PAS/H)

Method

1. Follow AB method (B) steps 1-3.
2. Continue with PAS/H method (A) steps 2-11.

D. ALCIAN BLUE, pH 1.0/ALCIAN YELLOW, pH 2.5 (AB, 1.0/AY)
(After Ravetto, 1964; Lev and Spicer, 1964)

Method

1. Dewax and bring sections to water.
2. Stain in 0.5% Alcian Blue 8GX in 0.1N HCl, pH 1.0 for 10 min.
3. Blot sections dry with filter paper (do not wash).
4. Stain in 0.5% Alcian Yellow GX in 3% acetic acid, pH 2.5 for 10 min.
5. Rinse in tapwater.
6. Counterstain as desired (e.g. with H&E; option).
7. Dehydrate, clear and mount.

APPENDIX 4

METHODS FOR ENZYME HISTOCHEMISTRY

A. THE CALCIUM COBALT SULPHIDE METHOD FOR ALKALINE PHOSPHATASE

(Recommended by E.W. van Lennep).

Incubation Medium

This is mixed, immediately before use, in the following order:

3% sodium β-glycerophosphate (substrate)	10 ml
2% sodium diethyl barbiturate, pH 9.4	10 ml
distilled water	5 ml
2% calcium chloride	20 ml
5% magnesium sulphate	1 ml

Procedure

1. Sections are either unfixed or fixed in phosphate buffered formalin solution for 10 min.
2. Incubate at 37°C for 30 min in incubation medium.
3. Rinse in 1% calcium chloride solution, 2 changes, 2 min each.
4. Transfer to 2% cobalt nitrate solution, $Co(NO_3)_2.6H_2O$, and leave for 4 min.
5. Rinse in distilled water, 3 changes, 2 min each.
6. Place in diluted ammonium sulphide solution for 2 min (in fume cupboard).
7. Rinse in running tapwater 5 min.
8. Mount in glycerin jelly or aqueous mounting media.

Control sections are incubated in the incubation medium without adding the substrate. Tissues from kidney are used as standard for alkaline phosphatase reaction.

Result

Black or brownish black deposits indicate alkaline phosphatase activity.

B. METHOD FOR ACETYCHOLINESTERASE ACTIVITY

(After Gerebtzoff, 1959)

Incubation Medium

This is mixed, immediately before use, in the following order:

0.1M acetic acid/acetate buffer, pH 6.0.....100.0 ml

(made up by adding 6.0 g glacial acetic acid to about 700 ml distilled water and 1M NaOH (about 110-120 ml) run in under a pH meter, stirring constantly to pH 6.0, then made up to 1 litre).

distilled water ..... 76.0 ml

3.75% glycine ..... 4.0 ml

0.1M cupric acetate ..... 4.0 ml

(4.99 g of cupric acetate/a total of 230 ml)

acetylthiocholine iodine solution (as substrate) 10.4 ml

(freshly prepared by adding 150 g acetylthiocholine iodide in 7.8 ml distilled water and 2.6 ml 0.1M cupric acetate).

Procedure

1. Cryostat sections (10-20 µm thick) are fixed for 10 min at room temperature in phosphate buffered formal saline solution.
2. Wash in buffer.
3. Incubate in the incubation medium (of appropriate substrate) for about 1-4 hours. (Usually pre-incubate for 30 min with the appropriate inhibitor before incubation in the medium containing both substrate and inhibitor).
4. Wash in distilled water.
5. Dip into diluted ammonium sulphide.

6. Counterstain with Mayer's haematoxylin (optional).

7. Mount with aqueous mountant.

Result

The reaction products appear dark brown.

C. THE SUCROSE-POTASSIUM-PHOSPHATE GLYOXYLIC ACID (SPG) METHOD FOR BIOGENIC-AMINE ACTIVITY
(de la Torre and Surgeon, 1976).

Preparation of SPG Solution

1. Dissolve sucrose (10.2 g) and KH_2PO_4 (4.8 g) crystals in 100 ml distilled water using a magnetic stirrer.

2. Add 1.5 g of glyoxylic acid crystals (98% free acid monohydrate, Sigma) and stir until solution is clear.

3. Titrate the solution to pH 7.4 with 1N NaOH.

4. Make up a final volume of 150 ml with distilled water.

5. Use at room temperature.

Procedure

1. Fresh tissues are directly frozen in cryostat at $-30^{\circ}C$ on a pre-cooled chuck and cut to 20-30 µm thickness.

2. Mount on clean slides and allow to air dry.

3. Quickly dip in room temperature SPG solution 3 times (1 dip/sec).

4. Sections are air dried for 3-5 min, using a hair dryer.

5. Place in an oven (C. Reichart AG., KT100, Germany), heated to 80 $\pm$ $1^{\circ}C$, for 5 min.

6. After removing from oven, mount with DPX mountant.

7. The coverslipped slides are then placed for 90 sec on a hot plate (C. Reichart AG, HK120, Germany) set at exactly 80°C to remove auto-fluorescent air bubbles.

8. Examine under a Leitz fluorescent microscope (E. Leitz, Inc., N.Y., N.Y.), using Schott BG12 or UG1 as a primary lamp filter and either Zeiss 41 + 47 or 44; or K530 as secondary barrier filters (Lindvall and Björklund, 1974).

9. Photograph on a high speed (400 ASA Ektachrome daylight) film.

APPENDIX 5

ANALYSIS OF EFFECTS OF PROPRANOLOL ON SECRETORY RESPONSES OF THE MANDIBULAR GLAND IN CONSCIOUS SHEEP DURING EATING

Analyses were carried out using the mean responses of individual animals ignoring within animal variation. That is, the error mean square in the analyses of variance is due to variation between animals within treatment groups. Similarity of the feeding responses (eight intervals from 0-10 through 120-130 min) for the control and treatment groups was examined by partitioning the between time, and time by treatment, mean squares using orthogonal polynomials for linear, quadratic and cubic effects of time.

The results can be summarized as follows:

		FLOW RATE		PROTEIN CONC		PROTEIN OUTPUT	
Source of Variation	df	MS	F	MS	F	MS	F
Between Treatment	1	0.0030		7.5600	146.9***	13.9080	42.6***
Between Time	7						
Linear effect	1	9.4711	68.2***	0.2225	4.3*	21.0950	64.5***
Quadratic effect	1	0.1683		0.0261		0.2647	
Cubic effect	1	0.2024		0.0213		0.4326	
(Remainder	4)						
Treatment x Times	7						
Linear deviation	1	0.0168		0.5382	10.5**	2.1480	6.3*
Quadratic deviation	1	0.0148		0.0135		0.2353	
Cubic deviation	1	0.0001		0.0042		0.1171	
(Remainder	4)						
Within Treatment	80	0.1389		0.0514		0.3265	

(MS = Mean Square)

Levels of significance are denoted:

* p <0.05

** p <0.01

*** p <0.001

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