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THE STORAGE AND SECRETION OF PROTEIN BY
THE RAT SUBMANDIBULAR GLAND

A thesis submitted to the University of
Durham by Stuart Macdonald Wilson, B.Sc.
(Dunelm) , a candidate for the degree of
Doctor of Philosophy in the faculty of
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ABSTRACT

The proteins secreted by the rat submandibular gland in response to various secretory stimuli have been investigated. The β -adrenergic agonist isoprenaline primarily caused the secretion of glycoproteins, although a number of other species were also present, and the saliva contained a moderate level of proteolytic activity. The α -adrenergic agent phenylephrine acted very differently, causing the secretion of a considerable amount of proteolytic activity, with only a very small amount of glycoprotein present in the secretion. Stimulation of the cervical sympathetic trunk caused the secretion of fluid at all frequencies from 1-20 Hz, and this was mediated via both α and β -adrenoceptors. However, at 20 Hz the proteins secreted were essentially similar to those obtained after phenylephrine, indicating that β -adrenoceptors were not involved in protein secretion. At the lower frequency of 5 Hz protein secretion appeared to be mediated via both α and β -adrenoceptors. Acetyl β -methyl choline and physalaemin were involved mainly with fluid secretion, although acetyl β -methyl choline caused the secretion of some proteolytic activity. Surprisingly this was not the case for parasympathetic nerve stimulation.

The effect of reserpine upon the carbohydrate histochemistry of the rat submandibular gland was also investigated. This agent was found to cause an accumulation of carboxylated glycoproteins within the acini, which normally contain a neutral glycoprotein population. This result contrasts with the findings of previous investigations, where reserpine caused a simple accumulation of the glycoproteins normally present in the acini, with no qualitative changes.

The investigations presented in this thesis were conducted by its author. No part of the work has previously been presented in fulfilment of the regulations for any degree or diploma, either at this University or any other institution. Some of the material presented has been previously published elsewhere (Jones, Smith and Wilson, 1982; Jones and Wilson, 1982; Wilson, 1982).

S.M.Wilson

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GENERAL INTRODUCTION

i) Historical introduction.

The first investigations of the salivary glands were conducted by the early anatomists, including Versalius, Nuck and Wharton (see Burgen and Emmelin, 1961). These early workers described the positions of the major salivary glands and of the ducts through which their secretions passed to the mouth. However, in this same period, very few physiological investigations were conducted, and so the means by which salivary secretions were formed, and the flow of saliva controlled, remained poorly understood. Perhaps the first investigation of the physiology of salivary secretion was conducted by Regner de Graaf, who provided dogs with chronic pancreatic and submandibular fistulae. Although his book "De Succo Pancreatico" (1677) deals almost exclusively with observations on the pancreas, in a short section on the submandibular gland he reports that either the sight or smell of food may cause a flow of saliva, and suggests that a major function of salivary secretions may be to facilitate swallowing. The use of chronic fistulae, a technique pioneered by de Graaf, is still a valuable physiological tool (see Gjørstrup, 1980a, 1980b).

The 19th Century was a period in which experimental physiology became an important branch of medical science and investigations of the physiology of salivary glands became more frequent. Tiedman and Gmelin (1826) investigated the chemical composition of dog parotid

saliva and noted the presence of potassium as the major cation; the fact that saliva was alkaline, and the ability of acetic acid to precipitate salivary proteins. They were also able to demonstrate the ability of sheep saliva to degrade the starch in crushed oats; this was the first demonstration of salivary amylase.

One of the most important discoveries was made by Ludwig (1851), who unambiguously showed that salivary secretion occurred in response to stimulation of the autonomic nerves. This observation was later confirmed by Bernard (1864), who also noted that prior section of the lingual nerve could abolish the reflex secretion normally obtained from the canine submandibular gland when sapid substances were placed on the tongue. These observations served to demonstrate that salivation was a process under active nervous control. Both Adrian and Eckhard (1860) and Heidenhain (1878) showed that although stimulation of either the sympathetic or parasympathetic nerves could cause secretion from the dog submandibular gland, the two nerves exerted different effects. The saliva induced parasympathetically was characteristically copious and watery whilst sympathetic saliva consisted of a few drops of very viscous secretion. To rationalise these observations Heidenhain proposed the existence of two classes of glandular nerve fibres. The first he termed "secretory fibres" which predominated in the parasympathetic nerves and controlled the flow of water and inorganic electrolytes into the saliva. The second class were termed "trophic fibres" and predominated in

the sympathetic nerves. These fibres were thought to somehow control the solubility of secretory proteins stored within the gland. According to Heidenhain's scheme the composition of salivary secretions would reflect the relative numbers of secretory and trophic fibres in the glandular nerves. Thus the sympathetic nerve supplying the cat submandibular gland contains more "secretory" fibres than its canine equivalent as the sympathetic saliva from the dog submandibular gland is much more viscous.

Heidenhain(1878) also conducted histological investigations using the rabbit parotid gland. He observed that the number of small dark granules present in the acinar cells was diminished by sympathetic nerve stimulation, but not in response to injections of pilocarpine (a parasympathomimetic compound). Heidenhain suggested that these granules ~~are~~ contained; secretory proteins ready to enter the saliva in response to "trophic" stimuli. Heidenhain's view of the innervation of the salivary glands was challenged by Langley (1878, 1879) who maintained that all glandular nerve fibres could cause the secretion of both the inorganic and organic components of saliva. He attributed the elevated levels of protein in sympathetically evoked saliva to the vasoconstriction which had been observed to accompany sympathetic stimulation (Bernard, 1858). Langley maintained that this vasoconstriction would compromise the flow of water into saliva and thus lead to the production of a secretion containing much more organic

material. Langley was also unable to confirm Heidenhain's observation that pilocarpine failed to cause degranulation of the rabbit parotid acinar cells, thus lending support to his own view of salivary innervation (Langley, 1879).

More recent investigations of salivary secretion have progressed in the light of much new information regarding the interactions between autonomic nerves and their effector organs. In particular the concept of distinct α and β -adrenoceptors, first introduced by Ahlquist (1948) has proved to be especially useful. The sympathetic vasoconstriction first described by Bernard (1858) is now known to be mediated via α -adrenoceptors. Sympathetic secretory responses may be mediated by β -adrenoceptors as in the cat submandibular gland (Emmelin, Schneyer and Schneyer, 1973); by α -adrenoceptors as in the dog submandibular (Emmelin and Holmberg, 1967) or by a combination of both as in the rat submandibular gland (Emmelin, Holmberg and Ohlin, 1967). Emmelin and Holmberg (1967) used α -adrenergic antagonists to abolish the sympathetic vasoconstriction in the dog submandibular gland and thus were able to study the β -adrenoceptor mediated secretion in the absence of any adverse vascular events (see also Emmelin, 1955 for an account of a similar study in the cat). Even under these conditions the secretory response to sympathetic nervous stimulation remained small. This observation indicated that the sympathetic nerves act more in the manner envisaged by Heidenhain than by

Langley. In general most modern authors now accept that stimuli which act via β -adrenoceptors exert a "trophic" effect in that they cause the secretion of a small volume of a fluid which contains a considerable amount of protein. Activation of muscarinic receptors or α -adrenoceptors causes a "secretory" response, with the secretion of a considerable quantity of fluid and relatively little protein (see Young and van Lennep, 1979). To a large extent this view is based upon studies of amylase secretion by the parotid glands of rats (see Schneyer and Hall, 1967; Schramm and Selinger, 1976) and rabbits (Asking and Gjørstrup, 1980a, 1980b).

ii) Morphology of the salivary glands.

In most mammalian species there are 3 pairs of major salivary glands, the submandibular, parotid and sublingual glands. A number of carnivores may have additional pairs of glands, e.g. the zygomatic gland (gland of Nuck) in the dog and the cat, and the molar gland of the cat. In addition to these major paired glands large numbers of "minor" salivary glands are found in the submucosa below the labial, lingual, palatine and pharyngeal epithelia (see Young and van Lennep, 1978).

Traditionally, salivary glands have been described as serous or as mucous on the basis of the consistency of their secretions. The secretions of the mucous glands, such as the major sublingual gland of the rat, are rather viscous and tend to draw out into long threads. Conversely the secretions of serous glands such

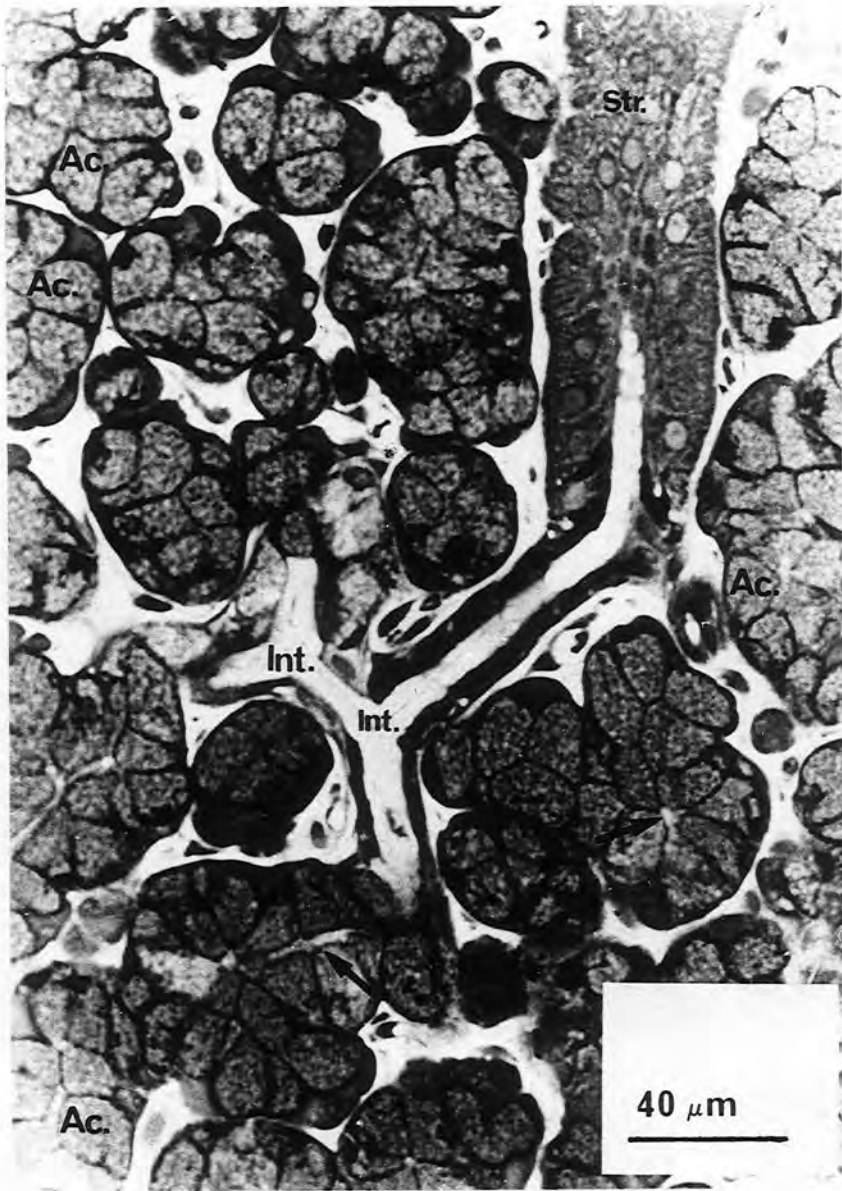
as the rat parotid gland, have a much more watery consistency. A number of glands are classified as seromucous as their secretions are of an intermediate viscosity (e.g. the rat submandibular gland).

All mammalian salivary glands have an acinar-lobular structure. In general they consist of numerous acini or secretory end pieces whose lumina drain to a common excretory duct via a dendritic ductal system. The acinus itself consists of a cluster of cells around a central lumen, and is the most distal unit of the secretory apparatus. The acinar cells of the serous glands. (e.g. the rat and rabbit parotid glands), may be observed to contain numerous dense granules, which both Heidenhain⁽¹⁸⁷⁸⁾ and Langley⁽¹⁸⁷⁸⁾ correctly interpreted as containing proteins destined for secretion. (Castle, Jamieson and Palade, 1975; Keller, Robinovitch, Iverson and Kauffman, 1975). The acinar cells of the mucous and seromucous salivary glands also contain secretory granules but these are of a rather different character to the typical serous granules (see Young and van Lennep, 1978, 1979). Typically they are much larger, more variable in shape and have a translucent granular appearance under the electron microscope.

The lumina of the acini are confluent with the lumina of the ductal system. All of the acini eventually lead to a common excretory duct, but considerable regional differentiation of the ductal epithelium exists. The most distal portion of the ductal tree consists of

small cuboidal cells, each almost entirely filled by its nucleus (see plate i.1). These ducts are called the intercalated ducts and typically are rather short, tending to be inconspicuous in histological sections. The intercalated ducts lead in turn to the striated ducts, which in most glands represent the most prominent duct type. These ducts are bounded by columnar epithelial cells containing prominent basal striations. In the submandibular glands of most adult rodents, and a number of other species, the distal regions of these ducts may become further modified by the inclusion of numerous small electron dense granules. Most authors now accept that these "granular ducts" have the ability to secrete protein (e.g. Matthews, 1974; Al-Gailani, Garrett, Kyriacou and Leite, 1980). In the mouse submandibular gland, the granular ducts are much more prominent in the male, this difference being abolished by castration (Junquera, Fajer, Rabinovitch and Frankenthal, 1949; Sreebny, Meyer, Bachem and Weinmann, 1957; Sreebny, Meyer and Bachem, 1958). The significance of this sexual dimorphism is not understood. Proximally, the striated ducts lead to the excretory ducts, through which the secretions pass to the mouth. Along most of their length these ducts consist of a simple columnar epithelium, but at the oral end this may become compound. The above description is of a "generalised" salivary gland, but considerable differences in morphology exist, both between equivalent glands in different species and between the different glands in the same species(see

PLATE 1.1 : LIGHT MICROGRAPH (1 μ m EPON EMBEDDED SECTION STAINED WITH TOLUIDINE BLUE) OF THE RAT MAJOR SUBLINGUAL GLAND, SEVERAL MUCOUS ACINI (Ac.) ARE VISIBLE, EACH CONSISTING OF A GROUP OF SECRETORY CELLS SURROUNDING A CENTRAL LUMEN (\rightarrow), THE ACINI DRAIN INTO THE INTERCALATED DUCTS (INT), WHICH IN TURN LEAD TO THE STRIATED DUCTS (STR).
PHOTOGRAPH BY COURTESY OF DR. C.J.JONES.



Young and van Lennep, 1978 for an extensive review of this subject).

iii) The innervation of the salivary glands.

Most salivary glands receive innervation from both branches of the autonomic nervous system. All glands appear to receive a dense parasympathetic innervation, however, the sympathetic innervation is much more variable. In some glands, such as the rat major sublingual, very few sympathetic fibres innervate the secretory elements, although the arteries supplying the gland are very well innervated by numerous adrenergic fibres (Jones, 1980). However, in the neighbouring submandibular gland the secretion structures are innervated by a dense plexus of sympathetic fibres.

In the rat the sympathetic fibres destined for the submandibular glands arise at the level of the 1st and 2nd thoracic vertebrae (Templeton, 1979) and run rostrally in the cervical sympathetic trunk to the superior cervical ganglion. From here, the postganglionic sympathetic fibres follow the arterial supply to the salivary glands themselves.

The parasympathetic fibres to the submandibular and major sublingual glands leave the skull with the facial nerve, and branch from it as the chorda tympani which then joins the lingual nerve. As the lingual nerve approaches the submandibular and sublingual ducts the glandular fibres leave the nerve and follow the ductal tree into the glands themselves. There is no discrete

parasympathetic ganglion, although several ganglion cells are located close to the junction of the duct and lingual nerve (Lichtmann, 1977). In the rat a number of parasympathetic nerve fibres are known to reach these glands via the lingual branch of the trigeminal nerve in addition to the route described above (Hellekant and Kasahara, 1973).

Parasympathetic fibres to the parotid gland run via the tympanic branch of the glossopharyngeal nerve to the otic ganglion. The postganglionic fibres then run to the gland itself in the auriculo-temporal nerve.

iv) The formation of saliva.

Most authors accept that saliva is formed by a process essentially similar to the "two stage" hypothesis originally proposed by Thaysen (1960), and which has since been extensively reviewed (Young 1979; Young and van Lennep 1979; Young and Schneyer, 1981). This hypothesis proposes that the acinar cells secrete a "primary" saliva whose ionic composition is essentially similar to that of plasma. As this secretion passes along the ductal system its composition is modified by the reabsorption of sodium and chloride, and the secretion of potassium and bicarbonate. Support for this hypothesis has come from a number of experimental approaches:

- a) Using a technique known as "micropuncture" it has proved possible to collect samples of saliva from the most distal regions of the ductal system

(Martinez, Holzgreve and Frick, 1966; Young and Schögel, 1966). Analysis of these samples has shown, at least in some glands, that the ionic composition of the primary saliva is similar to that of plasma. However, in all glands studied the composition of the primary saliva remains constant when the gland is stimulated, either sympathetically or parasympathetically (Young and Schogel, 1966; Martin and Young, 1971; Kaladelfos and Young, 1973, 1974), even though the composition of the final saliva may vary considerably.

- b) If one accepts that the secretion of potassium and bicarbonate and the reabsorption of sodium and chloride occur within the ductal system, then the final concentration of these electrolytes would depend upon their concentrations in the primary saliva, and upon the "contact time" between the secretion and the ductal epithelium. At high flow rates this contact time would be minimal and so the final saliva would resemble the primary secretion. At lower flow rates there would be considerable opportunity for the secretion to become modified and so the final saliva would contain considerably less sodium and chloride, and more potassium and bicarbonate than the primary saliva. Several investigators have demonstrated that this type of relationship does exist in a large number of salivary glands secreting in response to stimulation of the parasympathetic nerves (e.g.

Schneyer and Hall, 1965; Yoshida, Sprecher, Schneyer and Schneyer, 1967; Smaje, 1973); administration of parasympathetic agonists (e.g. Martin and Young, 1971; Young and Martin, 1971; Martinez, Quissell, Wood and Giles, 1975a), and administration of the α -adrenoceptor agonist phenylephrine (Martinez et al., 1975a; Young, Cook, Jones, McGirr and Thompson, 1979). Activation of β -adrenoceptors causes the production of a saliva containing more potassium and bicarbonate and less sodium than either "parasympathetic" or " α -adrenergic" saliva secreted from the same gland at a similar flow rate (Schneyer, 1962; Yoshida et al., 1967; Young and Martin, 1971; Martinez et al., 1975a). The "two-stage" hypothesis clearly cannot account for this difference unless the ion transporting activities of the ductal epithelium are under autonomic control. There is a considerable body of evidence from experiments on the artificially perfused rabbit submandibular duct which indicate that this is the case (Denniss and Young, 1975; see Young and van Lennep, 1979).

- c) The enzyme Na^+ , K^+ stimulated adenosine triphosphatase (Na^+ , K^+ ATPase) is considered to play a central role in the active movement of ions, and is almost invariably found in high concentrations at sites where such movements occur. This enzyme has been localised in the cat

submandibular gland using ^3H -labelled ouabain (Bungard, Møller and Poulsen, 1977). These results show that large amounts of this enzyme are present in the ^{basal} epithelium of the striated ducts, whilst only small amounts are found in the cells of the intercalated ducts and of the acini. This supports the view that it is the cells of the striated ducts ^{which} are responsible for the modification of the primary saliva. (see Young, 1979; Young and van Lennep, 1979; Young and Schneyer, 1981).

v) The secretion of proteins by salivary glands.

Since Tiedman and Gmelin (1826) demonstrated that addition of acetic acid to dog parotid saliva causes the precipitation of the viscous material it has been known that salivary glands may secrete considerable quantities of protein. The autonomic control of protein secretion has been investigated using the parotid glands of rats and, to a lesser extent, those of rabbits (Schneyer and Hall, 1967; Schramm and Selinger, 1976; Gjørstrup, 1979; Asking and Gjørstrup, 1980a, 1980b). In general, protein secretion appears to be mediated via β -adrenoceptors, whilst muscarinic and α -adrenoceptors control the secretion of fluid.

In a recent review, Young and Schneyer (1981) recognised two distinct classes of secreted proteins. Extrinsic proteins are synthesised outside the glands and enter the saliva by crossing the salivary epithelium; proteins such as serum albumin fall into this category.

However, by far the largest group of salivary proteins are the intrinsic proteins, which are synthesised within the glandular epithelia specifically for export. The means by which these proteins are synthesised and packaged into typical secretory granules has been the subject of much investigation. Blobel and Dobberstein (1975) proposed that the initial stage of this process is the synthesis of a "signal peptide". This stage occurs at the free ribosomes in the cytoplasm, and the short signal peptide in some way causes the ribosomal/messenger RNA complex to become bound to the membrane of the rough endoplasmic reticulum. The subsequent synthesis of the secretory protein itself occurs at this location, with the growing polypeptide chain projecting into the cistern of the rough endoplasmic reticulum. Once this process is complete, the polypeptide chain is released into the endoplasmic reticulum and the signal peptide removed by a specific protease. The nascent protein is now processed within the rough endoplasmic reticulum until it reaches the Golgi apparatus. The means by which this transfer occurs has been investigated by Palade and his co-workers (see review by Palade, 1977). They observed newly synthesised material (labelled with ^{14}C) to be transferred from the rough endoplasmic reticulum to the Golgi apparatus in a number of small "transitional vesicles". Studies using high voltage electron microscopy have suggested that these vesicles in fact represent a 3 dimensional network of fine tubules along which the newly synthesised material is transported by

some unknown mechanism (Rambourg, Marraud and Chretien, 1973; Rambourg, Clermont and Marraud, 1974). The secretory granules themselves are formed from "condensing vesicles" which "pinch off" from the Golgi, becoming more compact and homogenous as they migrate to the cell's apical surface. Protein secretion is thought to occur when the membrane bounding the secretory granule fuses with the secretory cell's apical membrane, thus discharging the contents into the lumen. As would be anticipated, stimulation of the sympathetic trunk, a process which causes the secretion of a large amount of amylase from the rat parotid gland, also causes an extensive loss of granules from the gland's acinar cells (Garrett and Thulin, 1975). However, an equally large amount of amylase may also be secreted upon prolonged stimulation of the parasympathetic nerves, but in this situation no loss of granules occurs. (Garrett and Thulin, 1975). Clearly then, at least in this gland, alternative pathways for the export of protein do exist.

There have been several studies of the protein populations secreted by a number of different salivary glands and these are discussed in an introduction to Chapter 1.

vi) The functions of saliva.

The main function of salivary secretions is to lubricate the epithelial surfaces of the buccal cavity, and thus facilitate chewing and swallowing. Saliva also plays an important role in protecting the teeth, and in

situations where the flow of saliva is prevented, (eg. surgical removal of the salivary glands), dental decay soon becomes established (see Burgen and Emmelin, 1961). Salivary secretions are known to contain the antibacterial enzyme lysozyme (Fleming, 1922; Fleming and Allison, 1922) which degrades certain carbohydrates present in bacterial cell walls, causing the bacterium to lyse. However, the function of this enzyme is unclear, particularly as glycoproteins present in saliva are known to reduce this enzyme's antibacterial actions.

Extensive licking of the fur is part of the grooming behaviour of most mammals, and it may be anticipated that at least some of the salivary glands may show specific adaptations to this behaviour. Al-Gailani et al. (1980) have proposed that the high levels of proteases found in the salivary glands of a number of species, including the cat, dog and guinea-pig may represent such an adaptation.

Perhaps the best known salivary enzyme is amylase which digests starch. It is tempting to ascribe a digestive function to this enzyme, however in humans it is unlikely that the food is retained in the mouth long enough for any significant digestion to occur. Several ruminant species "chew the cud" and thus retain the food in the mouth for prolonged periods. However, these species have only very low levels of amylase in the saliva. It therefore appears unlikely that this enzyme contributes significantly to the digestion of carbohydrates, especially as this process also occurs in

the duodenum catalysed by pancreatic amylase.

In several mammalian species, evaporation of saliva from the tongue and buccal cavity provides an important means of thermoregulatory cooling. In the dog, the flow of saliva in response to an elevated body temperature (induced by tetrahydronaphthylamine) appears to be mediated via the parasympathetic nerves (Aleksandrov, 1939).

vii) The purpose of the present study.

The aim of the present study is to investigate the population of proteins secreted by the rat submandibular gland in response to a range of autonomic stimuli.

Although the secretion of electrolytes by the major salivary glands of laboratory animals has been extensively studied, there have been surprisingly few investigations of the proteins secreted by these glands. The proteins present in rat parotid saliva have been studied to a certain extent (Hall and Schneyer, 1964a, 1964b; Robinovitch and Sreebny, 1969; Keller et al., 1975; Robinovitch, Keller, Johnson, Iverson and Kauffman, 1977) but studies of the proteins secreted by the rat submandibular gland have met with only limited success. (Hall and Schneyer, 1964a). The reason for this appeared to be, in part, that there is much less protein in submandibular saliva than in parotid saliva. However, it also seems that submandibular proteins do not separate out into such clearly defined bands as the

parotid proteins, as Hall and Schneyer (1964a) found that prior concentration of submandibular saliva did not greatly improve the resolution.

In the present study preliminary experiments were conducted using native gel electrophoresis (Davis, 1964). Although such techniques have given good resolution from parotid saliva (Robinovitch and Sreebny, 1969; Keller et al. , 1975; Robinovitch et al. , 1977) the results using submandibular saliva were poor, with the proteins separating out into broad, diffuse and rather faintly stained bands. A major problem of this project became to develop an electrophoretic technique which could give good resolution of submandibular proteins. The ideal technique proved to be sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis on gels incorporating a 3.3 - 24 % polyacrylamide gel concentration gradient. This technique gave good resolution from small amounts of protein, and enabled the molecular weights of secreted proteins to be estimated.

The first two chapters of this thesis give an account of the results of these studies. Chapter 1 describes the proteins secreted by this gland in response to pharmacological activation of α -adrenoceptors, β -adrenoceptors and muscarinic receptors. This work was then extended by investigating the proteins secreted in response to stimulation of the autonomic nerves, these results are presented in Chapter 2. The secretion of proteases, by the rat submandibular gland, is discussed in Chapter 3. The fourth Chapter presents the results of

a histochemical investigation of the effects of reserpine upon the glycoproteins present in the rat submandibular gland. The view that the reserpine-treated rat submandibular gland represents a potentially useful model of the human genetically transferred disease cystic fibrosis is discussed.

CHAPTER 1 :THE PROTEINS SECRETED IN RESPONSE TO
AUTONOMIC AGONISTS.

INTRODUCTION

The first electrophoretic studies of the proteins present in salivary secretions employed paper electrophoresis to fractionate whole human saliva. The resolution achieved was limited, but this work served to demonstrate that saliva contained a large number of protein species (Kinnersly, 1953). These proteins included some with antibiotic properties (Kinnersly and Hogberg, 1955); certain blood group substances, and at least one protein which could bind calcium (Kinnersly and Leite, 1957).

Similar techniques have been applied to study the proteins secreted by individual salivary glands (Drevon and Donikian, 1956; Köstlin and Rauch, 1957; Ferguson, Krahn and Hildes, 1958), and, in the case of human parotid and submandibular saliva, different populations of proteins were secreted by each gland. Ferguson et al. (1958) noted that the relative amounts of the various proteins present in human parotid saliva depend upon whether the sample was collected during a period of basal secretion or during a period when the flow rate was elevated by placing dilute acetic acid in the mouth.

In an attempt to further improve the separation of human salivary proteins, moving boundary electrophoresis was employed. Using this technique, in

which proteins are separated in free solution, up to 12 different components could be resolved from parotid or submandibular saliva (Patton and Pigman, 1957; Zipkin, Adamik and Saroff, 1957). In comparable studies using paper electrophoresis 5-9 bands were distinguished (Köstlin and Rauch, 1957; Ferguson et al. , 1958). These secretions have also been examined using the analytical ultracentrifuge (Patton and Pigman, 1957, 1958), where parotid and submandibular saliva were respectively resolved into 3 or 4 components. The major peak in parotid saliva (sedimentation coefficient 4.1 S) has been identified as amylase.

Most of the studies described have indicated that the proteins found in salivary secretions are quite different to those found in serum. However, the presence of small quantities of 4 serum proteins in human parotid saliva has been demonstrated by immunodiffusion, a technique which is very sensitive to small quantities of protein (Ellison and Mashimo, 1958; Ellison, Mashimo and Mandel, 1960).

Subsequent electrophoretic studies of human saliva have used increasingly sophisticated techniques to reveal a steadily more complex population of secretion proteins. Hoerman (1959) used starch gel electrophoresis to demonstrate 12 protein species in parotid saliva, whilst D'Silva and Ferguson (1962) were able to resolve 17 using a very similar technique. Caldwell and Pigman (1965) employed polyacrylamide gel electrophoresis and were able to resolve 21 bands from human submandibular

saliva. In a recent study, the use of SDS - polyacrylamide gel electrophoresis enabled 24 different species to be resolved from human parotid saliva (Shiba, Sano, Nakao, Yoshida, Cho and Hayashi, 1980).

Experiments carried out using saliva collected from human volunteers have revealed a complex population of protein species, some of which have been isolated chromatographically and partially characterised (e.g. Bennick and Connell, 1971; Hay, 1973, 1975; Bennick, 1975; Hay and Oppenheimer, 1974; Juriaanse and Booiij, 1979a, 1979b). However, these experiments have not investigated the way in which the protein population secreted by a particular gland may be dependant upon the nature of the secretory stimulus, although the results of Ferguson et al. (1958) suggest that this may be important.

There have been surprisingly few studies of the proteins secreted by the salivary glands of laboratory animals. The first such investigation was conducted by Sweeney, Shaw, Childs and Weissberger (1962), who used paper electrophoresis to fractionate whole rat saliva secreted after injections of pilocarpine. These authors found a very large number of protein species to be present and, by selective sialoadenectomy, showed that most of them originated from the parotid glands. Similar experiments showed the submandibular gland complex to secrete most of the fluid.

The first study of the proteins present in the secretions of particular salivary glands was conducted by

Hall and Schneyer (1964a). Like Sweeney et al. (1962) they employed paper electrophoresis to study pilocarpine evoked saliva, but collected the secretions from the exposed ducts of the parotid, submandibular and major sublingual glands. Unfortunately, however, these authors were only able to obtain good resolution with parotid saliva. It was apparent that a number of protein species were present in the secretions of the submandibular and major sublingual glands but these did not separate into clearly defined bands. In a subsequent study, the authors confined their attentions to the secretions of the parotid gland (Hall and Schneyer, 1964b).

As with the studies of human saliva, the use of polyacrylamide gel electrophoresis resulted in improved resolution. Robinovitch and Sreebny (1969) used this technique to fractionate rat parotid saliva. They were able to resolve a total of 23 different species. These authors also employed substrate digestion techniques to locate the secreted enzymes amylase, deoxyribonuclease and ribonuclease on the gels after electrophoresis.

Abe and Dawes (1978) used polyacrylamide gels to fractionate rat parotid and submandibular proteins secreted in response to stimulation of the parasympathetic nerves or administration of autonomic agonists. Whilst the rat parotid gland appeared to secrete a constant population of proteins, the protein population secreted by the rat submandibular gland was

dependant upon the nature of the secretory stimulus.

In the present chapter, the proteins secreted by the rat submandibular gland in response to administration of autonomic agonists have been studied by SDS polyacrylamide gel electrophoresis on gels incorporating a 3.3 - 24% polyacrylamide gel gradient. This technique has enabled good resolution to be obtained from relatively small amounts of material, and thus permitted fairly small doses of sialogogues to be administered by intravenous (IV) injection. This approach has avoided the prolonged maximal secretory responses obtained when autonomic agonists are administered by intraperitoneal (IP) injection.

MATERIALS AND METHODS

i) Animals and anaesthesia.

Male rats of a Wistar strain weighing 220-380g were anaesthetised with sodium pentobarbitone (May and Baker "Sagatal"; 60 mg/kg, IP) and the trachea and a femoral vein cannulated. Supplementary anaesthetic was administered IV in doses of 2-5 mg as required and rectal body temperature was maintained at 37-38°C.

ii) Collection of saliva.

The right submandibular duct was exposed below the mylohyoideus and transversus mandibularis muscles as described by Ohlin (1965) and a polythene cannula (Portex PE-10) introduced at a point rostral to the junction

between the duct and the lingual nerve. Salivation was evoked by the IV injection of acetyl β -methyl choline chloride (20-40 $\mu\text{g}/\text{kg}$), carbamyl β -methyl choline chloride (50-100 $\mu\text{g}/\text{kg}$), phenylephrine hydrochloride (100-200 $\mu\text{g}/\text{kg}$) or L-isoprenaline hydrochloride (30-60 $\mu\text{g}/\text{kg}$). Aliquots of saliva were collected either directly into small, ice-cold polythene test tubes or into 20 μl capillary micropipettes (Drummond "Microcaps") which were then emptied into polythene tubes. Three samples, each corresponding to a different agonist, were collected from most animals used. Each animal preparation was allowed to rest for at least 40 minutes between doses of different agonists and care was taken to ensure that the saliva in the dead space of the duct and cannula was discarded. In the case of phenylephrine and isoprenaline the volume of saliva secreted was fairly small, and so a preliminary dose, approximately 50% of the main dose, was given to flush out the cannula. In most animals phenylephrine caused a respiratory arrest, and so the animal was artificially ventilated via the tracheal cannula until spontaneous respiration returned.

Some samples were processed immediately, but most were stored at -18°C for up to 6 weeks pending analysis.

iii) Protein estimation.

The concentration of total protein in some of the samples was determined by the method of Lowry, Rosenbrough, Farr and Randal (1951) using bovine serum albumin (BSA; Cohn fraction V) as the standard.

iv) Electrophoresis.

Secreted proteins were examined by electrophoresis on 16 cm x 20 cm x 0.18 cm SDS-polyacrylamide gels incorporating a 3.3-24% polyacrylamide gel concentration gradient. These gels were prepared using a multichannel peristaltic pump (Watson-Marlow 501) and a homemade gradient-forming device. A linear gradient was manufactured by slowly diluting an acrylamide solution (initially 24%) with a second dilute (3.3%) acrylamide solution. The mixing vessel was vigorously stirred and the contents simultaneously withdrawn at a steady rate to the gel mould, where polymerisation took place. A full account of this method is given by Thomas (1979). Figure 1.1 illustrates the gradient maker diagrammatically. The polymerisation of the gel was catalysed with ammonium persulphate and N,N,N',N' tetramethylethylenediamine (TEMED). The gels were left to polymerise overnight at 5°C in a polythene box containing a wet paper towel to prevent them drying out. In some cases gels were stored in these conditions for up to 7 days. The exact composition of the two acrylamide solutions was as follows:

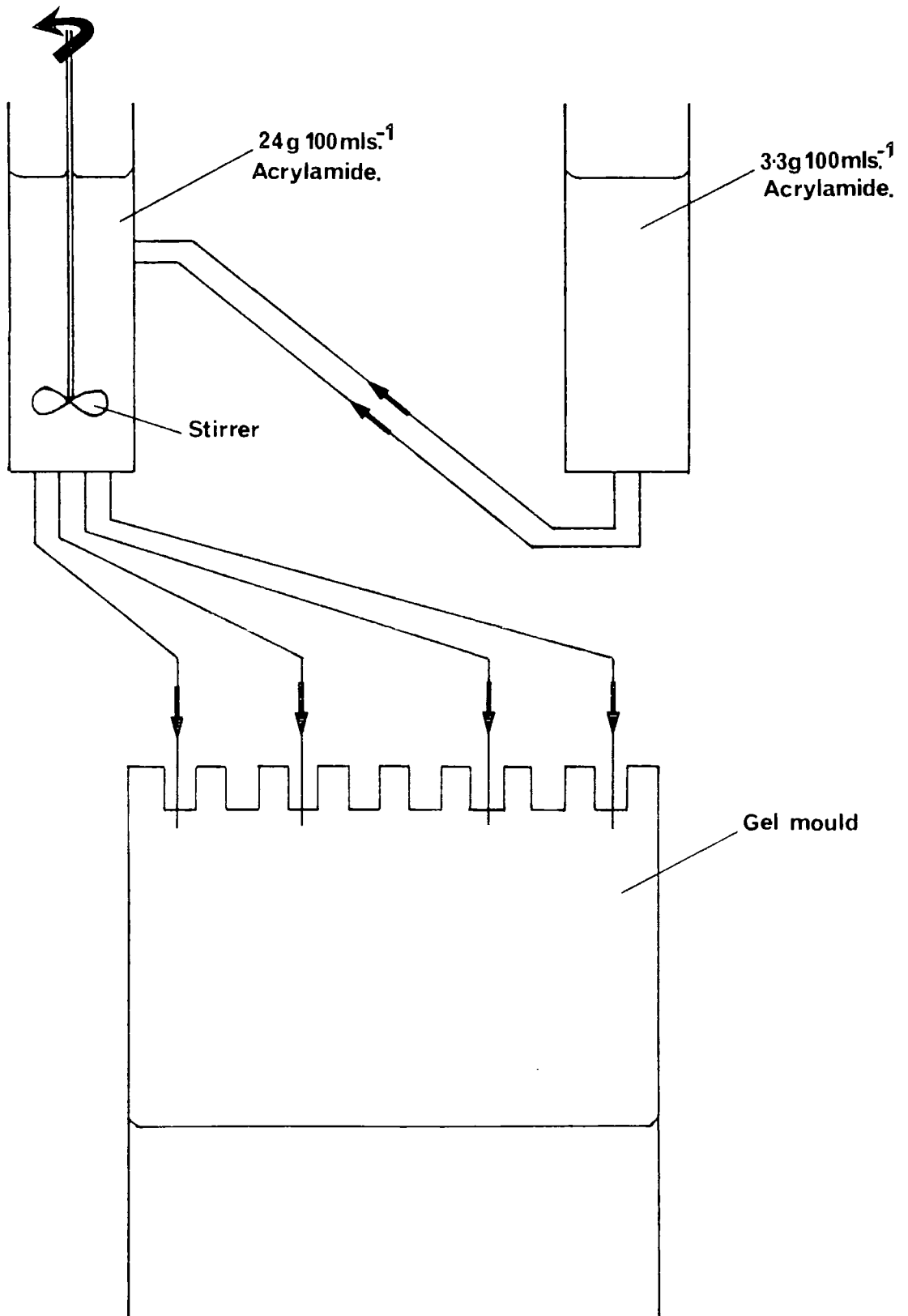
24% acrylamide....24% (w/v) acrylamide

0.96 (w/v) N,N' methylene bis
acrylamide

75% µg/ml ammonium persulphate

0.012% (v/v) TEMED

Fig.1.1:-Diagram showing the method used to produce S.D.S. gels incorporating a polyacrylamide concentration gradient. Details of the solutions used are given in the text.



0.1% (w/v) SDS

540 mM tris/HCl, pH 8.9

3.3% acrylamide...3.3% (w/v) acrylamide

0.13% (w/v) N,N' methylene bis
acrylamide

500 µg/ml ammonium persulphate

0.012% (v/v) TEMED

0.1% (w/v) SDS

540 mM tris/HCl, pH 8.9

Prior to electrophoresis sample proteins were simultaneously reduced with 2-mercaptoethanol and complexed with SDS using a buffer of the following composition: 26 mM tris/glycine, pH 8.9; 2.5% (w/v) SDS; 5% (v/v) 2-mercaptoethanol; 20% (w/v) sucrose; 0.1% (w/v) bromophenol blue, and 0.25-8 mg/ml sample protein. Aliquots of this buffer were placed in small polythene tubes which were sealed and immersed in a boiling water bath for 3-5 minutes.

Electrophoresis was performed in a "Raven" vertical gel apparatus. The buffer system employed was a slight modification of that described by Laemmli (1973) for single concentration gels. All working strength solutions were prepared by dilution of the appropriate stock solutions. These stock solutions were stored at room temperature and contained sodium azide (0.5-1 mg/ml) to prevent bacterial growth. Details of the buffers used are given below.

Upper tank buffer.... Stock solution

260 mM tris/glycine, 0.5% (w/v) SDS, pH 8.9 (approx 500 mM tris, 1% (w/v) SDS titrated to pH 8.9 with conc. glycine and diluted to 260 mM tris)

Working solution

52 mM tris/glycine, 0.1% (w/v) SDS, pH 8.9

Lower tank buffer.... Stock solution

500 mM tris/HCl, 0.5% (w/v) SDS, pH 8.1 (approx. 750 mM tris, 0.75% (w/v) SDS titrated to pH 8.1 with conc. HCl and diluted to 500 mM tris)

Working solution

100 mM tris/HCl, 0.1% (w/v) SDS, pH 8.1

Gel buffer..... Stock solution

1.5 M tris/HCl, 0.28% (w/v) SDS, pH 8.9 (approx. 2 M tris, 0.37% (w/v) SDS titrated to pH 8.9 with conc. HCl and diluted to 1.5 M tris)

Working solution

540 mM tris/HCl, 0.1% (w/v) SDS, pH 8.9

This buffer system differs from that described by Laemmli (1973) in two respects. The pH 6.8 large pore stacking gel was omitted as it did not improve the resolution and often prevented large protein species from entering the main gel, and the upper tank buffer was adjusted to ^{pH 8.9 rather than} pH 8.3, the higher pH giving slightly better resolution and considerably shorter running times.

Samples were loaded onto the gel in individual "sample wells" moulded into the gel's upper surface. Each sample consisted of 20-80 µg of protein, which was carefully transferred to the sample well using a 50 µl "Hamilton" microsyringe. The samples were loaded in the SDS/2-mercaptoethanol buffer, where the sucrose in this solution greatly increased its density and facilitated this process. Each gel could accommodate 14 different samples. Electrophoresis was carried out at a current of 35 mA per gel (lower tank positive) using a "Vokam" constant current source. The bromophenol blue in the sample mixture migrated ahead of the proteins as a clearly defined band, and electrophoresis was continued until this reached the gel's lower surface.

v) Staining of gels.

After electrophoresis, gels were removed from the apparatus and most stained for protein using a solution of Coomassie Brilliant Blue R-250 (0.25%, w/v) in water/methanol/glacial acetic acid (13:5:2; V:V:V). The gels were gently shaken in this solution for 18-24 hours. Excess stain was then removed by shaking in

several changes of water/methanol/glacial acetic acid (18:1:1; V:V:V).

Some gels were stained for glycosylated proteins using the periodic acid/Schiff's reagent technique described by Zaccarius, Zell, Morrison and Woodlock (1969). As the gels used in the present study were considerably thinner than those employed by Zaccarius et al. (1969) the oxidation and staining times were reduced. The following protocol was adopted:

- 1) Overnight in 8% (w/v) trichloroacetic acid.
- 2) 30 minutes in distilled water.
- 3) 30 minutes in 0.5% (w/v) periodic acid, 3% glacial acetic acid.
- 4) 6-7 hours in several changes of distilled water, with shaking.
- 5) 30 minutes in Schiff's reagent in the dark.
- 6) 3 x 10 minutes in freshly prepared 0.5% (w/v) sodium metabisulphite.
- 7) Destain in distilled water.

Schiff's reagent was prepared as described by McManus and Mowry (1960).

Phosphorylated proteins were detected using the methyl green / ammonium molybdate staining procedure of Cutting and Roth (1973). The procedure is outlined below:

- 1) Overnight in 10% (w/v) sulphosalicylic acid.

- 2) 1 hour in 10% (w/v) sulphosalicylic acid, 500 mM CaCl₂.
- 3) Rinse in distilled water.
- 4) 30 minutes in 500 mM sodium hydroxide at 60 C.
- 5) 2 x 10 minutes in 1% (w/v) ammonium molybdate.
- 6) 30 minutes in 1% (w/v) ammonium molybdate, 1 M nitric acid.
- 7) 30 minutes in 0.5% (w/v) methyl green, 7% (v/v) glacial acetic acid.

vi) Estimation of molecular weights.

A major advantage of SDS gel electrophoresis over "native" techniques is that the molecular weights of sample proteins may be estimated by comparing their electrophoretic mobilities with those of a number of proteins of known molecular weight (Shapiro, Viñuela and Maizel, 1967; Weber and Osborne, 1969; Lambin, 1978). The proteins employed as molecular weight markers in the present study are listed in table 1.1. The molecular weights quoted are for the constituent subunits of each protein.

Figure 1.2 shows a typical electrophoretic separation of these proteins as well as typical standard curve showing the relationship between the logarithm of molecular weight and electrophoretic mobility. Previously this relationship has been described as linear, both for single concentration (Shapiro et al., 1967; Weber and Osborne, 1969) and gradient gels (Lambin, 1978). In the present study, the higher molecular weight

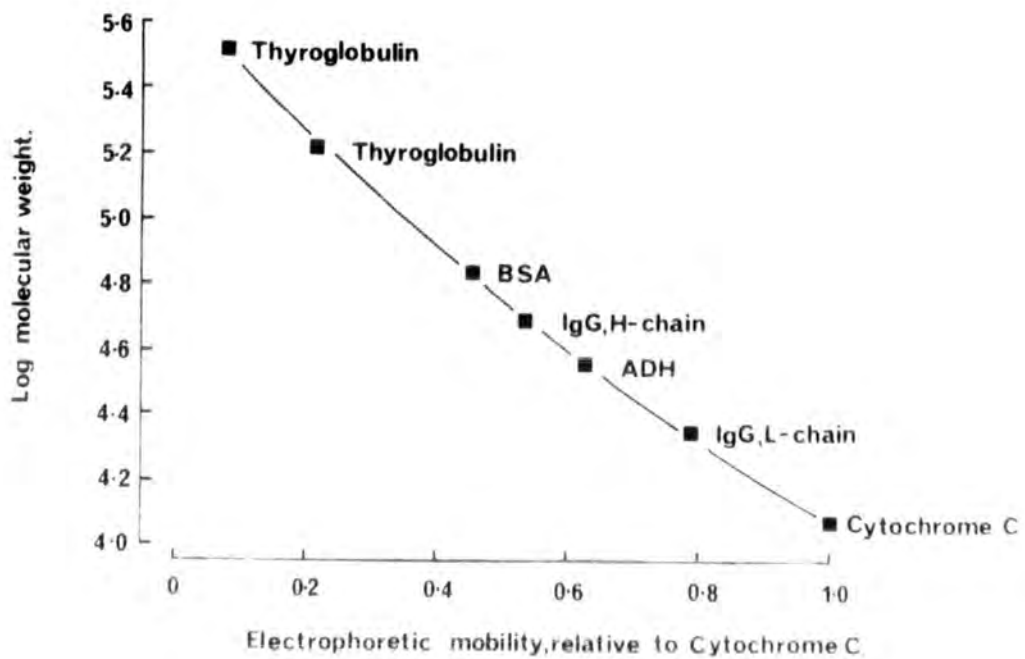
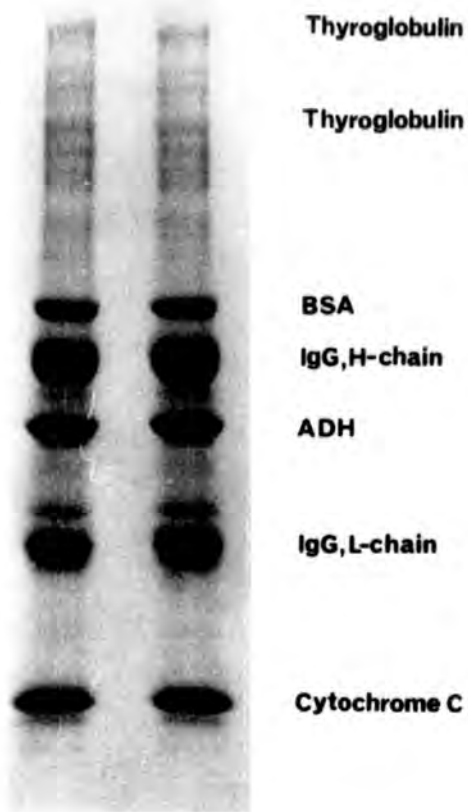
TABLE 1.1

Proteins employed as molecular weight markers in SDS-polyacrylamide gel electrophoresis

<u>Protein</u>	<u>Molecular Weight</u>	<u>Ref.</u>
Cytochrome C (Equine heart)	12,384	1
Immunoglobulin G L-chain (Bovine serum)	23,500	2
Alcohol dehydrogenase (Yeast)	37,000	3
Immunoglobulin G H-chain (Bovine serum)	50,000	2
Albumin (Bovine serum)	69,000	4
Thyroglobulin (Porcine)	165,000 & 330,000	5,6,7

1) "Sigma" product information; 2) Putnam, 1975;
3) "Pharmacia" product information; 4) Pappenheimer
et al., 1951; 5) de Crombrugge et al.; 6) Edelhoch and
de Crombrugge, 1966; 7) Pitt-Rivers, 1976.

Fig.1.2:-Determination of molecular weights by S.D.S. polyacrylamide gel electrophoresis.



species consistently showed greater mobilities than would be predicted from a linear relationship. The reason for this is unknown but it may imply that the technique used to manufacture the gradient gels tends to produce a slightly "concave" gradient.

As very small changes in running conditions may produce considerable changes in the absolute electrophoretic mobilities of particular protein species, it was important that each gel was internally controlled by the inclusion of standards.

vii) Inhibition of proteolytic activity.

The rat submandibular gland is known to contain (Bhoola and Dorey, 1971) and secrete (Matthews, 1974) proteolytic enzymes. Control experiments were undertaken to ensure that these enzymes did not hydrolyse other proteins within the sample and thus cause anomalous banding patterns. In one group of three animals, the saliva samples were processed for analysis as soon as they were collected. The procedure employed (see section iv above) would certainly inactivate any enzymes present. In a second group of 4 experiments, an aliquot of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF, 25 mg/ml in propan-2-ol; Fahrney and Gold, 1963) was added to each sample as soon as it was collected. These samples were then stored at -18°C pending analysis.

viii) Adrenergic and muscarinic antagonists.

In some animals atropine sulphate, propranolol

hydrochloride or dihydroergotamine mesylate (Sandoz) were administered IV. Doses quoted in the text refer to these salts.

RESULTS

i) Isoprenaline.

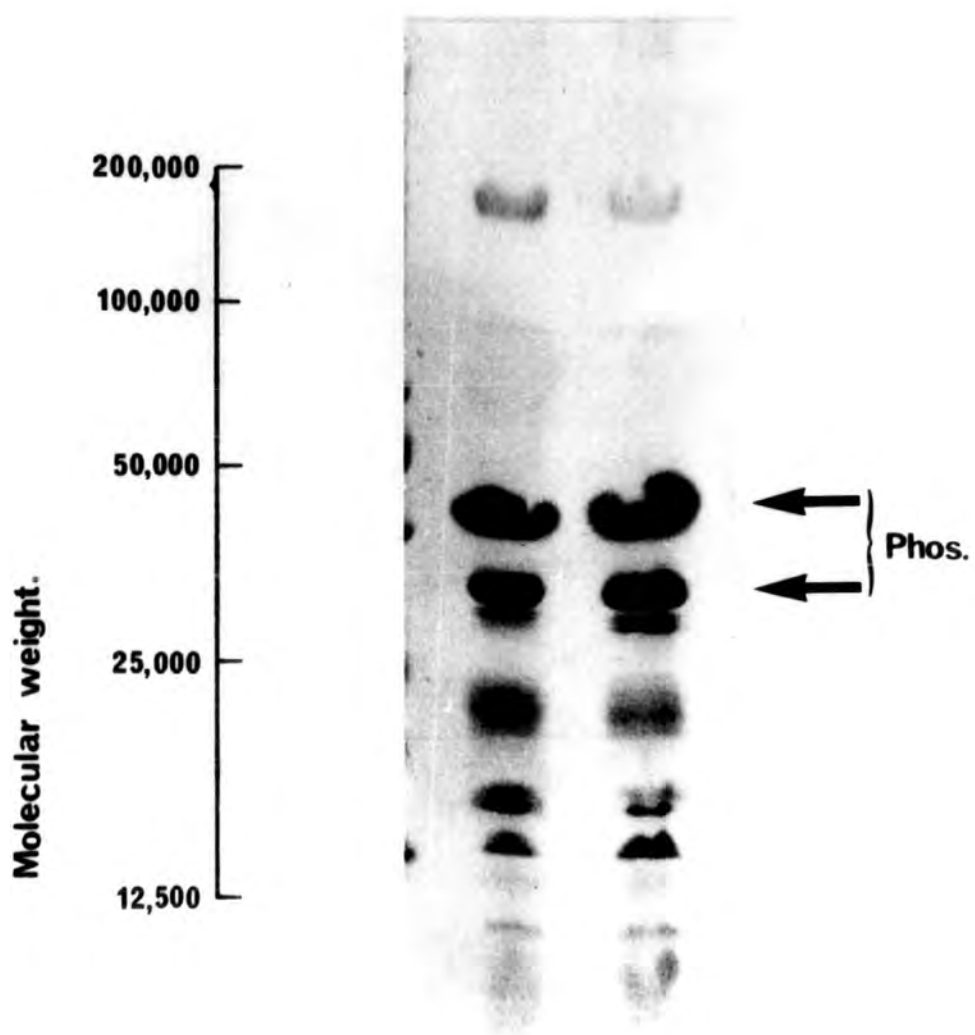
The β adrenergic agonist isoprenaline caused the secretion of a saliva containing a considerable amount of protein (41.95 ± 1.84 mg/ml, mean \pm S.E.; n=6), which, upon fractionation by gradient gel electrophoresis, yielded a complex population of individual protein species.

Figure 1.3 shows typical electrophoretic separations of isoprenaline-induced saliva from 2 individuals. The isoprenaline-evoked saliva always contained a prominent band with a molecular weight of 150,000 and, in some animals, 1 or 2 rather faint bands of higher molecular weight could be observed. Another distinguishing feature was the band with a molecular weight of 38,000-45,000, this species always migrating as a very distinctively shaped band. A number of lower molecular weight species were also present.

Staining with the PAS procedure showed that 4 or 5 glycosylated protein species were present (see figure 1.6). The prominent 150,000 molecular weight species always gave a strong reaction, as did the higher molecular weight species when they were present.

The results of the methyl green / ammonium

Fig. 1.3:- Electrophoretic separations of proteins secreted in response to the β adrenergic agonist isoprenaline.



molybdate procedure indicated that two of the protein species secreted in response to isoprenaline were phosphorylated. These are indicated on figure 1.3 (Phos.).

The population of proteins secreted in response to isoprenaline was not affected by prior administration of atropine (1 mg/kg) or dihydroergotamine (1 mg/kg), given either alone or in combination. Propranolol (2 mg/kg) totally abolished the secretory response to the doses of isoprenaline employed.

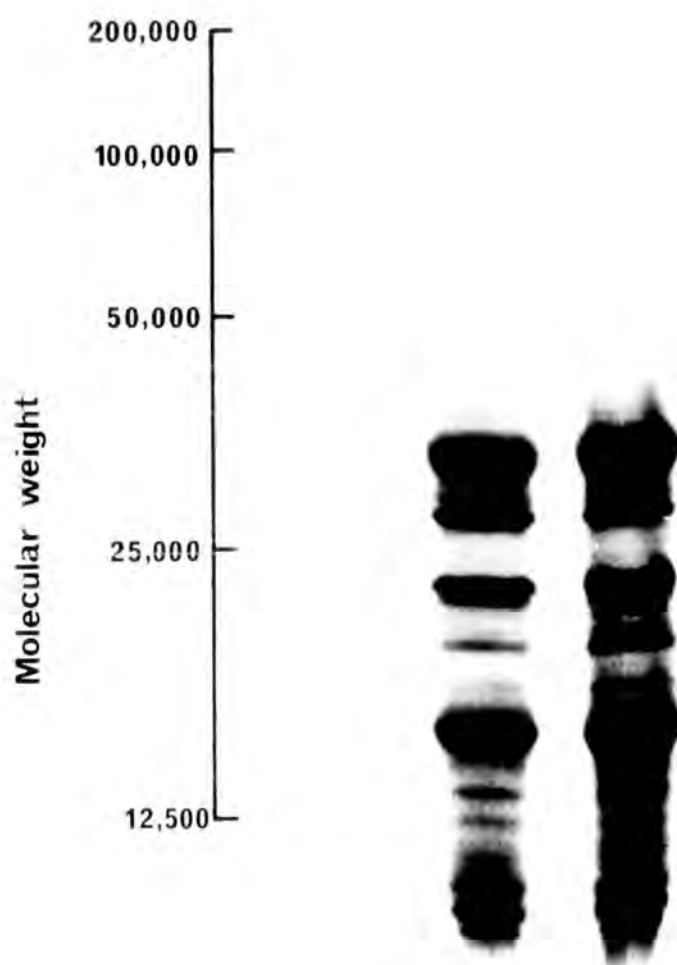
ii) Phenylephrine.

The volume of saliva secreted in response to this agent was fairly small, but it was possible to collect a 20 μ l aliquot from every animal. The saliva contained a considerable quantity of protein (41.95 ± 9.48 mg/ml, mean \pm SE; n=5) but there was more variability between animals than was the case for isoprenaline.

Typical electrophoretic separations of the proteins secreted in response to phenylephrine are shown in figure 1.4. In the best gels a total of 13 species could be resolved after staining with Coomassie Brilliant Blue, these ranged in molecular weight from about 10,000 to 38,000 and, in general, appeared to be of a rather different character to those secreted after administration of isoprenaline, as they separated out into bands which were much more sharply defined.

The band with a molecular weight of 38,000 appeared to represent a very small quantity of the species which

Fig. 14:- Electrophoretic separations of proteins secreted in response to the α adrenergic agonist phenylephrine.



constituted the broad (38,000-45,000 molecular weight), distinctively shaped band observed after isoprenaline. Similarly staining with the PAS technique demonstrated that a small amount of the 150,000 molecular weight glycoprotein was also present in the phenylephrine-evoked saliva (see figure 1.6). This species was never detected by staining with Coomassie Brilliant Blue, thus demonstrating the great sensitivity of the PAS technique for detecting glycoproteins.

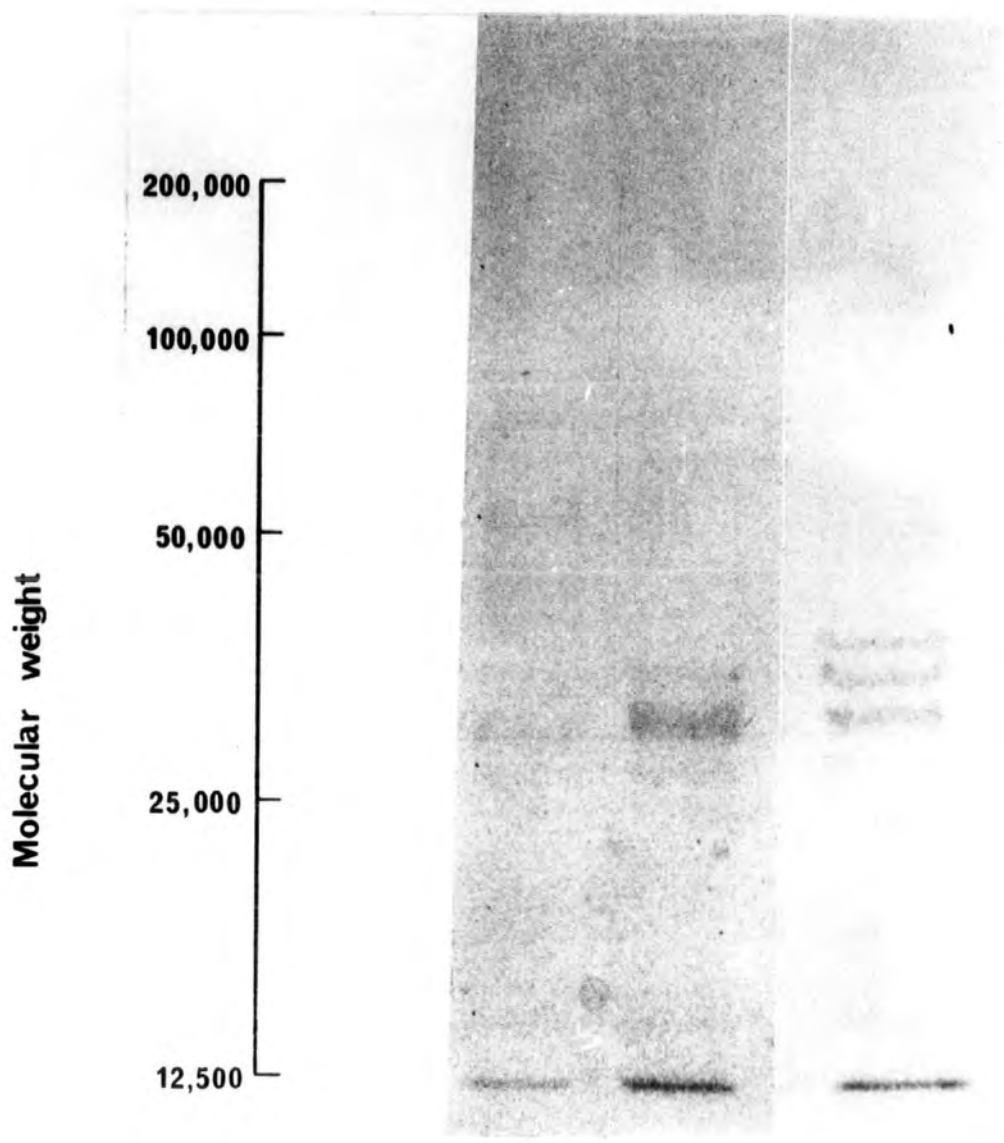
Staining with methyl green / ammonium molybdate always failed to detect any phosphorylated proteins in the saliva collected after administration of phenylephrine.

Dihydroergotamine (1 mg/kg) always totally blocked the secretory response to the doses of phenylephrine employed. Conversely, administration of propranolol (2 mg/kg) and/or dihydroergotamine exerted no effect upon the response to this sialogogue. These blockers also failed to modify the population of secreted proteins as revealed by staining with either Coomassie Brilliant Blue or by the PAS technique.

iii) Acetyl β -methyl choline.

The saliva secreted in response to this agent contained only a very small amount of protein (0.69 ± 0.07 mg/ml, mean \pm SE; n=7) and so, unlike the saliva secreted after isoprenaline or phenylephrine, it was difficult to visualise the proteins on the gels after electrophoresis. It also was apparent that a considerable

Fig. 1.5 Electrophoretic separations of proteins secreted in response to the muscarinic agonist acetyl β methyl choline.



amount of variation existed between individual animals, a problem not encountered with isoprenaline or phenylephrine. A band of 12,500 could always be detected and, in most animals a group of three bands (molecular weights 31,000, 34,000 and 38,000) was also present. A few samples contained a rather faint band with a molecular weight of 27,000, and in some cases a band of 150,000 molecular weight could be very faintly observed.

Staining with the PAS technique gave much more consistent results. In all samples examined, a single band with a molecular weight of 150,000 could be resolved (see figure 1.6). The methyl green / ammonium molybdate technique always gave negative results for the saliva secreted in response to acetyl β -methyl choline.

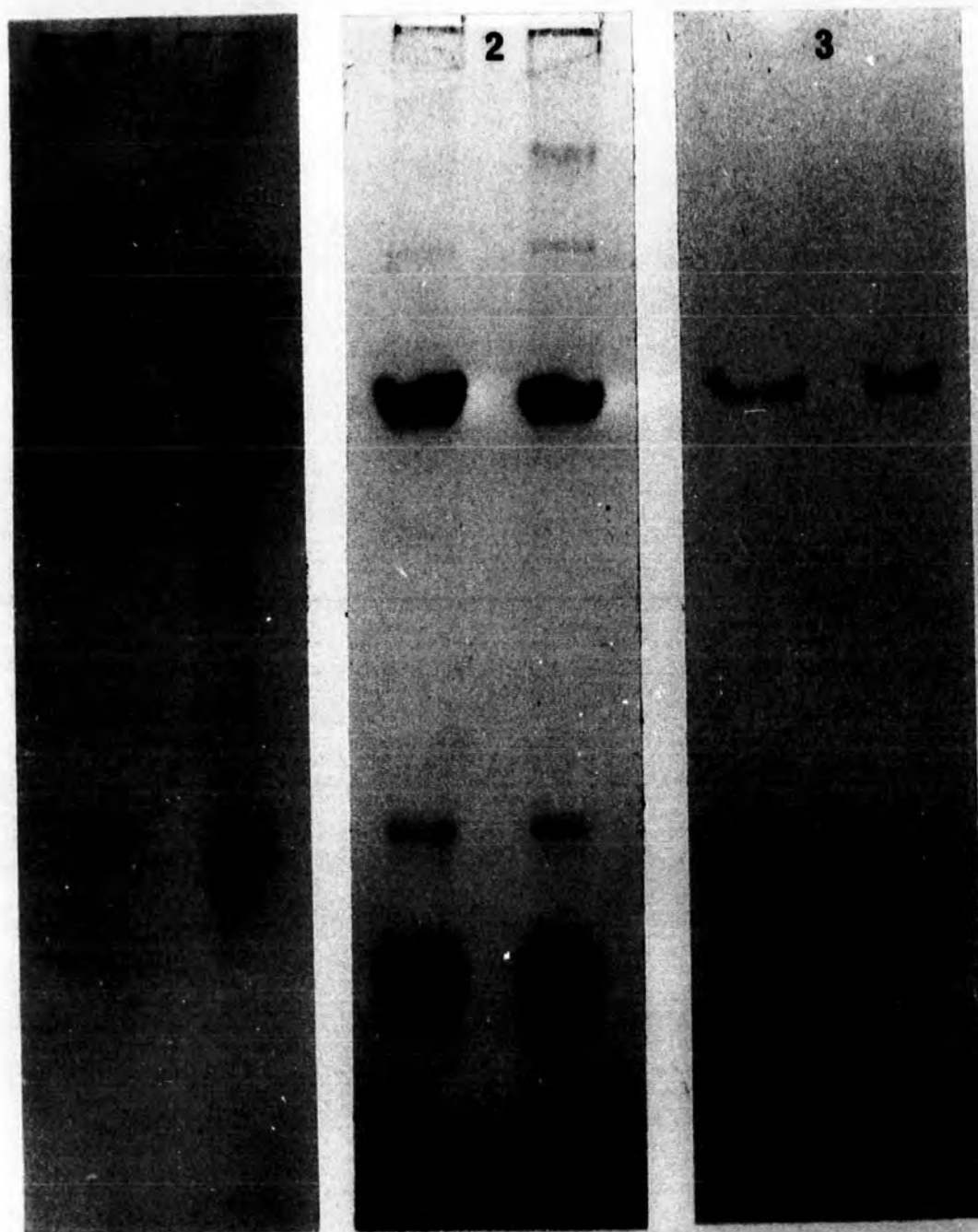
iv) Carbamyl β -methyl choline.

The saliva secreted in response to this agent was indistinguishable from that secreted in response to acetyl β -methyl choline. However the secretory response to this agent was always much more prolonged, making it unsuitable for the purposes of the present study. This may be due to the fact that carbamyl β -methyl choline is not a substrate for acetylcholinesterase, thus giving it a much longer half-life in the body (see Taylor, 1980).

DISCUSSION

The proteins secreted by the rat submandibular gland in vivo have previously been studied using

FIG 1.6 : GELS STAINED WITH THE PAS TECHNIQUE TO SHOW GLYCOSYLATED PROTEINS SECRETED IN RESPONSE TO 1) PHENYLEPHRINE, 2) ISOPRENALINE AND 3) ACETYL β METHYL CHOLINE. THE BAND COMMON TO ALL PATTERNS HAS A MOLECULAR WEIGHT OF 150,000.



electrophoretic techniques (Hall and Schneyer, 1964a; Abe and Dawes, 1978, 1980; Abe, Fujita, Yokota and Dawes, 1979; Abe, Yoneda, Fujita, Yokota and Dawes, 1980). However the present study is the first in which such techniques have been used to investigate the chemical properties of the secreted proteins.

Both Hall and Schneyer (1964a), and Abe and his co-workers (Abe and Dawes, 1978, 1980; Abe et al., 1979, 1980) employed different electrophoretic techniques to those employed here, and so it is not possible to compare banding patterns directly. In particular Abe and his co-workers employed polyacrylamide gels with a high (15%) acrylamide concentration. Polyacrylamide gel electrophoresis relies upon the ability of the monomeric components of the gel to form a molecular "sieve" upon polymerisation. The mean pore size of this sieve is governed by the monomer concentration, high concentration gels having a low mean pore size. The larger protein species observed in the present study (molecular weight 150,000) would probably be totally excluded from the pores of a 15% gel. In their product information "Shandon" Products limited recommend gels with a 15% monomer concentration as being suitable for fractionating proteins with molecular weights of between 10,000 and 20,000, proteins larger than 100,000 requiring gels with an acrylamide concentration of about 3%.

Abe and Dawes (1978) reported that the rat submandibular gland secreted a constant population of proteins in response to β -adrenergic, α -adrenergic and

muscarinic agonists; parasympathetic nerve stimulation and administration of pilocarpine. However these authors found that stimulation of the chorda lingual (parasympathetic) nerve or administration of α -adrenergic agonists also caused the secretion of a number of "additional" protein species. As the secretion of these additional proteins was abolished by the α -adrenergic antagonist phentolamine, it was concluded that they were secreted as a response to activation of α -adrenoceptors. Clearly the findings of these authors are not supported by the results of the present study, where α -adrenergic and β -adrenergic agonists caused the secretion of markedly different protein populations with very few features in common. However, the results presented by Abe and his colleagues in subsequent publications (Abe and Dawes, 1980; Abe et al. , 1980) do not support their original statements as they show the protein populations secreted in response to phenylephrine and isoprenaline to be markedly different.

The findings of the present study conflict with those of Abe and his colleagues in a number of other respects. In the present study the α -adrenergic agonist phenylephrine caused the secretion of a saliva containing a considerable quantity of protein (41.95 ± 9.48 mg/ml; $n=5$). Abe and his co-workers found much smaller amounts of protein present in the saliva evoked by the α -adrenergic agonists methoxamine (Abe and Dawes, 1978 : 4.92 ± 1.1 mg/ml, $n=9$; Abe et al. , 1979 : 4.53 ± 0.65 mg/ml, $n=10$; Abe and Dawes, 1980 : 4.59 ± 0.74

mg/ml, n=8) and phenylephrine (Abe et al. , 1980 : 12.6 ± 1.3 mg/ml, n=10). Also Abe et al. , 1980 found phenylephrine to act as a β -adrenergic agonist at doses below 2 mg/kg (IP), whilst in the present study the response to 100-200 μ g/kg (IV) of phenylephrine was totally abolished by dihydroergotamine (1 mg/kg, IV) and unaffected by propranolol (2 mg/kg, IV). These observations indicate that in the present study phenylephrine acts exclusively as an α -adrenergic agonist. These differences may be due to the use of different routes of drug administration. Abe and his co-workers administered large doses of autonomic agonists by intraperitoneal injection, whilst in the present study much smaller doses were administered intravenously. The former approach means that the glands secrete at a maximal rate for prolonged periods, whilst in the latter situation the secretory response lasts for only a few minutes. This brief secretory response means that several samples, each corresponding to a different agonist, may be collected from a single gland which is not fatigued by prolonged periods of maximal secretion.

In the current investigation the rat submandibular gland was found to secrete glycosylated proteins of high molecular weight, particularly in response to isoprenaline. The secretion of such proteins has been investigated using enzymatically dispersed rat submandibular gland cells (Quissell and Barzen, 1980; Fleming and Sturgess, 1981; Quissell, Barzen and Lafferty, 1981). The results of these experiments

indicated that the secretion of glycoproteins occurred only in response to stimulation of β -adrenergic receptors, but that the secretory response was augmented when β and α -adrenoceptors were stimulated simultaneously. In the present in vivo study isoprenaline was certainly found to be the most potent stimulus of glycoprotein secretion, but small quantities of the 150,000 molecular weight glycoprotein were also secreted in response to phenylephrine and acetyl β -methyl choline. The reason for this discrepancy may be that when working with dispersed cells in vitro, there is a slow, continuous basal leakage of secretory material which may obscure a small secretory response. In addition it is worth noting that Fleming and Sturgess (1981) observed dispersed rat submandibular gland acini to show some morphological evidence of glycoprotein secretion in response to muscarinic as well as β -adrenergic agents.

Quissell and Barzen (1980) employed gel filtration to estimate the molecular weight of the glycoproteins secreted during their in vitro experiments. The greater part of the material was found to have a molecular weight in excess of 200,000 (the upper resolving limit of their technique), but one fraction was found to have a molecular weight of 140,000. This latter figure compares favourably (within 10%) with the estimate of 150,000 for the major glycoprotein band observed for the present study. However whilst Quissell and Barzen (1980) found most of their secreted material to be of a high molecular

weight, the present author observed only one or two very faint bands of higher molecular weight. The reason for this may be that in the present study the secreted proteins were reduced with 2-mercaptoethanol and denatured with SDS prior to electrophoresis. This would rupture disulphide bridges, causing proteins to dissociate into their constituent subunits. Quissell and Barzen (1980) employed much milder reducing conditions (1 mM dithiothreitol) which may permit higher molecular weight aggregates to exist. Also these authors used trichloroacetic acid and phosphotungstic acid to precipitate the secreted material. These agents may cause certain proteins to aggregate (P.W.Kent, personal communication).

The rat submandibular gland contains only one type of acinar cell (Shackleford and Klapper, 1962; Young and van Lennep, 1978) but a second population of cells which secrete protein, the granular duct cells, exists within the gland (Matthews, 1974; Young and van Lennep, 1978). Histochemical studies have shown that the granular duct cells contain a number of proteolytic enzymes which are not present in the acinar cells (Junquera, Toledo and Saad, 1964; Cunningham, 1974; Ørstavik, Brandtzaeg, Nustad and Halvorsen, 1975; Brandtzaeg, Gautvik, Nustad and Pierce, 1976; Ørstavik and Glenner, 1978; Al-Gailani et al. , 1980). There is a considerable body of evidence to suggest that secretion from these cells occurs only in response to activation of α -adrenoceptors (Matthews,

1974; Orstavik and Gautvik, 1977; see reviews Young and van Lennep, 1979; Barker, 1980). The results of the present study are consistent with the view that two populations of secretory cells exist within the rat submandibular gland, and that these cells have differing sensitivities to α -adrenergic and β -adrenergic agonists. It is difficult to envisage a means by which one cell type may secrete such markedly different protein populations in response to different secretory stimuli. It may be that the proteolytic enzymes present in the saliva contribute, at least in part, to these differences by hydrolysing other proteins in the secretion. The results of experiments undertaken to test this possibility (see page 31), indicate that this is not the case. The patterns of proteins secreted ^{by} the gland were not affected by addition of the protease inhibitor PMSF (final concentration 2.3 mg/ml), or by heating the samples close to 100° C as soon as they were collected.

Brandtzaeg et al. (1976) found that rat submandibular gland kallikrein (an esteroprotease) existed as 4 isoenzymes, each with a molecular weight of about 34,000. Similarly the trypsin-like proteases from the rat submandibular gland are thought to have molecular weights of 25,000-30,000 (Ekfors, Riekinen, Malmiharjo and Hopsu-Havo, 1967; see also Barka, 1980). As the secretion of these enzymes occurs in response to activation of α -adrenoceptors (Matthews, 1974; Orstavik and Gautvik, 1977), they should be present in the saliva secreted in response to phenylephrine. Examination of

the proteins secreted in response to this agent certainly revealed bands which may correspond to these enzymes. However, positive identification is not feasible with the electrophoretic technique employed, particularly as enzymatic activity is lost after SDS treatment (see methods). However this may be achieved in the future using an immunochemical method.

In summary the proteins secreted by the rat submandibular gland have been investigated electrophoretically. Excitation of β -adrenoceptors caused the secretion of a considerable amount of protein, as did administration of α -adrenergic agonists. Muscarinic agonists, on the other hand, caused the secretion of only small quantities of protein. Electrophoresis showed that very different patterns of proteins were secreted upon excitation of β -adrenergic and α -adrenergic receptors. Isoprenaline caused the secretion of a considerable quantity of a high (150,000) molecular weight glycoprotein as well as several other protein species. Phenylephrine caused the secretion of only a very small amount of the 150,000 molecular weight glycoprotein, but a considerable number of lower molecular weight species were also secreted. These lower molecular weight bands were not observed after isoprenaline. The muscarinic agonist acetyl β -methyl choline caused the secretion of a saliva with a low concentration of protein. Electrophoresis revealed a small quantity of the high (150,000) molecular weight glycoprotein as well as a rather variable number of lower

molecular weight species. These results are consistent with the generally held view (see Young and van Lennep, 1979; Young and Schneyer, 1981) that protein secretion from the salivary glands is controlled ^{by} the sympathetic nervous system, whilst the parasympathetic nerves control the flow of fluid. However, most previous studies have indicated that, in the rat submandibular gland, β -adrenoceptors are mainly concerned with fluid secretion, whilst the present study suggests that they may be important in regulating protein secretion. The relative importance of α -adrenoceptors and β -adrenoceptors in mediating nerve-induced protein secretion is the subject of the next chapter.

CHAPTER 2:- THE PROTEINS SECRETED IN RESPONSE TO
STIMULATION OF THE AUTONOMIC NERVES.

INTRODUCTION

All salivary glands respond to stimulation of the parasympathetic nerves with a vigorous secretion of saliva (see Emmelin, Schneyer and Schneyer, 1973; Young and van Lennep, 1978; Young and Schneyer, 1981), but the magnitude of the response to sympathetic nervous stimulation is much more variable (ibid.). In those glands which do show a sympathetic secretory response the saliva is often described as containing much more protein than the parasympathetic saliva from the same gland (see Babkin, 1945; Young and van Lennep, 1979; Young and Schneyer, 1981). Young and van Lennep (1979) stated that "...this ability to stimulate protein release independantly of the secretion of large volumes of fluid is mediated via β -adrenoceptors...". This view has largely arisen from studies of amylase secretion by the parotid glands of rats and rabbits (Schneyer and Hall, 1967; Schramm and Selinger, 1976; Asking and Gjørstrup, 1980a, 1980b; Gjørstrup, 1979, 1980a, 1980b) as, in these glands β -adrenoceptor excitation causes the secretion of a considerable amount of amylase (the major secretory protein in these glands) whilst α -adrenoceptor and muscarinnic receptor excitation mainly causes the secretion of fluid. There have been fewer studies of the protein secretion by the submandibular gland, but the

results of these investigations do tend to support the view of Young and van Lennep (1979). Thus Martinez et al. (1975a) found that phenylephrine appeared primarily to cause fluid secretion, whilst isoprenaline caused the secretion of only a small volume of saliva with a very high protein concentration. Abe and his co-workers obtained similar results (Abe and Dawes, 1978, 1980; Abe et al. , 1979, 1980).

However, the data presented in the previous chapter showed that, when given intravenously, phenylephrine may be as effective a stimulus of protein secretion as isoprenaline. This finding suggested that the role of α -adrenoceptors in the control of glandular protein secretion may have been previously underestimated.

The electrophoretic studies described in the previous chapter indicated that stimulation of α -adrenergic and β -adrenergic receptors caused the secretion of markedly different populations of secreted proteins. It was therefore decided to extend these experiments by investigating the population of proteins secreted by the rat submandibular gland in response to sympathetic nervous stimulation. If, as Young and van Lennep (1979) suggested, sympathetically induced protein secretion is mediated by β -adrenoceptors then it would be anticipated that the pattern of secreted proteins would be similar to that observed after administration of isoprenaline. Similarly, involvement of α -adrenoceptors would be reflected by the secretion of a protein population which resembled that previously observed after

phenylephrine.

MATERIALS AND METHODS

i) Animals and anaesthesia.

Male rats of a Wistar strain (210-360 g) were anaesthetised and prepared as described in the previous chapter. In addition, either the cervical sympathetic trunk or the plexus of parasympathetic nerve fibres surrounding the submandibular duct (chorda lingual nerve) was stimulated electrically.

ii) Nerve dissection and stimulation.

The sympathetic trunk ipsilateral to the cannulated duct was carefully dissected free of the vagus nerve, ligated as far centrally as possible and divided immediately caudal to the ligature. The peripheral portion of the nerve was then laid across bipolar silver wire electrodes and covered with warm (37⁰ C) liquid paraffin which had previously been equilibrated with 0.9% (w/v) sodium chloride.

To stimulate the chorda lingual nerve the entire connective tissue bundle containing the submandibular and major sublingual ducts was laid across bipolar silver wire electrodes.

Both nerves were stimulated with supra-maximal pulses (usually 4V) of 2 milliseconds duration at frequencies between 1 and 20 Hz. The cervical sympathetic trunk was stimulated in 23 animals and the

chorda lingual nerve in 6.

In an additional series of 3 experiments the effect of stimulating the cervical sympathetic trunk was investigated in rats which had been anaesthetised with chloralose (100 mg/kg). To administer this anaesthetic the rat was first anaesthetised with ether, and a femoral vein cannulated. The chloralose was dissolved in warm 0.9% (w/v) saline (approximately 50 mg/ml) and slowly infused via the venous cannula whilst the animal was still under ether anaesthesia. Administration of ether was discontinued once most of the chloralose had been given. Additional doses of chloralose (5-10 mg) were given IV as required.

iii) Electrophoresis.

Secreted proteins were examined electrophoretically as described in the previous chapter.

RESULTS

i) Sympathetic nerve stimulation.

Stimulation of the cervical sympathetic trunk caused a flow of saliva at all of the frequencies employed. At 1 Hz. there was a 25-35 second latency between the beginning of stimulation and the onset of secretion, at 5 Hz this latency was 3-5 seconds whilst at 20 Hz it was only 1-2 seconds. The volumes of saliva secreted are shown in table 2.1. At frequencies below 5 Hz the flow rate could generally be well maintained for

several minutes, however at higher frequencies there was a marked tendency for the flow rate to decline after an initial period of rapid secretion. The α -adrenergic antagonist dihydroergotamine (1 mg/kg) caused the volume of saliva secreted in response to sympathetic stimulation at 5-20 Hz to be reduced by about 50%. In 8 animals the response to stimulation at 1 Hz, ^{was abolished} but in the remainder a trace of secretion was observed. The latency period between switching on the stimulator and observing the start of secretion was 4-6 times longer after dihydroergotamine, and at higher stimulation frequencies the flow rate did not show such a marked tendency to decline with time. Higher doses of dihydroergotamine (up to 4 mg/kg) were without any further effects. Propranolol (2 mg/kg) also reduced the volume of saliva secreted in response to stimulation of the cervical sympathetic trunk by about 50%. However the tendency of the secretory rate to decline with time became much more marked and, at frequencies above 5 Hz the flow often ceased altogether after about 30 seconds. This made the effect of this drug hard to quantify. Propranolol did not influence the latency period and, as was the case with dihydroergotamine, higher doses (up to 6 mg/kg) were without any further effects. When propranolol and dihydroergotamine were given together the response to sympathetic nerve stimulation was totally abolished. Atropine (1 mg/kg) exerted no effect upon the response to sympathetic nerve stimulation.

The response to stimulation of the cervical

sympathetic trunk was investigated in 3 animals which had been anaesthetised with chloralose. Barbiturate anaesthetics are known to inhibit ganglionic transmission (Harvey, 1980) and so may depress the response to sympathetic nerve stimulation. The results from rats anaesthetised with sodium pentobarbitone were compared with the data obtained using chloralose (see table 2.1), a carbohydrate based anaesthetic which does not display this property (N. Emmelin, personal communication). At all frequencies, except 20 Hz, more saliva was obtained from the rats under chloralose anaesthesia. However this difference is statistically significant only at 15 Hz. This finding indicates that sodium pentobarbitone does not cause any significant blockade at the superior cervical ganglion.

Electrophoretic examination showed that the protein population secreted by the rat submandibular gland in response to sympathetic nervous stimulation depended upon the stimulus frequency employed. At the higher frequency of 20 Hz the proteins secreted were essentially similar to those obtained after administration of the α -adrenegic agonist phenylephrine. Figure 2.1 shows electrophoretic separations of the proteins secreted in response to this high frequency of stimulation. As can be seen by comparison with figure 1.4 (proteins secreted in response to phenylephrine), the resemblance between the two patterns is striking. In both patterns there is a prominent group of low molecular weight species (below 12,000) as well as major bands with molecular weights of

TABLE 2.1

(in μ l)

Volumes of saliva/secreted during 2 minutes of sympathetically-evoked secretion in rats anaesthetised with sodium pentobarbitone (60 mg/kg) or with ether/chloralose (100 mg/kg). Values are mean \pm SE, n=number of animals.

<u>STIMULUS FREQUENCY</u>	<u>PENTOBARBITONE</u>	<u>CHLORALOSE</u>	<u>P</u>
1Hz	1.92 \pm 0.20(10)	2.52 \pm 0.63(3)	NS
5Hz	6.58 \pm 0.28(16)	6.67 \pm 0.90(3)	NS
10Hz	6.42 \pm 0.31(4)	7.81 \pm 1.00(3)	NS
15Hz	6.84 \pm 0.50(5)	12.40 \pm 1.27(3)	.0033
20Hz	12.87 \pm 0.64(7)	11.56 \pm 0.79(3)	NS

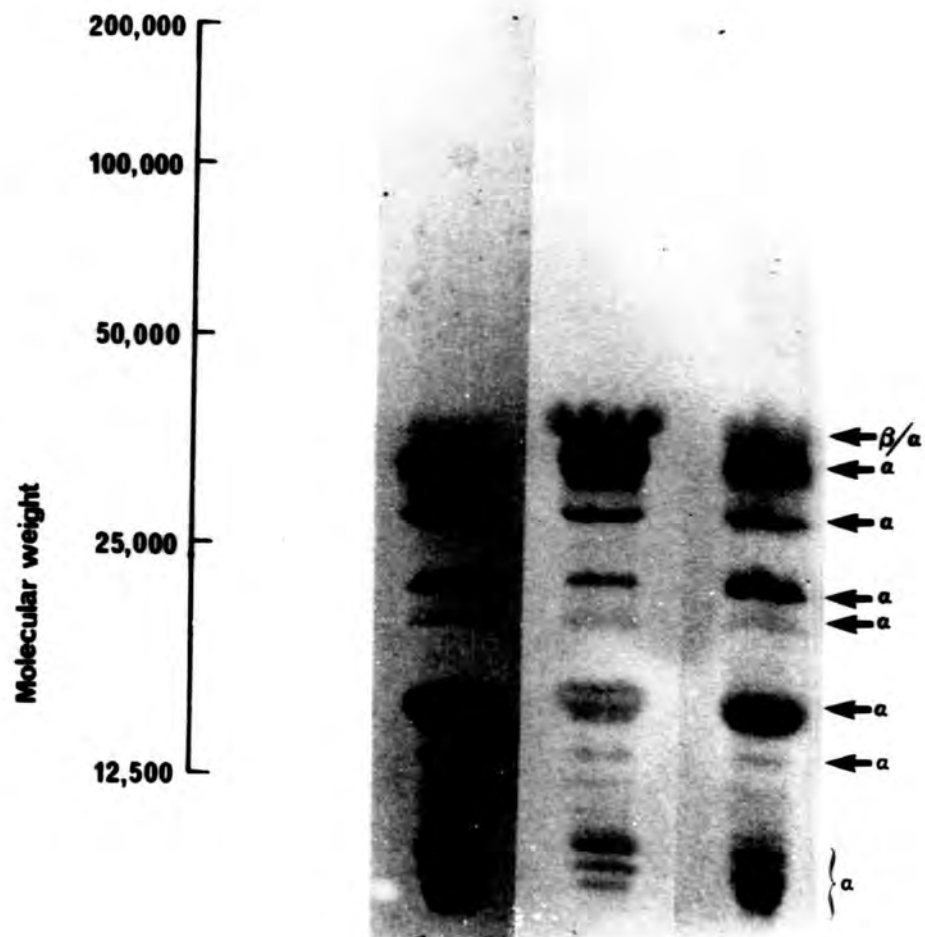
15,000-17,000, 23,000, 28,000, and 38,000. Considerably more of the latter species was present in the saliva evoked by high frequency sympathetic nerve stimulation than was present in the saliva obtained after phenylephrine. This protein was also present in the saliva secreted in response to isoprenaline as the broad (38,000-45,000) band which migrated with a characteristic shape. Staining with the PAS technique demonstrated that the 150,000 molecular weight glycoprotein observed previously (see previous chapter) was also present but remained undetected by staining with Coomassie Brilliant Blue. Although the protein population in the saliva obtained after administration of phenylephrine or high frequency sympathetic nerve stimulation appeared very similar they did differ in one important respect. Upon electrophoresis the proteins in the nerve-induced saliva showed a marked tendency to "clump" together into aggregates of very high molecular weight. These would often fail to enter the gel matrix and cause longitudinal streaks of protein to run down the gels. This problem was never encountered with the phenylephrine evoked saliva and its significance is unknown.

When the lower stimulus frequency of 5Hz was employed the population of secreted proteins became considerably more complex. Figure 2.2 shows proteins secreted in response to sympathetic stimulation at this frequency. The pattern now contains several features which previously had only been observed after administration of the β -adrenergic agonist isoprenaline.

The most obvious such feature is the 150,000 molecular weight species, which is now present in a large enough quantity to be detected by staining with Coomassie Brilliant Blue. Similarly, considerably more of the strangely shaped protein species (molecular weight 38,000-45,000) is now present. Other features such as the broad band whose molecular weight lay just below 25,000 and the species with a molecular weight of 100,000 had also previously been observed only amongst the proteins secreted in response to isoprenaline. However a number of proteins associated with the α -adrenoceptor agonist phenylephrine, or with stimulation at 20 Hz were also present. However, a number of the protein species present in the saliva secreted in response to sympathetic stimulation at 5 Hz had not been observed in any of the previous experiments.

All of the proteins on figures 2.1 and 2.2 have been classified as α , β or as neither (?). This classification was performed by comparing the typical profiles for the the saliva secreted in response to stimulation of the cervical sympathetic trunk with those obtained after administration of autonomic agonists. Thus, if a protein appeared after isoprenaline it is marked with a β symbol. These figures serve to summarise the results of this part of the investigation, showing that at 20 Hz most of the species fall into the α classification; at 5 Hz both α and β species are present, as well as a number of species which fall into neither category, these being detected only upon

Fig 2.1 :- Electrophoretic separation of protein secreted in response to stimulation of the sympathetic trunk at 20Hz



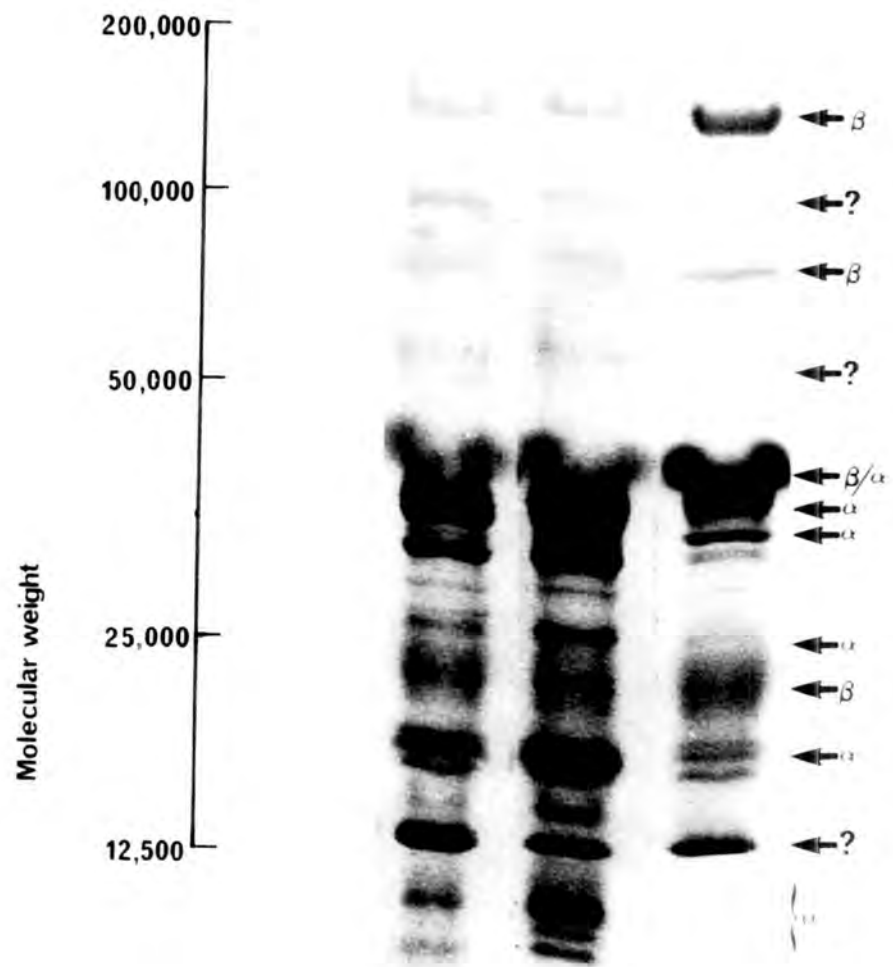
sympathetic nervous stimulation.

ii) Parasympathetic nervous stimulation.

The secretory response to stimulation of the parasympathetic (chorda lingual) nerve supply was very much greater and of a different character to the typical sympathetic secretory response. At 1 Hz the flow rate was $5.25 \pm 0.2 \mu\text{l}/\text{min}$ (mean \pm SE, n=6), at 10 Hz it was $47.67 \pm 5.52 \mu\text{l}/\text{min}$ (mean \pm SE, n=6) and at 20 Hz it was still larger but much more variable ($61.63 \pm 22.04 \mu\text{l}/\text{min}$, mean \pm SE, n=6). The flow rates could be well maintained compared to the typical sympathetic response, although at 20 Hz the flow rate tended to decline with time in some animals.

Administration of dihydroergotamine (1 mg/kg) caused approximately a 40% reduction in the volume of saliva secreted upon stimulation of the chorda lingual nerve. However, this effect was transient and normal responses returned within 30-40 minutes, in contrast to the sympatholytic effects of this drug, which persisted for several hours. Atropine (1 mg/kg) totally abolished the secretory response to chorda lingual nerve stimulation at 1 and 5 Hz, at higher frequencies a trace of secretion always persisted. This response could not be abolished by further doses of atropine (up to 5 mg/kg), by dihydroergotamine (1 mg/kg) or by propranolol (2 mg/kg), given either alone or in combination. Propranolol (2 mg/kg) did not affect the response to chorda lingual nerve stimulation.

Fig 2.2:- Electrophoretic separation of protein secreted in response to stimulation of the sympathetic trunk at 5Hz



Electrophoretic examination of the proteins secreted in response to stimulation of the parasympathetic nerves revealed that these were similar to those obtained after injections of acetyl β -methyl choline. There was, however, much less variation between individual animals, bands with molecular weights of 12,500, 31,000 and 38,000 being always observed. As would be anticipated, staining with the PAS procedure demonstrated that the glycoprotein of high molecular weight (150,000) was also present, but in an insufficient amount to be detected by staining with Coomassie Brilliant Blue.

DISCUSSION.

There have been very few investigations of the proteins secreted by salivary glands in response to nervous stimulation. Abe and Dawes (1978) investigated the proteins secreted by the rat submandibular gland upon stimulation of the parasympathetic nerves, and very recently the proteins secreted by the rat parotid gland upon stimulation of the autonomic nerves were studied by electrophoresis (Anderson, Garrett, Johnson, Kauffman, Keller and Thulin, 1982). The present chapter describes the first study in which the protein population secreted by the rat submandibular gland in response to sympathetic nervous stimulation has been investigated.

Stimulation of the cervical sympathetic trunk caused the submandibular gland to secrete a population of

proteins which changed according to the stimulation frequency employed. At the higher frequency of 20 Hz the protein population was essentially similar to that observed after administration of the α -adrenergic agonist phenylephrine. At the lower frequency of 5 Hz the population also contained features typically observed after isoprenaline, as well as a number of species which had not been observed previously. This result suggests that whilst at 20 Hz protein secretion is mediated almost exclusively by α -adrenoceptors, at lower frequencies β -adrenoceptors become important.

Abe et al. (1980) examined the proteins secreted by the rat submandibular gland in response to various doses of a number of different sympatholytic agents. These authors noted that adrenaline (2 mg/kg, IP), noradrenaline (2 mg/kg, IP) and phenylephrine (6 mg/kg, IP) all caused the secretion of a protein population identical to that observed after administration of methoxamine (an α -adrenoceptor agonist; see Weiner, 1980). At lower doses (less than 1 mg/kg for adrenaline and noradrenaline, and less than 2 mg/kg for phenylephrine) these same agents acted very differently, as the population of secreted proteins was now identical to that secreted in response to isoprenaline. These results agree, in part, with those presented in the present chapter. Both studies indicate that "intense" sympathetic stimuli (high frequency nerve stimulation or administration of large doses of agonists), cause the secretion of protein by acting upon the glandular

α -adrenoceptors. Involvement of β -adrenoceptors appears to require more "moderate" stimuli (lower stimulation frequencies or smaller doses of agonist).

Dr. C.J. Jones pointed out that both of these results suggest that the β -adrenoceptor is triggered by lower levels of neurotransmitter than the α -adrenoceptor, but that once the α -adrenoceptor is activated its characteristic response will predominate. Such a situation may serve to explain the characteristic response of the cat submandibular gland vasculature to sympathetic nerve stimulation. During the stimulus period itself there is a marked vasoconstriction which is mediated via the α -adrenoceptor. Once the stimulating current is switched off this response is replaced by a transient period of active vasodilation, which is mediated by β -adrenoceptors (Bhoola, Morley, Schachter and Smaje, 1966). The concentration of noradrenaline in the synaptic cleft would clearly be greatest whilst the nerve is being stimulated, and it is during this period that the response is mediated via α -adrenoceptors. Once the stimulating current is switched off, then the levels of transmitter will rapidly fall, giving a transient expression of the response evoked by stimulation of β -adrenoceptors.

A similar hypothesis has been proposed to explain the feedback control of noradrenaline release from the sympathetic nerve terminals. It has been suggested that this is effected via pre-synaptic α and β -adrenoceptors. After working with isolated guinea pig atria,

TABLE 2.2

Amounts of noradrenaline required to give a half maximal response to activation of α and β -adrenoceptors in various tissues (adapted from Adler-Grashinsky and Langer, 1975)

<u>TISSUE</u>	<u>ADRENOCEPTOR</u>	<u>CONCENTRATION</u>	<u>REF.</u>
Cat splenic strip	α	9.55×10^{-6} M	1
Cat nictating membrane	α	9.12×10^{-6} M	2
Rat vas deferens	α	5.89×10^{-5} M	3
Guinea pig vas deferens	α	3.31×10^{-5} M	4
Guinea pig tracheal strip	β	3.02×10^{-7} M	5
Guinea pig atria	β	3.02×10^{-7} M	2
Cat left atria	β	2.29×10^{-8} M	6
Cat papillary muscle	β	2.19×10^{-8} M	7
Rat adipocytes (lipolysis)	β	5.01×10^{-8} M	8

1)Granata and Langer, 1973; 2)Langer and Rubio, 1973; 3)Patil, LaPridus and Tye, 1967; 4)Nedergard and Westerman, 1968; 5)Furchgott, 1967; 6)Osorio, Stephanio and Langer, 1974; 7)Kaumann, 1970; 8)Fain, 1967.

Adler-Graschinsky and Langer (1975) proposed that low concentrations of neurotransmitter would activate pre-synaptic β -adrenoceptors, resulting in an increased amount of noradrenaline released per nerve impulse. Once the level of neurotransmitter in the synaptic cleft became high enough, it would activate the pre-synaptic α -adrenoceptors which inhibit neurotransmitter release.

Furthermore several studies have indicated that the concentration of noradrenaline required to trigger post-synaptic β -adrenoceptors is 30-100 times higher than that required to activate the post-synaptic α -adrenoceptors (see Adler-Graschinsky and Langer, 1975; this thesis, table 2.2)

However, in the rat submandibular gland β -adrenoreceptors may mediate the secretion of fluid even at high stimulation frequencies (Emmelin et al., 1967; Matthews, 1974; this study). Similarly Schneyer (1975) showed that at frequencies as high as 25 Hz sympathetic nerve stimulation could cause the secretion of a saliva containing high levels of potassium. As the ductal secretion of potassium is controlled via β -adrenoceptors (eg Yoshida et al., 1967), these results show that high stimulation frequencies per se do not inactivate glandular β -adrenoceptors, and thus make it rather difficult to understand why the proteins in response to stimulation at 20 Hz should not include typical " β " proteins.

Some of the protein species secreted in response to sympathetic nerve stimulation at 5 Hz were not observed

after administration of either phenylephrine or isoprenaline. The reason for this may lie in the fact that these agents cause almost exclusive stimulation of one particular class of adrenoceptors, an action which may not mimic the effect of sympathetic nervous stimulation, where both classes of adenoceptor would be stimulated simultaneously. Perhaps certain protein species are secreted only in response to simulataneous activation of both α and β -adrenoceptors. However it is hard to envisage how this may occur if proteins are secreted by exocytosis (see this thesis general introduction; Young and van Lennep, 1979) as this theory requires that all of the proteins contained within the secretory vesicles are released simultaneously. Alternative pathways for protein secretion are known to exist, at least in the rat parotid gland (see Garrett and Thulin, 1975) and so it may be possible that such pathways could become active in the submandibular gland.

An alternative explanation is that these proteins enter the saliva by crossing the secretory epithelium. Serum proteins are known to enter saliva in this way (Ellison and Mashimo, 1958; Ellison et al. , 1960; Junquera, Toledo and Ferri, 1965) and in the rat submandibular gland adrenaline substantially increases the amount of protein entering the saliva by this route (Junquera et al. , 1965). However, when rat serum proteins were fractionated on the same gels as the proteins secreted in reponse to stimulation of the sympathetic trunk (results not shown) there were no bands

in common between the two protein populations. It would seem therefore that serum ^{proteins} are not present in rat saliva in any great quantities, and that highly sensitive immunochemical techniques are required to detect these proteins.

There have been several studies of the pharmacology of fluid secretion by the rat submandibular gland (e.g. Emmelin et al. , 1965; Thulin, 1974, 1976a, 1976b). The results of the present study agree with the findings of these authors. The sympathetically evoked secretion is mediated via both α and β -adrenoceptors, whilst a small component of the parasympathetic secretion is resistant to the combined effects of atropine, propranolol and dihydroergotamine. The significance of this response, first described by Thulin (1976a) is unknown.

CHAPTER 3:- THE SECRETION OF PROTEOLYTIC ENZYMES
IN RESPONSE TO PHYSALAEMIN AND OTHER
SECRETORY STIMULI.

Introduction.

Most salivary glands normally secrete only in response to stimuli reaching them via the autonomic nerves, consequently many agents with secretory actions imitate the effects of autonomic nervous stimulation (see Burgen and Emmelin, 1961; Emmelin et al. , 1973). However, at least one group of compounds act as potent sialogogues without acting via any of the "classical" autonomic receptors. These agents are all short chain peptides and are known collectively as the "tachykinins". The most potent representative of this group is the 11 amino acid peptide physalaemin, originally isolated from the skin of the South American amphibian Physalaemus fuscumaculatus (Erspamer, Bertaccini and Cei, 1962) but later synthesised (Bernadi, Bosisio, Goffredo and de Castiglione, 1964). This compound causes salivation in a number of species, being particularly potent in the dog and rat (Erspamer, Anastasi, Bertaccini and Cei, 1964; Bertaccini and de Caro, 1965; Emmelin and Lenninger, 1967). As prior administration of α -adrenergic, β -adrenergic and muscarinic antagonists, either alone or in combination, does not diminish the secretory response to physalaemin (Bertaccini and de Caro, 1965; Emmelin and Lenninger, 1967; Schneyer and

Hall, 1968; Thulin, 1976b), this agent is thought to act via a specific "peptide" receptor on the surface of the secretory cell (Putney, 1977; Putney, van de Waale and Wheeler, 1980).

There have been very few studies of the composition of the saliva evoked by physalaemin, or any other of the tachykinins. Schneyer and Hall (1968), investigated the levels of sodium, potassium and amylase in rat submandibular and parotid saliva secreted in response to this agent. They found the saliva's composition to be essentially similar to that obtained upon parasympathetic nervous stimulation. This finding agreed with the subjective observation, made by Bertaccini and de Caro (1965), that the saliva secreted by the rat in response to injections of physalaemin resembled that collected after injection of parasympathomimetic drugs.

Recently Coroneo, Denniss and Young (1979) conducted a more detailed investigation of the composition of the saliva secreted by the rat submandibular and major sublingual glands in response to physalaemin, which they administered by close arterial injection. These authors found that the physalaemin-evoked saliva closely resembled that secreted in response to acetylcholine, thus confirming the findings of Schneyer and Hall (1968). However Coroneo et al. (1979) also observed that the relationship between the concentrations of sodium and potassium in the saliva, and the secretory rate differed for the two agents. These results suggested that whilst acetylcholine

stimulated the ductal reabsorption of sodium, this process was inhibited by physalaemin. This finding confirmed the results of experiments conducted using the isolated, perfused excretory ducts of rat and rabbit submandibular glands (Denniss and Young, 1978).

The aim of the present study was to investigate the effects of physalaemin upon protein secretion from the rat submandibular gland. As the proteins present in the secretions of this gland have previously been characterised electrophoretically (this thesis chapters 1 and 2) the use of these techniques would seem the logical approach to the problem. However preliminary experiments indicated that the amount of protein present in the physalaemin-evoked saliva was very small, and electrophoresis revealed very little detail. As the author had already investigated the secretion of proteolytic enzymes by the rat submandibular gland in response to a range of autonomic agonists, it was decided to extend this study to include the physalaemin-evoked secretion. The original purpose of this investigation was to examine the possibility that enzymatic hydrolysis of the secreted proteins may cause anomalous banding patterns upon electrophoresis (see chapter 1). The present chapter gives an account of the secretion of these enzymes by the rat submandibular gland.

MATERIALS AND METHODS

i) Animals and anaesthesia.

Male rats of a Wistar strain were anaesthetised and prepared as described in chapter 1.

ii) Collection of saliva.

Samples of saliva secreted in response to acetyl β methyl choline (20-40 $\mu\text{g}/\text{kg}$, IV), phenylephrine (100-200 $\mu\text{g}/\text{kg}$, IV), isoprenaline (30-60 $\mu\text{g}/\text{kg}$, IV) or physalaemin (3-5 $\mu\text{g}/\text{kg}$, IV) were collected as described in chapter 1. The physalaemin employed ("Sigma") was supplied in sealed ampoules, each containing 100 μg of the drug together with 20 mg of dextran. The entire contents of one vial were dissolved in 10 mls of ice cold 0.9% saline and this solution stored for up to 7 days at 4^o C.

As Abe and Dawes (1978) suggested that chorda lingual nerve stimulation may stimulate both muscarinic and α -adrenergic receptors experiments were undertaken to investigate the effects of α -adrenoceptor blockade upon the levels of proteolytic activity present in samples of saliva collected upon stimulation of this nerve. In these experiments the submandibular duct was cannulated and the chorda lingual nerve stimulated as described in chapter 2. Five samples of saliva were collected into preweighed tubes and the secretory flow rate estimated by assuming the secretion to have a specific gravity of unity. The stimulation frequencies

were chosen to give as wide a range of flow rates as possible. Dihydroergotamine (1mg/kg, IV) was then given and 30 minutes later efficacy of α -adrenoceptor blockade tested by injecting phenylephrine (200 μ g/kg, IV). After a further 20 minutes chorda lingual stimulation was repeated and further samples of saliva were collected. At the end of this period the extent of α -adrenergic blockade was again tested. Saliva samples were assayed as described below.

iii) Protein estimation.

Total protein was assayed by the method of Lowry et al. (1951) using BSA as a standard.

iv) Estimation of proteolytic activity.

The level of proteolytic activity present in each sample was estimated using the chromogenic trypsin pseudosubstrate N α benzoyl DL-arginineparanitroanilide (BAPA). The method employed was a slight modification of that described by Bhoola and Dorey (1971) for the assay of glandular homogenates.

The reaction was carried out in a 3 ml plastic cuvette containing 2.6 mls of 200 mM tris buffer (approximately 240 mM tris, 30 mM CaCl₂ adjusted to pH 7.8 with conc HCl and diluted to 200 mM tris, 25 mM CaCl₂), and 200 μ l of 10 mM BAPA (250 μ moles of BAPA dissolved in 12.5 mls of N,N dimethylformamide and then made up to 25 mls with 200 mM tris buffer. The cuvette was then placed in the thermostatically controlled

heating-block of a Pye-Unicam SP8-100 spectrophotometer and its temperature monitored. Once thermoequilibrium was established ($37 \pm 0.1^{\circ}$ C) an aliquot of saliva (2-50 μ l) was added and the cuvette contents thoroughly mixed. The optical density at 405 nm was monitored and recorded continuously using the instrument's integral chart recorder. This absorbance was then related to the concentration of paranitroaniline (the coloured reaction product) in the reaction mixture by means of a standard curve. Reaction velocities were calculated from the initial rate of substrate hydrolysis and were expressed either as moles of substrate hydrolysed / minute / μ l of saliva (mole/min./ μ l), or as moles of substrate hydrolysed / minute / μ g of salivary protein (mole/min/ μ g).

The substrate activation kinetics of this reaction were not investigated, however a 2 fold increase in the amount of BAPA present in the reaction medium did not cause any increase in the rate at which the substrate was hydrolysed. It was therefore considered that the enzyme was working at maximum velocity under the assay conditions.

v) Statistics.

Student's two tailed t-test was used to test for significant differences between groups of data, P values of less than 0.05 were considered as significant. Critical values of the regression coefficient, R, were taken from the tables in Rohlf and Sokal (1969).

RESULTS

The levels of proteolytic activity present in samples of saliva secreted in response to the sialogogues employed are presented in figure 3.1. In this figure the data are expressed as moles/min./ μ l. Quite clearly the greatest level of activity is found in the saliva secreted in response to phenylephrine, this level being 129 times greater than that observed after isoprenaline ($t=4.196$; $df=15$; $P=0.0011$); 682 times greater than the level in the saliva evoked by acetyl methyl choline ($t=4.467$; $df=17$; $P=0.006$), and 3,280 times the activity found in the saliva secreted in response to physalaemin ($t=4.706$; $df=17$; $P=0.0004$).

In previous studies of enzyme secretion by salivary glands the results have usually been expressed as units of activity per unit volume (eg Matthews, 1974; Ørstavik and Gautvik, 1977; Asking and Gjørstrup, 1980a, 1980b; Gjørstrup, 1979, 1980a, 1980b). However in the case of the rat submandibular gland the amount of protein present in the secretion is known to vary over a wide range according to the secretory stimulus employed (eg Martinez et al. , 1975a). The levels of protein found in the saliva samples collected during the present study are shown in figure 3.2. Furthermore in this gland, two distinct populations of secretory cells contribute to the protein in the final saliva. Of these cells only the granular ducts are thought to secrete proteolytic enzymes (Junquera et al. , 1964; Cunningham, 1967; Matthews,

Fig. 3.1 Levels of proteolytic activity (as moles BAPA hydrolysed $\text{Min.}^{-1} \mu\text{l.}^{-1}$) present in samples of saliva secreted in response to physalaemin (Ph.), acetyl β methyl choline (Ac.), isoprenaline (Iso.) and phenylephrine (Phe.). Bars delimit ± 1 standard error.

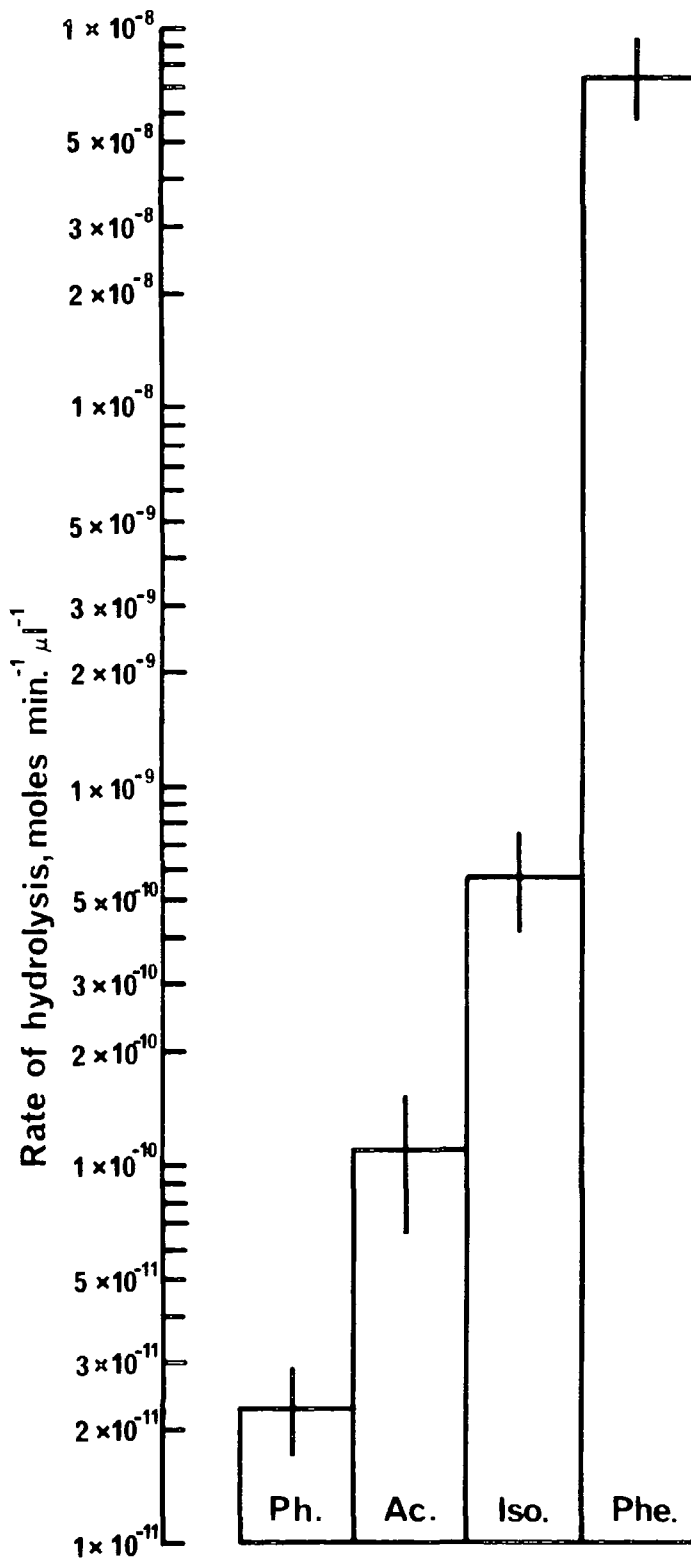
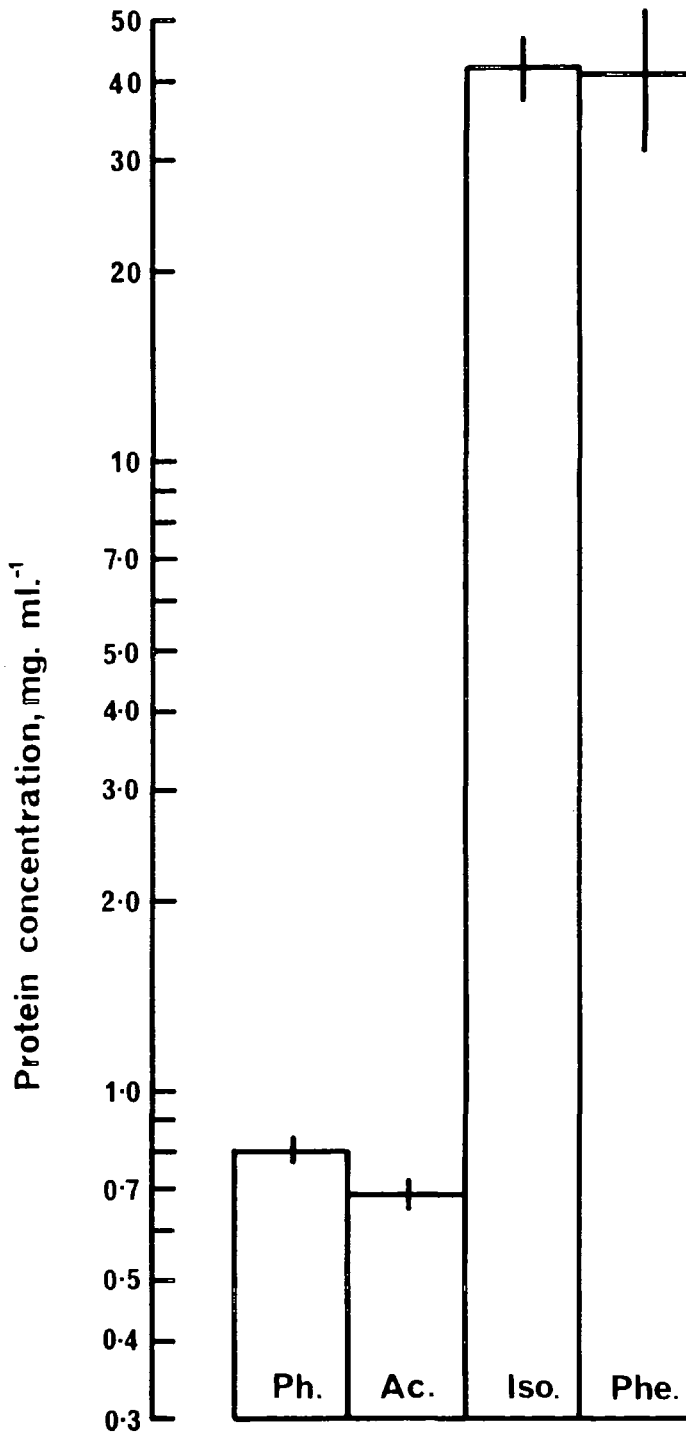


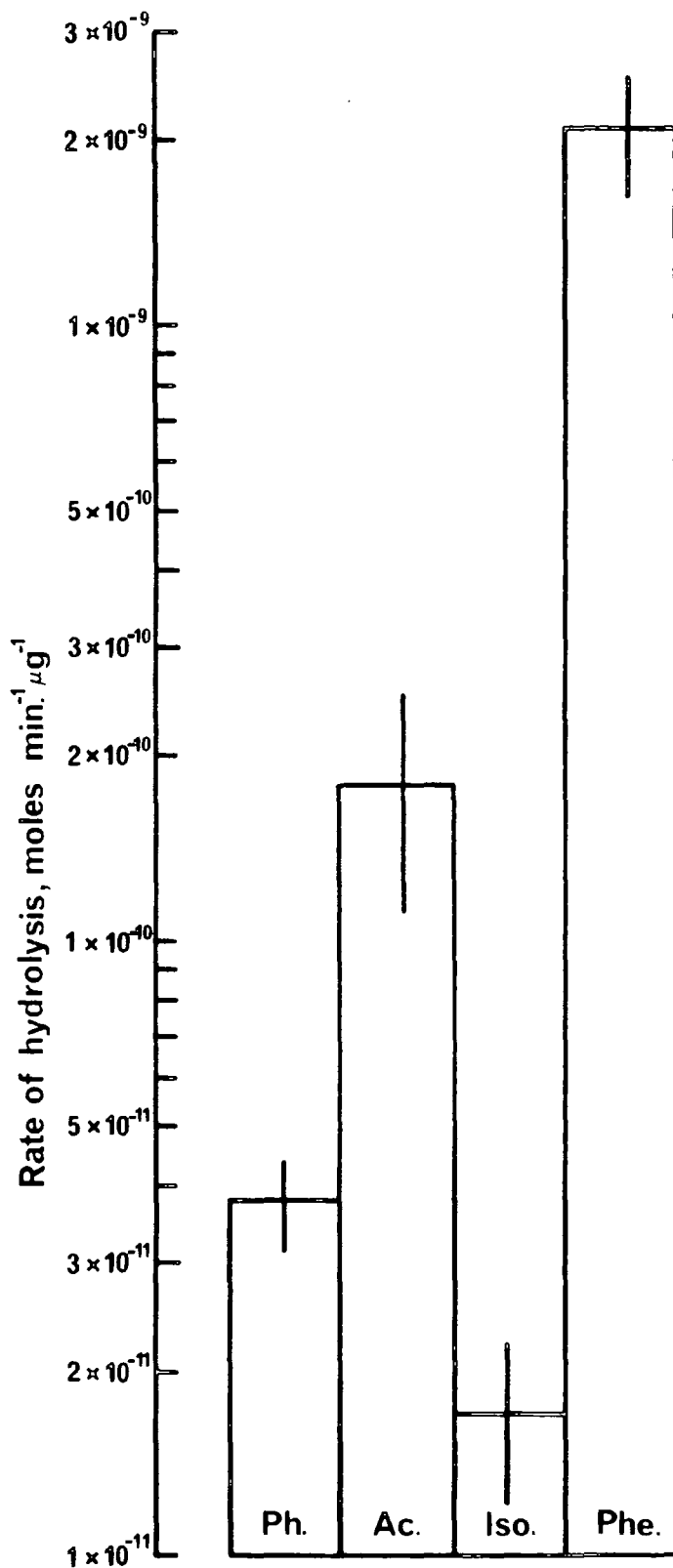
Fig. 3.2 Levels of total protein (estimated by the method of Lowry et al., 1951) present in samples of saliva. Abbreviations as for fig. 3.1. Bars delimit ± 1 standard error.



1974). Under these conditions enzyme activities should be corrected for differences in the total amount of protein present in each sample. Without this correction it would be impossible to tell whether a difference in the level of proteolytic activity was due simply to differences in the amount of protein in the secretion or due specifically to a change in the amount of enzyme secreted into the saliva.

When the data are corrected for the differences in the level of total protein in the saliva (i.e. expressed as moles/min./ μ g) the values do not cover such a wide range as when the data are expressed as moles/min./ μ l. More importantly however the various sialogogues now show a rather different "order of potency" (see figure 3.3). The highest activity is still associated with phenylephrine, and this value was 11.5 times the level found in the saliva evoked by acetyl methyl choline ($t=5.255$; $df=10$; $P=0.0006$); 74 times the level observed after physalaemin ($t=7.100$; $df=13$; $P=0.00005$), and 121 times that for the saliva secreted in response to isoprenaline ($t=5.375$; $df=9$; $P=0.0007$). It is interesting that although isoprenaline caused the secretion of a considerable amount of enzyme activity, the specific activity (activity as moles/min./ g) of the secreted enzyme was very low. Conversely acetyl methyl choline caused the secretion of only a very small amount of activity (1/5 th the amount in the saliva collected after administration of isoprenaline : $t=2.886$; $df=17$; $P=0.01$), but the specific activity of this enzyme

Fig. 3.3 Specific activities (as moles BAPA hydrolysed $\text{Min}^{-1} \mu\text{g}^{-1}$) of the salivary proteases. Abbreviations as for fig. 3.1. Bars delimit ± 1 standard error.



was 10 times greater than the value observed after isoprenaline ($t=2.195$; $df=11$; $P=0.048$). Physalaemin caused the secretion of only a small amount of enzyme which had a very low specific activity, not significantly different from that calculated for isoprenaline ($t=1.216$; $df=14$; $P=0.243$).

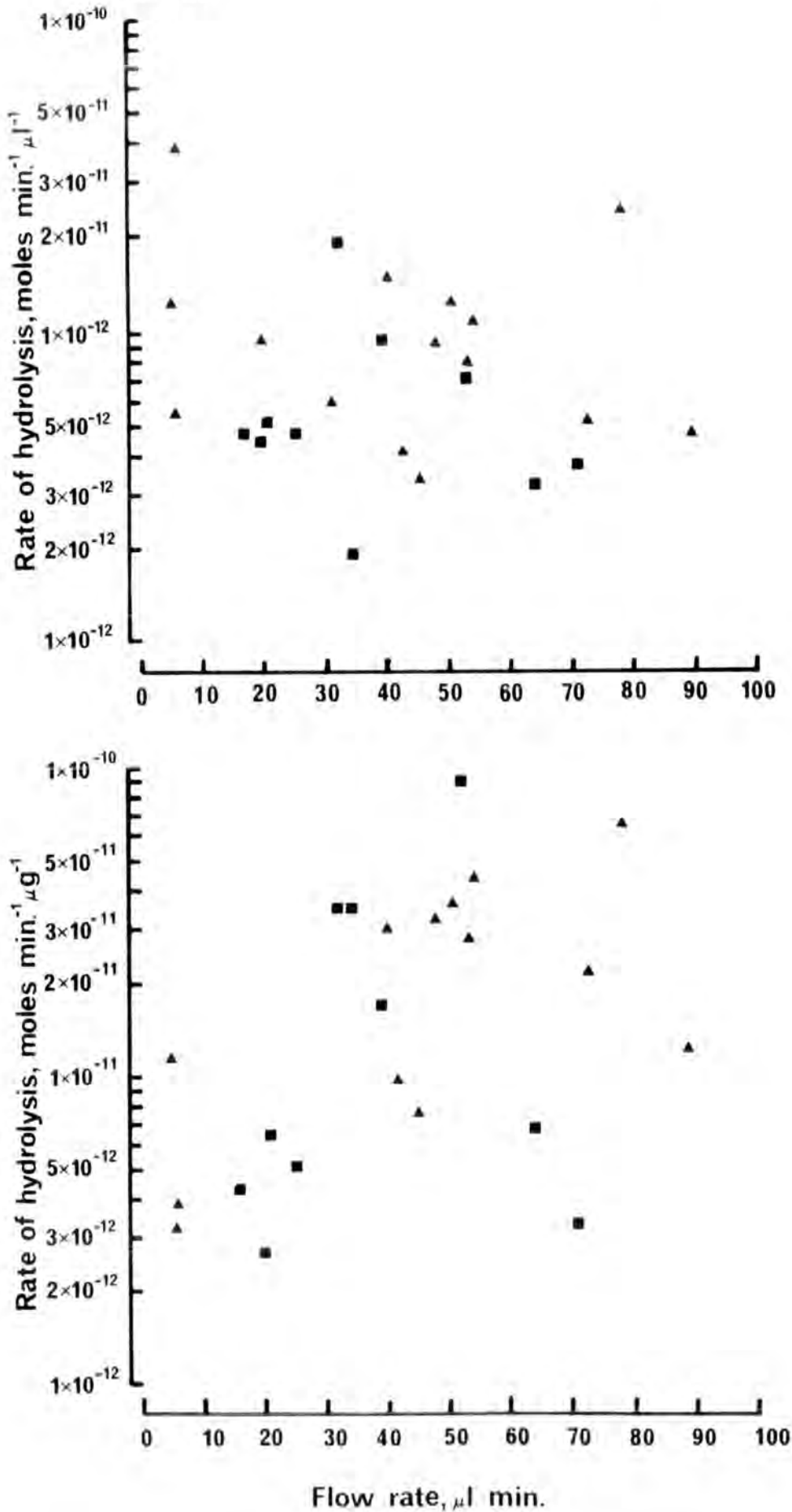
The level of proteolytic activity present in the samples of saliva secreted in response to stimulation of the chorda lingual nerve was always very low, both before and after dihydroergotamine. The mean values are presented in table 3.1. Dihydroergotamine did not cause any statistically significant change, either in the amount of proteolytic activity secreted or in the specific activity of the secreted enzyme. There was a considerable amount of variation in the amount of enzyme present in different samples of saliva, even between those secreted by the same animal. As the samples were all collected whilst the gland was secreting at a steady rate (estimated by collecting into pre-weighed tubes), the data were analysed to determine whether any correlation existed between the flow rate and either, the amount, or the specific activity of the secreted proteases. The results of this analysis are presented in figure 3.4. There was no significant correlation between the salivary flow rate and the amount of enzyme present in the saliva, either before ($R=0.043$; $n=15$; $P>0.05$) or after ($R=0.146$; $n=10$; $P>0.05$) administration of dihydroergotamine. Similarly the specific activities of the secreted enzyme showed no significant correlation

TABLE 3.1

Effects of DHE (1 mg/kg) upon the levels of proteolytic activity present in samples of saliva secreted in response to stimulation of the chorda-lingual nerve. Values are mean \pm SE.

	<u>Moles BAPA/min/μl</u>	<u>Moles BAPA/min/μg</u>
Control	$1.268 \times 10^{-11} \pm 0.37 \times 10^{-11}$	$2.357 \times 10^{-11} \pm 0.506 \times 10^{-11}$
DHE	$8.236 \times 10^{-12} \pm 1.949 \times 10^{-12}$	$2.055 \times 10^{-11} \pm 0.873 \times 10^{-11}$
t	0.929	0.315
dt	23	21
P	0.365	0.687

Fig.3.4 Proteolytic activity present in samples of saliva secreted upon chorda lingual stimulation both before (\blacktriangle) and after (\blacksquare) D.H.E. (1 mg/kg).



with flow rate either before ($R=0.543$; $n=13$; $P>0.05$) or after ($R=0.227$; $n=10$; $P>0.05$) blockade of α -adrenoceptors.

DISCUSSION.

The present study is the first in which the effects of physalaemin upon the secretion of protein by salivary glands has been investigated in vivo. Spearman and Pritchard (1980) have recently investigated the effects of this agent upon the secretion of proteases by the rat submandibular gland in vitro. Their results indicate that physalaemin is not an effective stimulus of protease secretion, a finding supported by the results of the present experiments. As has been previously described (Erspamer et al., 1964; Bertaccini and de Caro, 1965; Emmelin and Lenninger, 1967; Schneyer and Hall, 1968; Thulin, 1976b; Coroneo et al., 1979) physalaemin was a very potent stimulus of fluid secretion, a dose of 5 $\mu\text{g}/\text{kg}$ (IV) caused the secretion of 5-6 drops of saliva (approximately 100 μl) within 45-60 seconds. There has been very little speculation as to why such a potent sialogogue should be found in the skin of an amphibian. One possible explanation is that this agent may make the toads themselves unpalatable to mammalian predators.

The secretion of proteases by the rat submandibular gland in response to stimulation of the autonomic nerves was investigated by Matthews (1974). In this study saliva secreted in response to sympathetic nervous

stimulation was found to contain 18 times the proteolytic activity (expressed as activity per unit volume) found in samples of parasympathetic saliva. After α -adrenoceptor blockade (phentolamine) this figure was reduced to 3 times the parasympathetic level. Whilst the β -adrenoceptor antagonist propranolol reduced the activity to 9 times the activity found in parasympathetic saliva. The experiments described in the present chapter parallel those conducted by Matthews (1974) as secretion was evoked by administration of autonomic agonists rather than by stimulation of the autonomic nerves in the presence of specific blockers. The most immediate contrast between the results of the two studies is that the data obtained from the present experiments cover a very much wider range than the comparable figures obtained by Matthews (1974). The reason for this probably lies in the fact that the present investigation made use of a synthetic substrate in the assay, whilst Matthews (1974) employed casein, a naturally occurring protein possessing a large number of phosphorylated peptide chains. The results suggest that the salivary proteases are capable of digesting the synthetic substrate at a very much higher rate, thus making the present assay far more sensitive.

The data presented in figure 3.1 agree with the findings of Matthews (1974). Both investigations indicate that the secretion of proteolytic enzymes is mediated primarily via α -adrenoceptors, but that β -adrenoceptor activation also caused the output of a

considerable amount of enzyme activity. Similarly both studies have shown muscarinic stimuli to be relatively ineffective. Matthews did not consider the specific activities (expressed as mole/min./ μg) of the secreted enzymes. These data (presented in figure 3.3) are interesting in that quite marked differences in specific activity exist between the different agonists. This result indicates that different protein populations are secreted in response to the various agonists, thus confirming the results of the electrophoretic studies presented in chapter 1. The proteolytic enzymes in the rat submandibular gland are known to be confined almost exclusively to the cells of the granular ducts (Junquera et al. , 1964; Cunningham, 1967; Matthews, 1974; Al-Gailani et al. , 1980). Al-Gailani et al. (1980) detected proteases within the acini of the rat submandibular gland, but did not consider that this activity was due to proteins destined for secretion. If it is accepted that the secreted proteases originate mainly from the granular ducts then a high specific activity means that a sialogogue causes protein secretion primarily from these cells. Conversely a low specific activity means that the proteins present in the saliva are principally of acinar origin. On this basis, phenylephrine acts almost exclusively upon the granular ducts whilst isoprenaline acts upon the acinar cells. Isoprenaline caused the secretion of a considerable amount of proteolytic activity (expressed as moles/min./ μl), but this activity represents a considerable amount

of protein (see figure 3.2) with a very low specific activity (see figure 3.3).

Acetyl β methyl choline caused the secretion of only a very small amount of enzyme activity (see figure 3.1) but this represented a small amount of protein with a specific activity considerably higher than that observed after isoprenaline. This result suggested that the proteins secreted in response to acetyl β methyl choline originate from both the granular ducts and acini. The "peptidergic" agonist physalaemin, like acetyl β methyl choline, caused the secretion of very little protein. However consideration of the specific activities of the secreted proteins indicated that this agent only caused proteins to be secreted from the acini (see fig 3.3) When Abe and Dawes (1978) investigated the proteins secreted by the rat submandibular gland, their results indicated that stimulation of the chorda lingual nerve did not cause exclusive stimulation of muscarinic receptors. They noted that a number of the proteins secreted corresponded to species previously observed only after administration of α -adrenergic agonists. Furthermore, as the secretion of these proteins was abolished by administration of α -adrenergic antagonists, these authors concluded that stimulation of this nerve excited α -adrenoceptors as well as muscarinic receptors. This is a surprising observation as stimulation of the chorda lingual nerve has previously been extensively employed as a simple means of stimulating the parasympathetic fibres supplying the submandibular gland

(eg Yoshida et al. ,1967; Muir and Templeton, 1976; Ørstavic and Gautvik, 1977). As stimulation of the glandular α -adrenoceptors has been shown to cause the secretion of proteolytic enzymes, it was decided to investigate the levels of proteolytic activity in saliva evoked by stimulation of the chorda lingual nerve, both before and after administration of dihydroergotamine. If Abe and Dawes (1978) were correct, then saliva evoked by chorda lingual nerve stimulation should contain elevated levels of proteolytic activity, and this would be decreased by α -adrenoceptor blockade. The results of these experiments (see figure 3.4 and table 3.1) showed that the level of activity present in the nerve-mediated saliva was always very low. Similarly the specific activities of these proteases were also low. Administration of dihydroergotamine did not cause any significant reduction in these values. The results, therefore contradict the findings of Abe and Dawes (1978) as they indicate that chorda lingual nerve stimulation does not activate the glandular α -adrenoceptors. The reason for this discrepancy is unknown. In this part of the study the amount of enzyme secreted varied considerably even between successive samples from the same animal. The reason for this was probably that the amount of enzyme present was always very small, and the values recorded (see table 3.1) are approaching the smallest amounts detectable with this technique.

In the present study the amount and specific activity of the enzymes secreted in response to acetyl

β -methyl choline, were greater than the values recorded for stimulation of the parasympathetic nerves. This indicates that whilst muscarinic agonists may cause secretion from the granular ducts, parasympathetic nervous stimulation does not. The reason for this discrepancy is unknown. The most obvious possibility is that the granular ducts are provided with muscarinic receptors, yet receive no functional parasympathetic innervation. However, histological studies clearly indicate that these structures are innervated by cholinergic fibres (Garrett, 1972, 1974). Morphological evidence of secretion from the granular ducts has been observed following large intraperitoneal injections of the muscarinic agonist pilocarpine (Scott, 1964; Heins and Tamarin, 1968; Cutler and Chaudry, 1973), although not by all authors (Junquera et al., 1964; Matthews, 1974). Matthews (1974) suggested that this secretion may be caused by pilocarpine acting upon the post-synaptic nicotinic receptors in the superior cervical ganglion, rather than directly upon the ductal cells themselves, Schneyer and Hall (1965, 1966) having originally shown that pilocarpine may activate such pathways. It is unlikely that acetyl β -methyl choline would act in this manner, as this compound is not thought to retain the nicotinic properties of acetyl choline (see Taylor, 1980).

In summary the secretion of protein and of proteases by the rat submandibular gland in response to administration of physalaemin and other sialogogues as

well as to stimulation of the chorda lingual nerve has been investigated in vivo . Physalaemin was found to be a relatively ineffective stimulus of protease secretion, an observation which agrees with previous findings in vitro (Spearman and Pritchard, 1980). Of the "classical" autonomic agonists, phenylephrine was found to be the most effective stimulus for protease secretion and acetyl β -methyl choline the least. Isoprenaline was found to be of an intermediate potency. These findings confirm the results of parallel experiments conducted by Matthews (1974). Consideration of the specific activities of the secreted enzymes suggested that isoprenaline and physalaemin caused the secretion of proteins which originated primarily from the acini, whilst the proteins secreted in response to phenylephrine originated primarily from the granular ducts. These results agree with the findings of the electrophoretic studies described in chapter 1. Acetyl β -methyl choline appeared to cause some ductal secretion, a rather surprising observation as this did not appear to be the case for parasympathetic nerve stimulation.

CHAPTER 4:- THE EFFECT OF RESERPINE UPON THE
CARBOHYDRATE HISTOCHEMISTRY OF THE RAT
SUBMANDIBULAR GLAND.

INTRODUCTION.

Reserpine is one of the many alkaloids which may be extracted from the roots of the climbing shrub Rauwolfia serpentina . The major pharmacological action of this compound is to deplete the stores of noradrenaline, adrenaline and serotonin in the sympathetic nerve terminals, adrenal medula and cerebral cortex respectively. The subsequent reaccumulation of these compounds is also inhibited, although this is not a permanent effect. The principal physiological actions of this agent may be explained in terms of a reduction or removal of sympathetic tone. Thus the drug causes a reduction in peripheral vascular resistance; a reduction in cardiac output; an increase in gastric motility and tone, and an increase in the rate of gastric acid secretion. Central effects such as sedation, and in some cases depression, are also observed and these are attributed to the depletion of serotonin in the cerebral cortex (see Wiener, 1980b for a full review of the pharmacology of reserpine).

Taylor, Richardson, Roddy and Titus (1967) observed the quantity of trichloroacetic acid soluble glycoproteins present in the rat submandibular gland to increase 4-5 fold 18-24 hours after a single injection of

reserpine (0.25-2 mg/kg). As removal of the superior cervical ganglion or section of the cervical sympathetic trunk did not produce this effect, it was not simply a consequence of sympathectomy. Similarly, the effect was observed in chronically decentralised glands (both lingual nerve and cervical sympathetic trunk divided), and so the effect was not due to the central actions of the drug. Taylor et al. (1967) suggested that this hitherto undescribed effect was the result of a direct action of reserpine upon the submandibular gland itself. The effects of reserpine upon this gland have since been investigated by Martinez and his co-workers (Martinez et al. , 1975a; Martinez, Adelstein, Quissell and Barbero, 1975b; Martinez, Adshead, Quissell and Barbero, 1975c; Martinez and Quissell, 1977; Quissell, Martinez and Giles, 1977; Cutler, Baccuzi, Yaeger, Bottaro, Christian and Martinez, 1981). Martinez et al. (1975b) noted the accumulation of glycosylated proteins first described by Taylor et al. (1967), as an increase in the amount of material visible in histological sections after staining with the PAS technique. This material was found both in the acinar cells and in the lumina of the ductal system. In this publication the authors point out that a number of similarities exist between the morphology of the salivary glands in the human genetically-transferred disease cystic fibrosis, and the morphology of the rat submandibular gland after 7 days of reserpine treatment. They propose that the reserpine-treated rat submandibular gland may represent a potentially useful experimental

model of this disease.

Additional support for this view came from a study of the composition of the saliva secreted by the reserpine-treated rat submandibular gland. Whilst the maximum secretory rate was found to be depressed, the saliva from these glands was found to contain elevated levels of sodium, calcium, protein and carbohydrate (Martinez et al. ,1975c). These changes certainly resemble those described in cystic fibrosis (Chernick, Barbero and Parkins, 1961; Chernick and Barbero, 1963; Chernick, Eichel and Barbero, 1964; Mandel, Kutscher, Denning, Thompson and Zegarelli, 1967). Perhaps more significantly the saliva from the reserpine-treated rat submandibular gland was found to cause disorientation and eventual abolition of the cilliary beat in the gills of the freshwater mussel Unio . Both saliva (Doggett and Harrison, 1973) and serum (Spock, Hieck, Cress and Logan, 1967; Bowman, Lockhart and McCombs, 1969) from patients with cystic fibrosis have been shown to contain a similar "ciliotoxic factor". Evidence of a more general similarity between the effects of reserpine and cystic fibrosis has been obtained from studies of the pancreas (Perlmutter and Martinez, 1978) and lung (Thompson, Quissell, Williams and Martinez, 1976) of the reserpine-treated rat.

Martinez et al. (1975b) found reserpine to cause a simple increase in the amount of PAS-positive material present within the submandibular gland, the accumulated material being apparently the same as that normally

stored within the gland. This observation agrees with the findings of Taylor et al. (1967) who detected no significant differences between the carbohydrate/protein ratios in the glycoproteins extracted from either the control or reserpine-treated animals. However, Taylor et al. (1967) did suggest that qualitative differences may exist which remained undetected by their experimental approach.

The aim of the present study was to investigate the carbohydrate histochemistry of the reserpine-treated rat submandibular gland to determine:

- i) To what extent do the morphological changes caused by reserpine resemble those changes described in cystic fibrosis.
- ii) Whether reserpine causes any qualitative change in the population of glycoproteins present within the gland.

The author wishes to thank Miss P.M. Smith (Department of Histopathology, Dryburn Hospital, Durham) for her help with the experiments described in this chapter.

MATERIALS AND METHODS

i) Treatment of animals.

A total of 15 male Wistar rats were housed under identical conditions in 5 groups each of 3 animals. Each animal in 3 of these groups received a total of 7 daily injections of reserpine (free base, 0.5 mg/kg). The

injectate was prepared by first dissolving the drug in glacial acetic acid at a concentration of 50 mg/ml, this solution was then diluted about 80 fold with 0.9% (w/v) saline and its pH adjusted to 4.5 with sodium hydroxide. More saline was then added to give a final concentration of 0.5 mg reserpine/ml. This solution was always prepared immediately before use, as the drug tended to precipitate after a few hours. One group of animals received 7 daily injections of the solvent buffer alone, whilst the fifth served as untreated controls. All animals were starved for 18-24 hours prior to removal of the glands, but allowed water ad libitum.

ii) Fixation and Embedding.

All glands were fixed with 4% (v/v) formaldehyde buffered to pH 7.2 with 50 mM phosphate buffer. In most cases the animals were anaesthetised with sodium pentobarbitone (60 mg/kg, IP), the submandibular glands dissected free and placed in the fixative. These glands were processed after 48-72 hours of fixation. In some cases the glands were fixed by perfusion via the aorta. These animals were also anaesthetised with sodium pentobarbitone and a polythene cannula (Portex PE 50) introduced into the aorta via the left ventricle. The descending aorta was clamped off and, after flushing out the vasculature with 0.9% (w/v) saline containing heparin (10 i.u./ml), the fixative was gently infused using a 20 ml syringe. Back pressure was relieved by severing both jugular veins high in the neck. In all cases the glands

were flushed free of blood almost immediately. The glands fixed in this manner were processed after a further 2-4 hours in fixative.

All tissues were conventionally dehydrated through graded ethanols, infiltrated and wax embedded. 5 μ m thick sections were cut from the blocks as required.

iii) Histological techniques.

Sections cut from the embedded blocks were stained for glycoproteins using a number of different techniques:

a) The Alcian Blue / PAS technique was employed as described by Mowry (1956). The sections were stained with 1% (w/v) Alcian Blue 8GX in 3% (v/v) glacial acetic acid (pH 2.5). After washing in distilled water they were then oxidised (1% (w/v) aqueous periodic acid for 2 minutes), and then stained with Schiff's reagent for 8 minutes. A nuclear counterstain was not employed in this technique.

b) Sections were also stained with the Alcian Blue / PAS technique after methylation of the terminal carboxyl groups of the glycoproteins within the tissue. This was achieved by incubating the sections for 4 hours at 37 C in absolute methanol containing 0.8% (v/v) concentrated hydrochloric acid. Control sections were incubated for the same length of time in distilled water at 37 C. The control and methylated sections were then stained using the Alcian Blue / PAS technique as described above.

c) The Alcian Blue / critical electrolyte

concentration technique (Scott and Dorling, 1965) was also employed. For this technique the exact magnesium chloride concentration of an approximately 2 M solution was determined by titrating a 1 ml aliquot against 100 mM mercuric nitrate to a diphenylcarbazone endpoint. On the basis of this result, 5 solutions of 0.05% (w/v) Alcian Blue 8GX (Hopkins and Williams) in 200 mM sodium acetate buffer pH 5.8 were prepared, containing 0 mM, 200 mM, 400 mM, 600 mM or 800 mM magnesium chloride respectively. Five representative sections from each gland were stained overnight in one of these solutions, and then counterstained with 0.5% (w/v) aqueous Neutral Red for 2 minutes.

d) Sections were also stained with the combined Alcian Blue / Alcian Yellow technique (Ravetto, 1964). For this technique sections are first stained with 1% Alcian Blue in 200 mM hydrochloric acid, and then with 1% Alcian Yellow in 3% glacial acetic acid. The sections were then counterstained with 0.5% aqueous Neutral Red.

For all histochemical techniques efficacy of staining was checked by running a control slide in parallel with the experimental material. This slide carried 5 μ m wax sections of various human tissues whose staining properties have been well characterised (see Cook, 1977). The following tissues were employed, umbilical cord (strongly sulphated glycoproteins, hyaluronic acid), stomach (neutral mucins) and submandibular gland (sialomucins).

In addition some sections were stained with

haematoxylin and eosin, for general tissue histology.

A full account of all staining procedures employed is given by Cook (1977).

RESULTS

No matter which staining procedure was employed no difference could be detected between the glands removed from untreated animals and those which had received the control injections of solvent buffer.

When staining with haematoxylin and eosin, glands removed from the reserpine-treated animals were of a very different appearance to those from control and untreated animals. The acini were always larger and stained much less intensely in the reserpine-treated animals, and in many cases the cytoplasm had a vacuolated appearance. Plate 4.1 shows typical sections, stained with haematoxylin and eosin taken from control and reserpine-treated animals.

Staining with Alcian Blue / PAS indicated that reserpine caused marked changes in the carbohydrate histochemistry of the rat submandibular gland. In the control glands the acini stained magenta, indicating that they contained a population of glycoproteins which was essentially neutral. However, after 7 days of reserpine treatment these cells stained blue, indicating that they now contained predominantly acidic glycoproteins (see plate 4.2). In many cases material with staining properties similar to the acinar material was seen

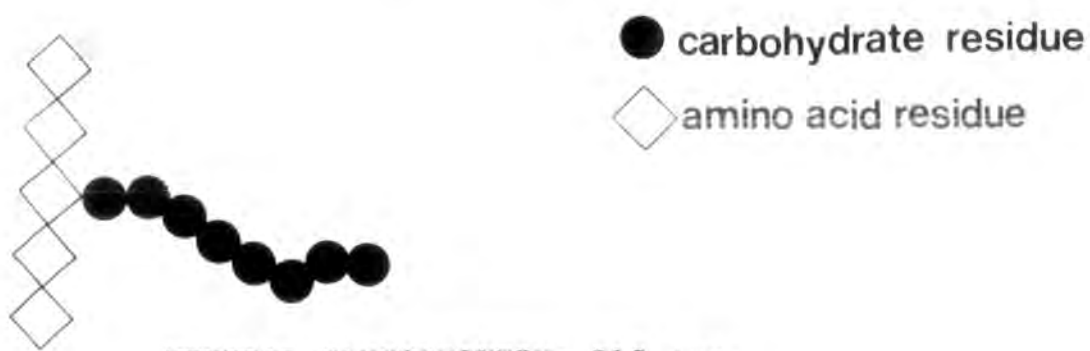
deposited in the lumina of some of the intercalated and striated ducts (see plate 4.4).

The epithelial cells of the granular ducts were weakly PAS positive (i.e. stained magenta), and contained numerous small PAS positive granules. These cells showed similar staining properties in the control, untreated and reserpine-treated animals. However in the control groups the granules present in these cells were generally confined to the luminal portion of the epithelium. In the reserpine-treated animals the number of granules present was far greater, so that they now extended right up to the cells basal border (see plate 4.3).

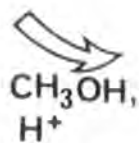
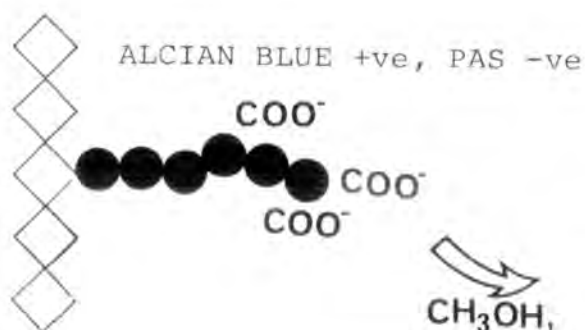
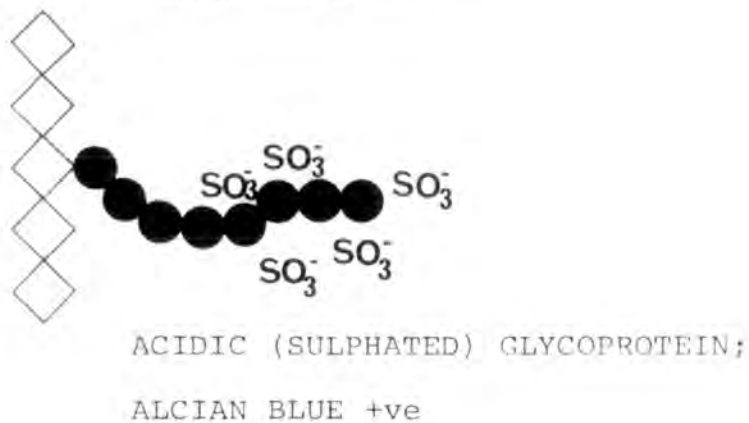
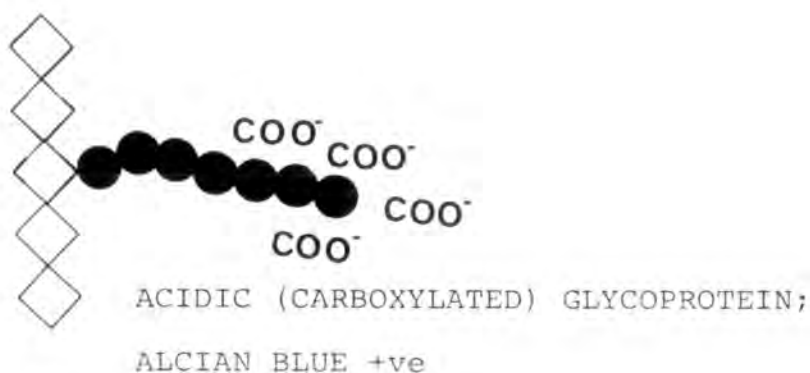
Acidic glycoproteins of epithelial origin generally fall into two subclasses, the sulphated glycoproteins (alternatively known as the sulphomucins) and the carboxylated glycoproteins (the sialomucins). Their structures are shown in figure 4.1. Attempts were made to determine into which of these subclasses the acidic glycoproteins accumulated by the reserpine-treated rat submandibular gland fell. This was achieved using a number of different histochemical techniques.

Methylation of the terminal carboxyl groups present within the section (see figure 4.1) prevented the Alcian Blue staining normally seen in the acini of the reserpine-treated animals after staining with the Alcian Blue / PAS technique (see plate 4.5). Blocking of Alcian Blue staining in this manner is a reaction characteristic of carboxylated rather than sulphated glycoproteins (Spicer, 1960), and so this result suggests that the

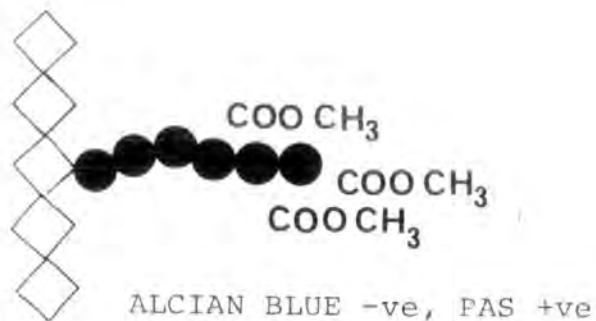
Fig 4.1:- Simplified structures of neutral and acidic glycoproteins.



NEUTRAL GLYCOPROTEIN; PAS +ve.



METHYLATION



acidic proteins accumulated by the reserpine-treated rat submandibular gland belong to this category. The rat submandibular gland contained numerous mast cells. These cells stained very intensely with Alcian Blue, both before and after methylation (see plate 4.5). As these cells contain heparin, a strongly sulphated glycoprotein (see Cook, 1977), this observation serves to confirm that this technique is capable of distinguishing between sulphated and carboxylated glycoproteins.

When sections from control animals incubated in distilled water were compared "side by side" with those which had been methylated by incubation in acidified methanol, it was apparent that a very small amount of Alcian Blue staining was present in the submandibular glands removed from the control and untreated animals. This observation indicated that small quantities of a carboxylated glycoprotein, similar to that accumulated by the reserpine-treated rat submandibular gland are also present in the control glands.

The combined Alcian Blue / Alcian Yellow technique was also employed. Using this procedure, any sulphated glycoproteins present in the section are initially stained with Alcian Blue at a very low pH (0.7). Carboxylated glycoproteins, whose staining is characteristically blocked at this low pH are then stained with Alcian Yellow at pH 2.5. Using this approach both the acinar and ductal epithelia of the control animals were unstained (except for the Neutral Red counterstain). In the reserpine-treated animals there

was faint yellow staining in the acinar cells, indicating the presence of carboxylated glycoproteins within these cells.

Sections from both the control and reserpine treated animals were also stained with Alcian Blue using the critical electrolyte concentration technique. This technique relies on the fact that certain cations will compete with the cationic dye Alcian Blue for the binding sites on the glycoproteins within the section. As the different glycoprotein subclasses display differing affinities for Alcian Blue, the electrolyte concentration at which staining is inhibited will also vary. In the present study the magnesium ion was employed as the competitive cation, and staining carried out at pH 5.8. In the absence of magnesium chloride there was extensive staining in both the ducts and acini of the submandibular glands removed from either control or reserpine-treated animals. The ductal staining was not as strong as that in the acini. Two hundred mM magnesium chloride effectively blocked the staining in both the ducts and acini of the glands removed from the control (see plate 4.6), and the reserpine treated (see plate 4.7) animals. Staining in the interlobular connective tissue persisted in the presence of up to 400-600 mM magnesium chloride. Strong granular staining persisted in the mast cells, even in the presence of 800 mM magnesium chloride, the highest concentration employed. Only sulphated glycoproteins would be expected to take up the Alcian Blue stain in the presence of 200 mM magnesium chloride,

Plate 4.1

Sections from control (upper panel) and reserpine-treated (lower panels) stained with haematoxylin and eosin. Note that in the reserpine-treated animals the acinar cells (Ac) are larger and stain less intensely, the cytoplasm of these cells often contains prominent vacuoles (arrows).

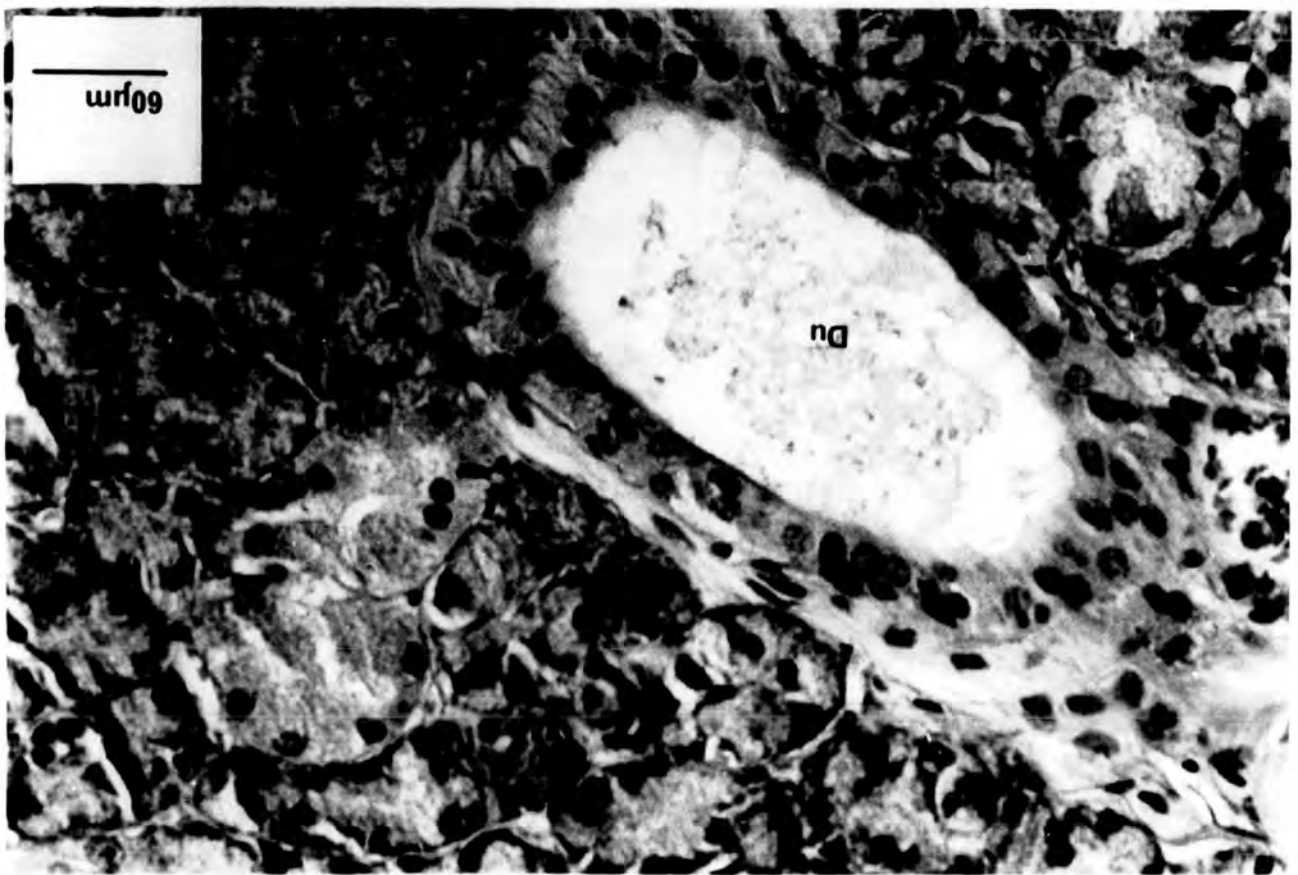


Plate 4.1

Plate 4.2

Sections from control (upper panel) and reserpine-treated (lower panel) animals which were stained simultaneously with the Alcian-Blue / PAS technique. Note that in the control animals the acinar cells (Ac) are stained magenta, whereas in the reserpine-treated animals these same cells are stained blue (ie. PAS negative). In both control and reserpine-treated animals the granules present in the granular ducts (Du) are PAS positive.

Plate 4.2

Ac

Ac

Du

30 μ m

Ac

Ac

Du

30 μ m

Plate 4.3

Alcian Blue / PAS stained sections from reserpine-treated (upper panel) and control animals (lower panel) showing details of granular ducts (G). In the reserpine-treated animals the granules in these cells are more prominent, often extending right to the cell's basal border (Arrows). In the control animals the granules are usually confined to the apical region of the cell (see also upper panel of plate 4.2), with the nucleus (n) placed against the cell's basal border (arrows).

Plate 4.3

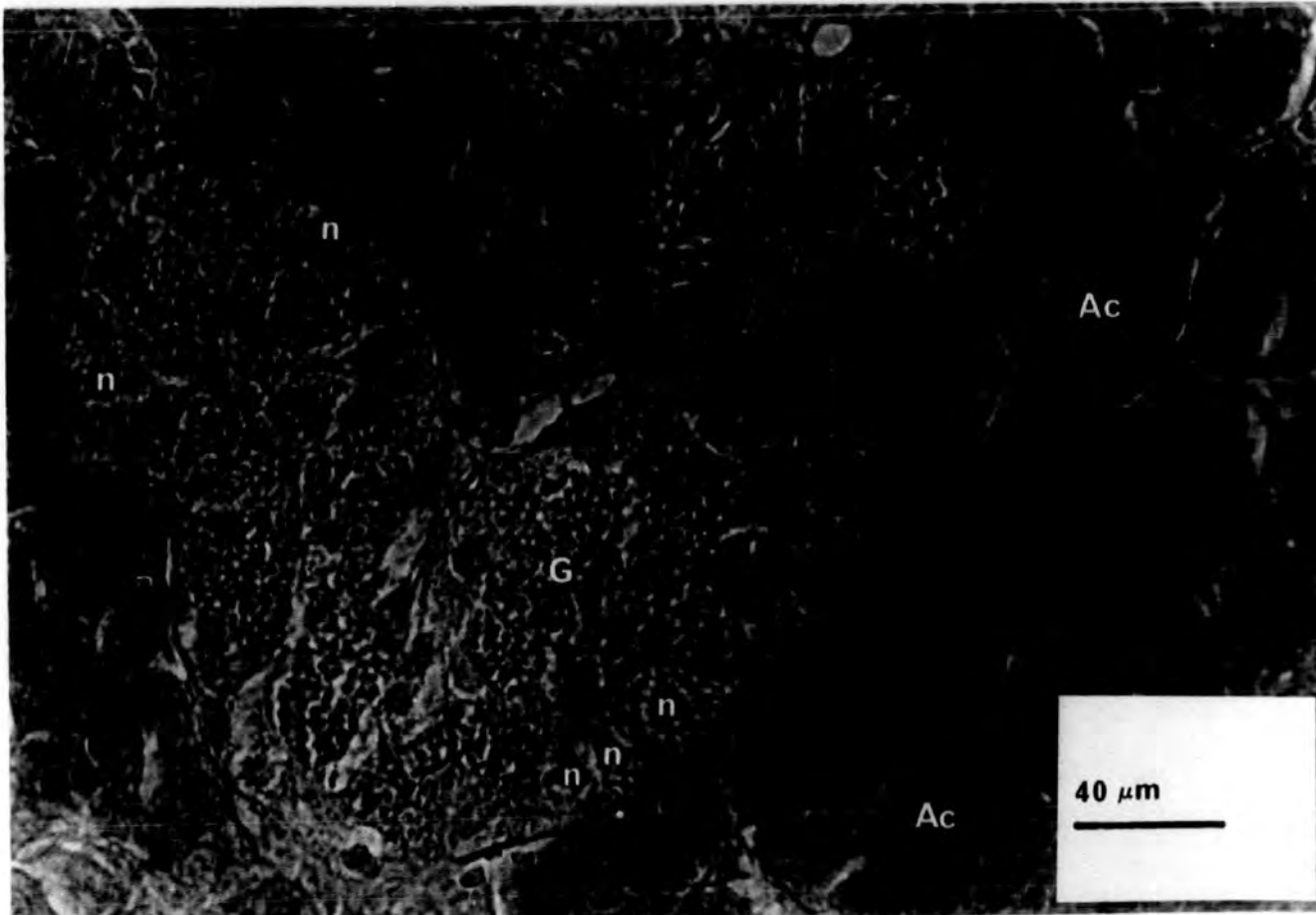
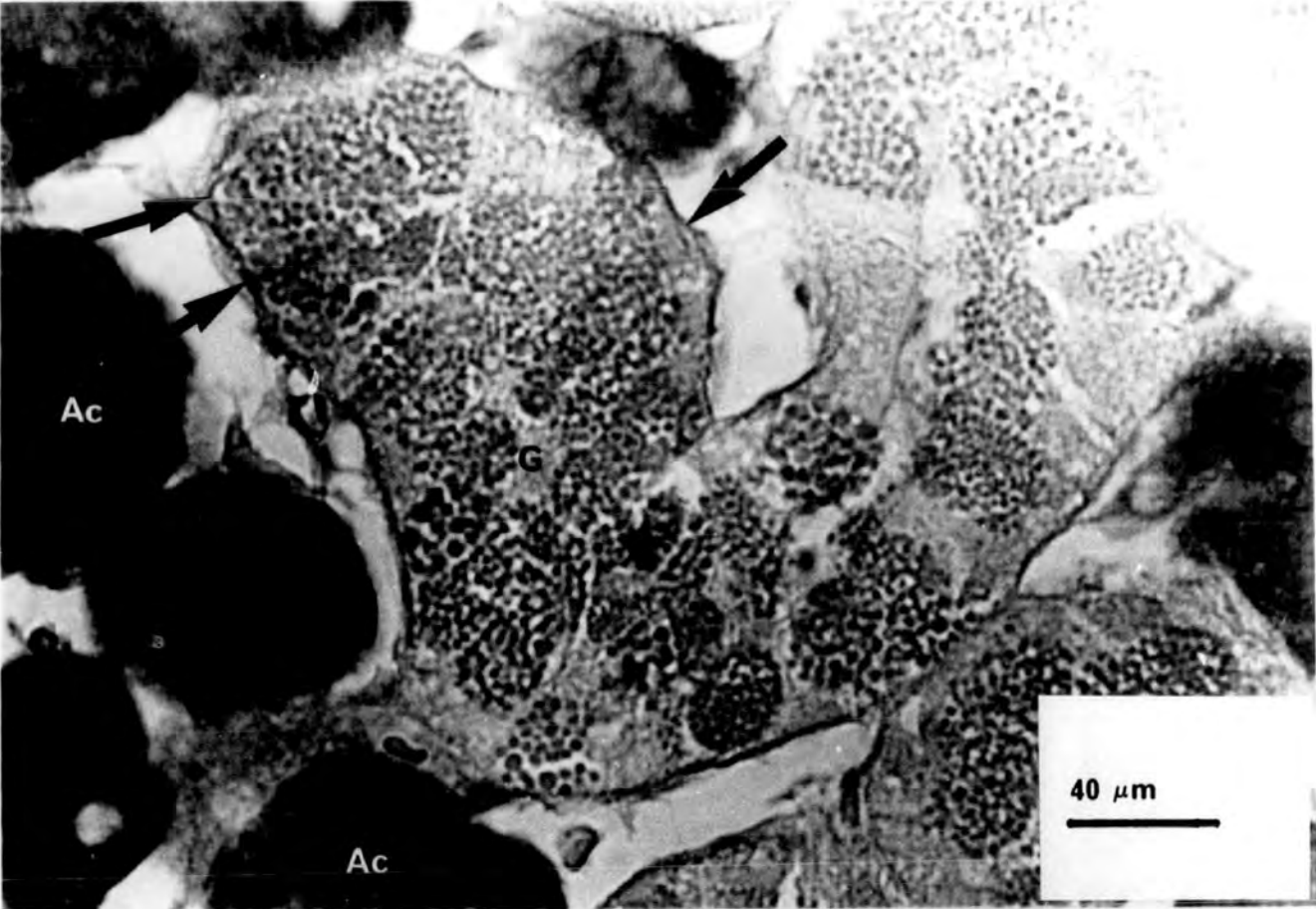


Plate 4.4

Sections from reserpine-treated animals stained with Alcian Blue / PAS showing the characteristic deposition of secretory material within the striated (St.) and interlobular (Int.) ducts (see also plate 4.4 upper panel). This showed similar staining properties to the material present in the acini (Ac). In a few cases very small amounts of secretory material were observed deposited in the intralobular ducts of control animals (see plate 4.1 upper panel).

Plate 4.4

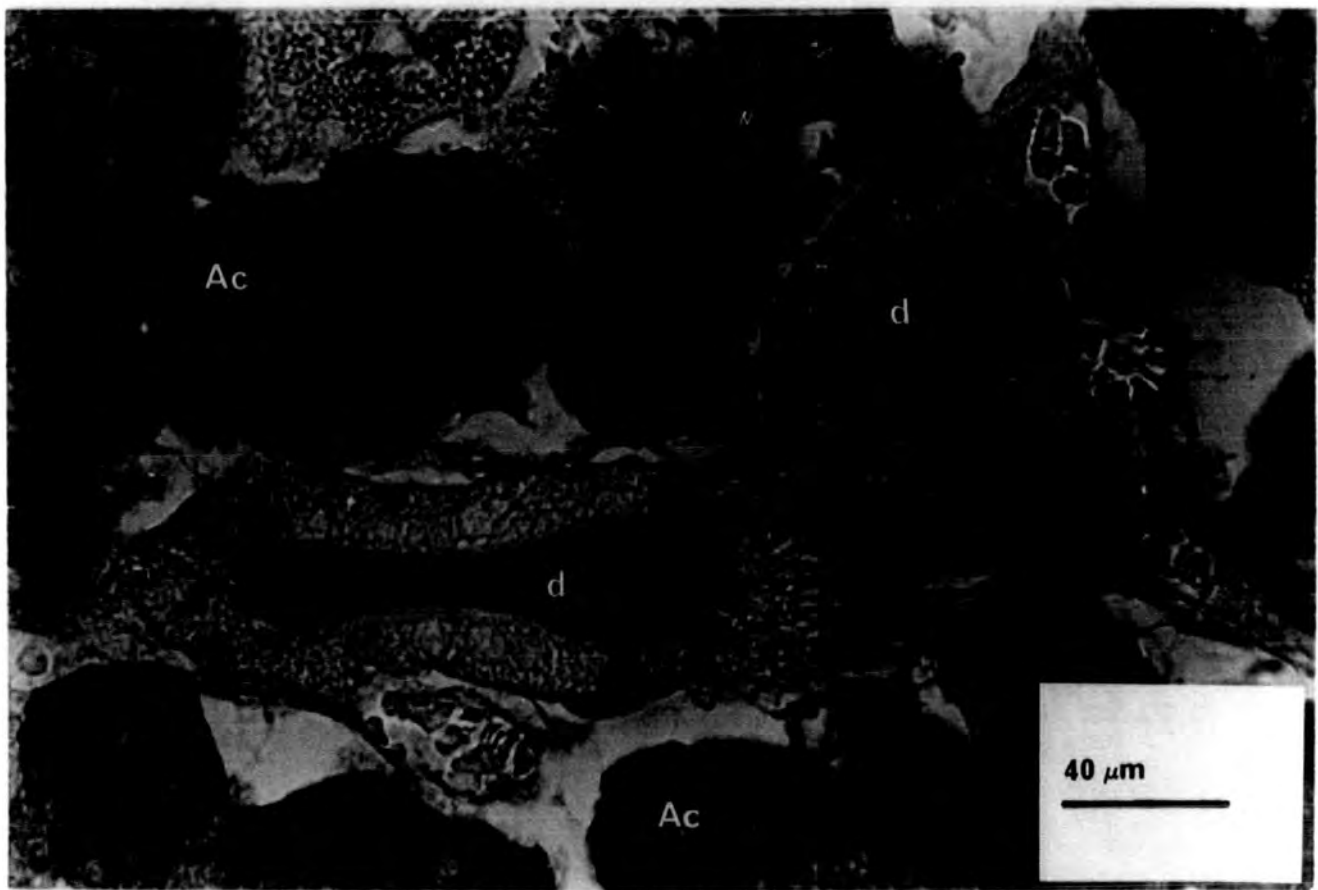
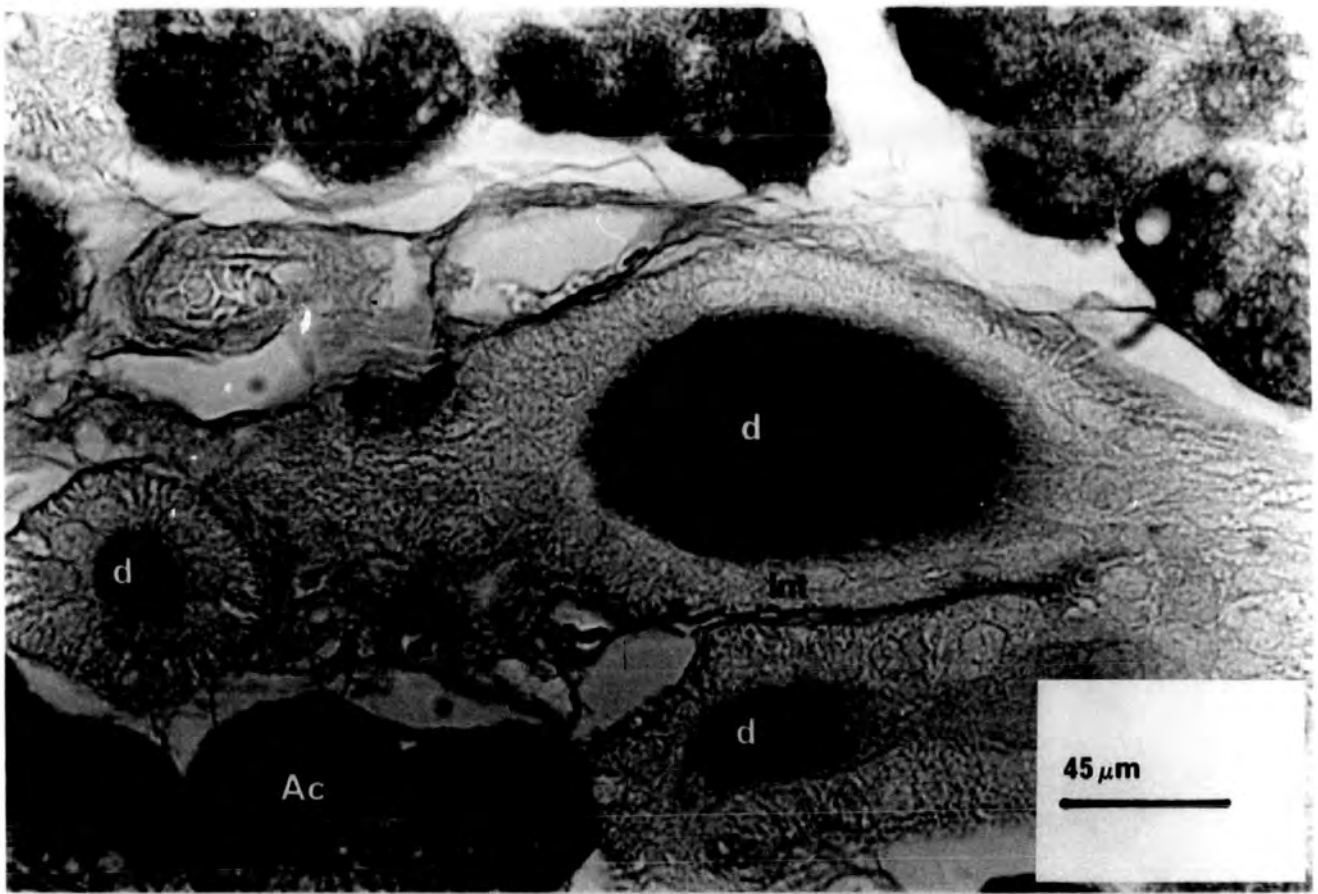


Plate 4.5

Sections taken from a reserpine-treated animal and stained with Alcian Blue / PAS. The upper panel shows a control section which was incubated in distilled water at 37 °C for 3 hours prior to staining. The lower panel shows a section which was incubated in acidified methanol at 37 °C for 3 hours. This treatment converts acidic carboxyl groups (Alcian Blue positive) to neutral methyl esters (PAS positive), indicating that the Alcian Blue staining in the reserpine-treated animals is due to the presence of carboxylated glycoproteins.

Both sections contain mast cells (MC) which are Alcian Blue positive due to the presence of a strongly sulphated glycoprotein (heparin). This staining is not prevented by methylation.

Note also the difference in staining between plate 4.2 (lower panel) and this plate (upper panel). This demonstrates the variability of Alcian Blue / PAS technique and illustrates the need to run control sections.

Plate 4.5

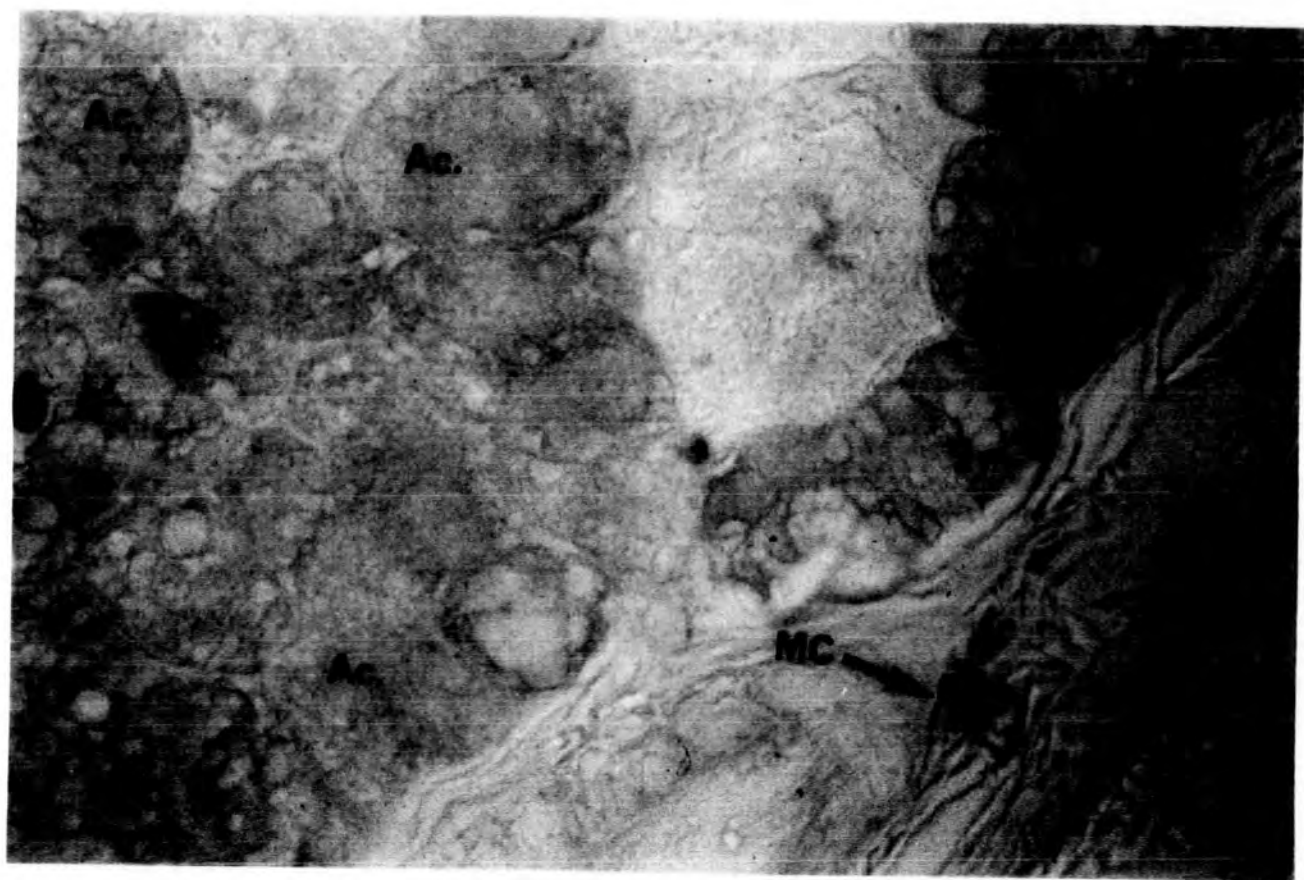
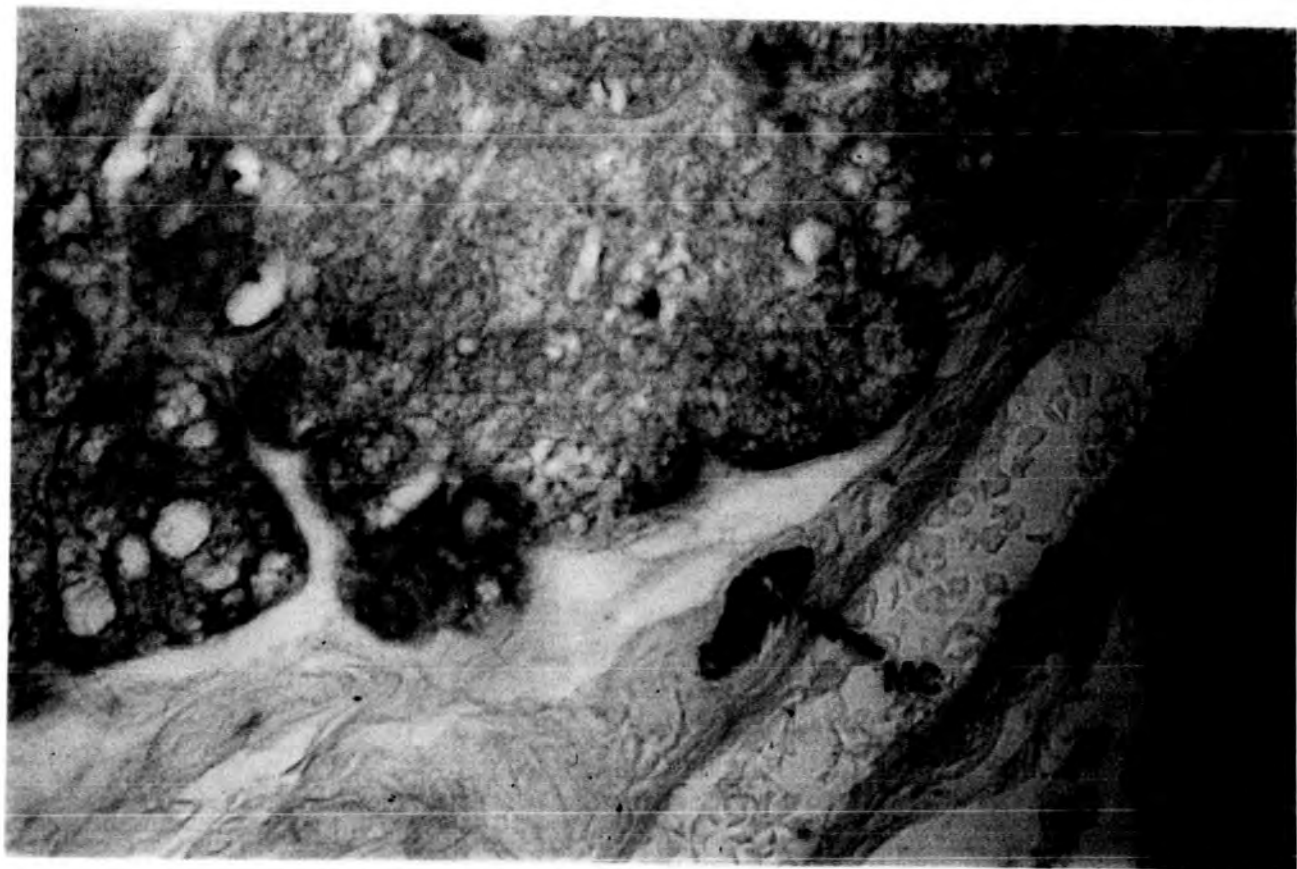


Plate 4.6

Sections from reserpine-treated animals stained with Alcian Blue / critical electrolyte concentration technique. Both sections were stained with Alcian Blue at pH 5.8. In the absence of magnesium chloride there was extensive staining in the acini (Ac). However, this was abolished by inclusion of 200 M magnesium chloride in the staining solution (lower panel). Staining in mast cells (M) persisted.

The nuclear staining is due to the Neutral Red counterstain.

Plate 4.6

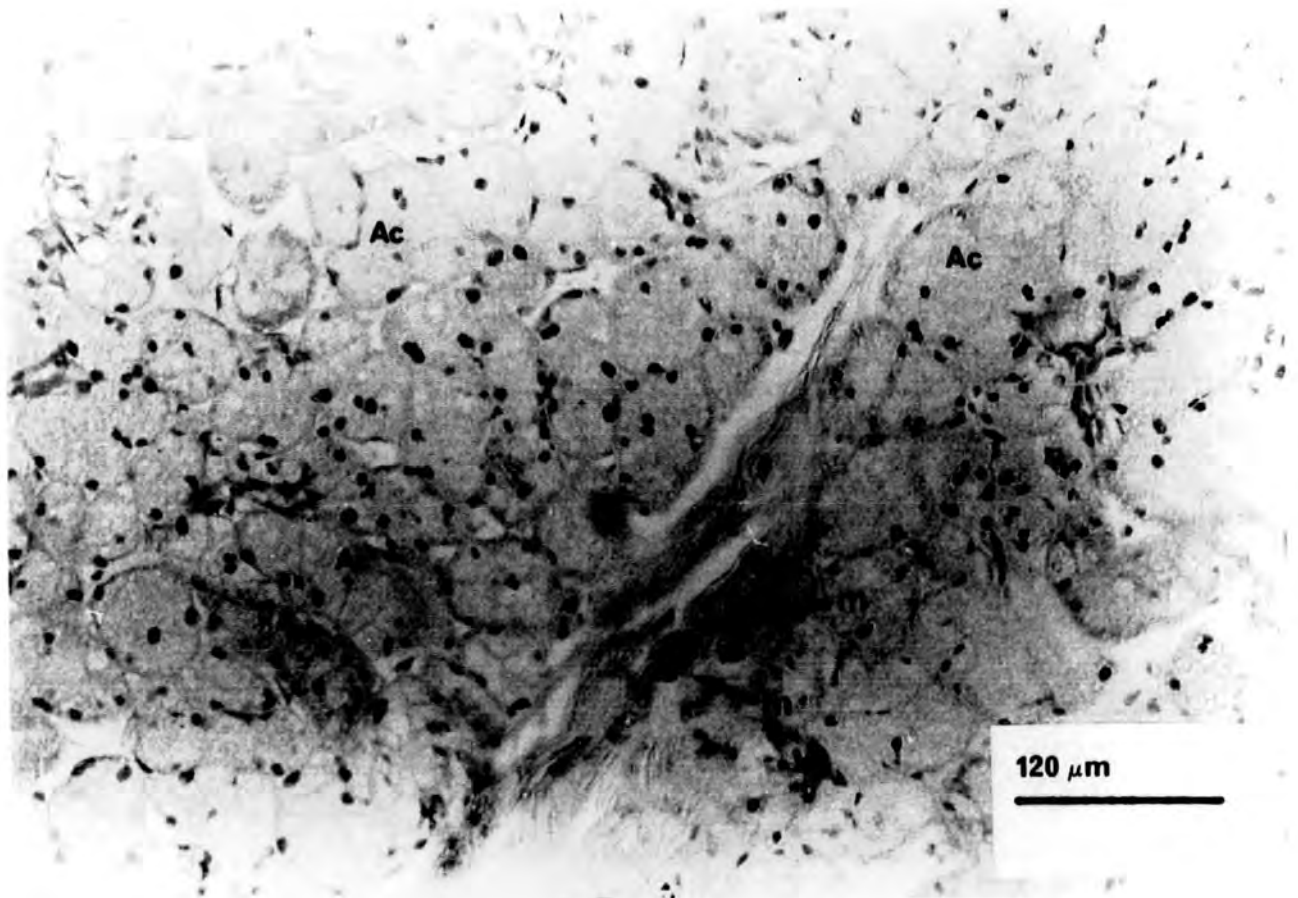
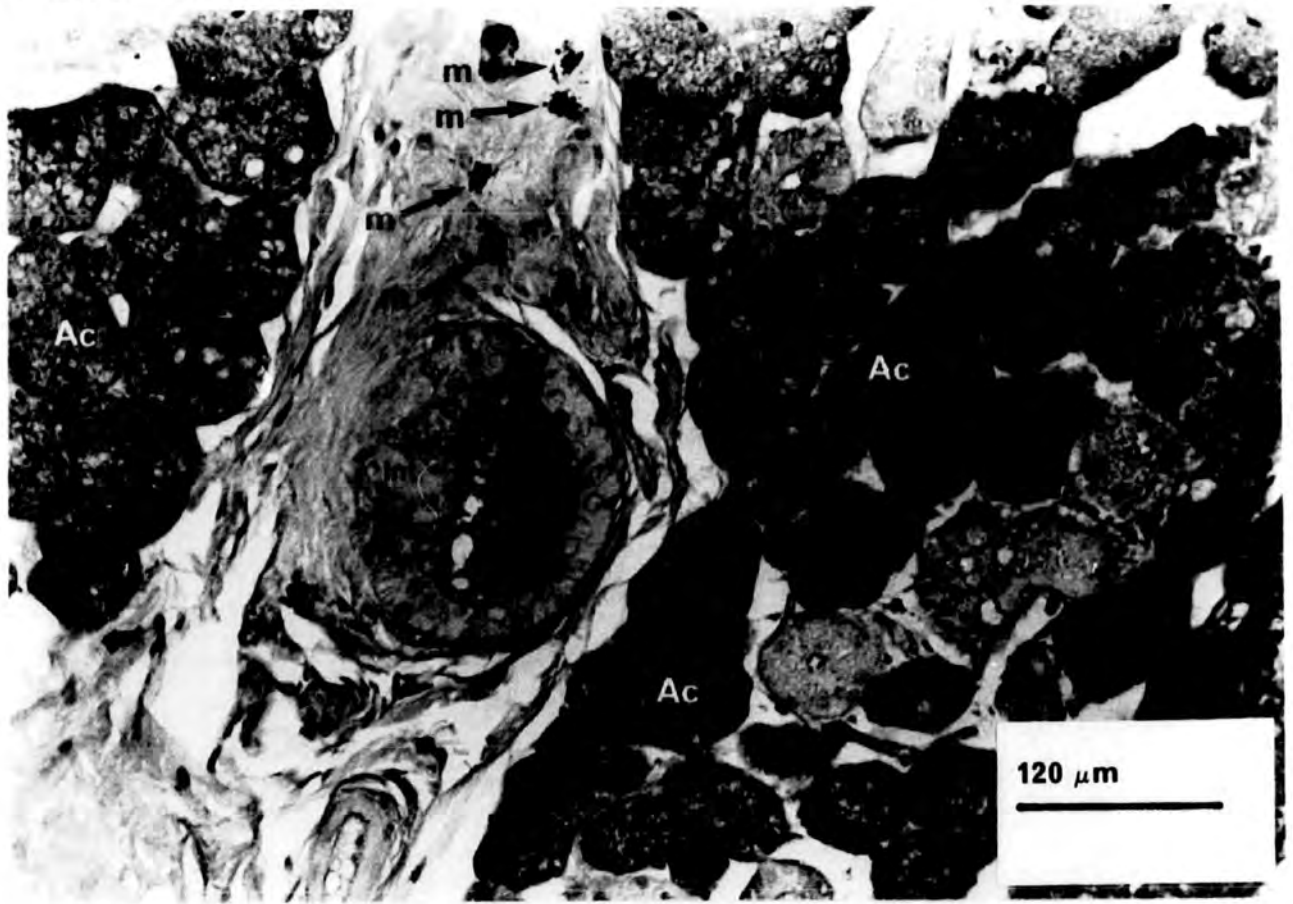
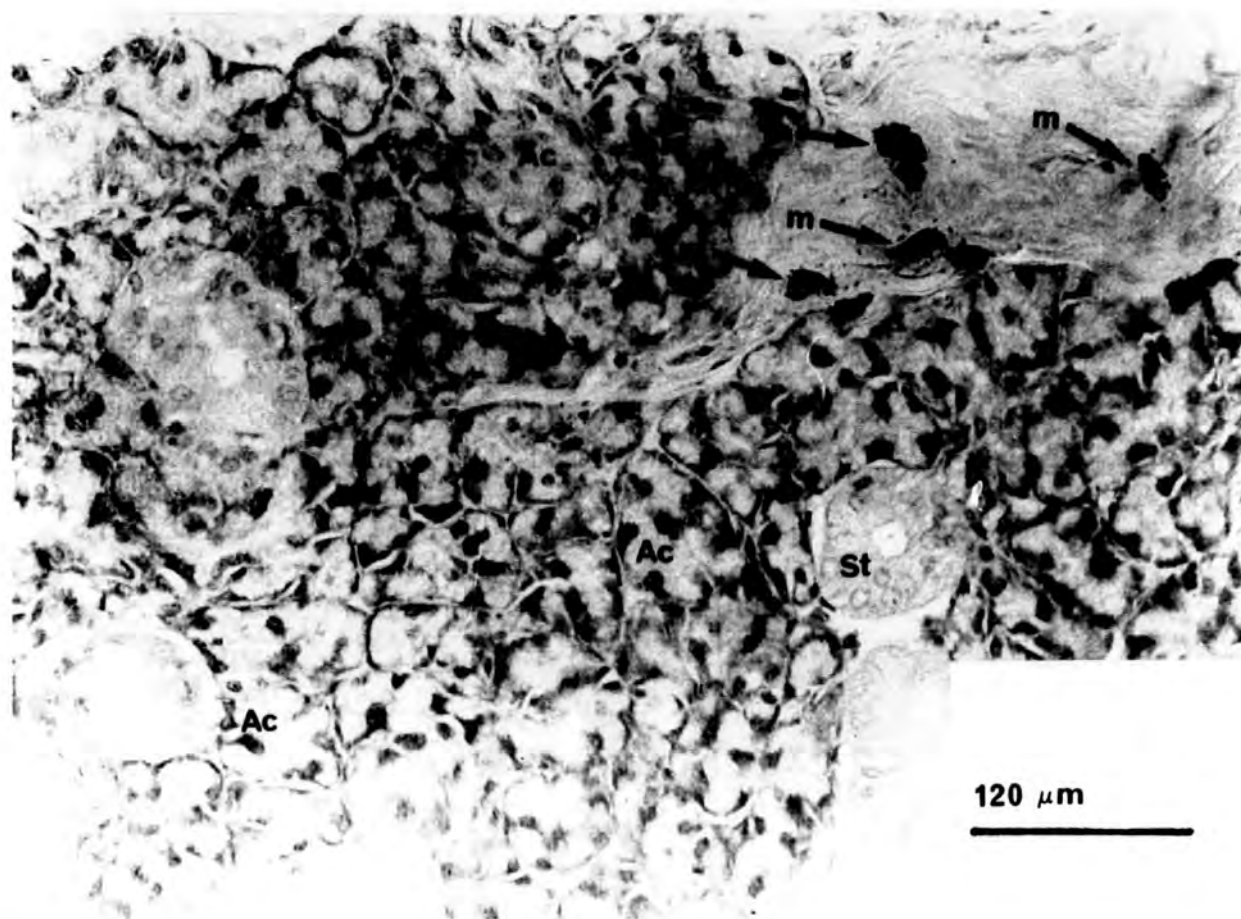
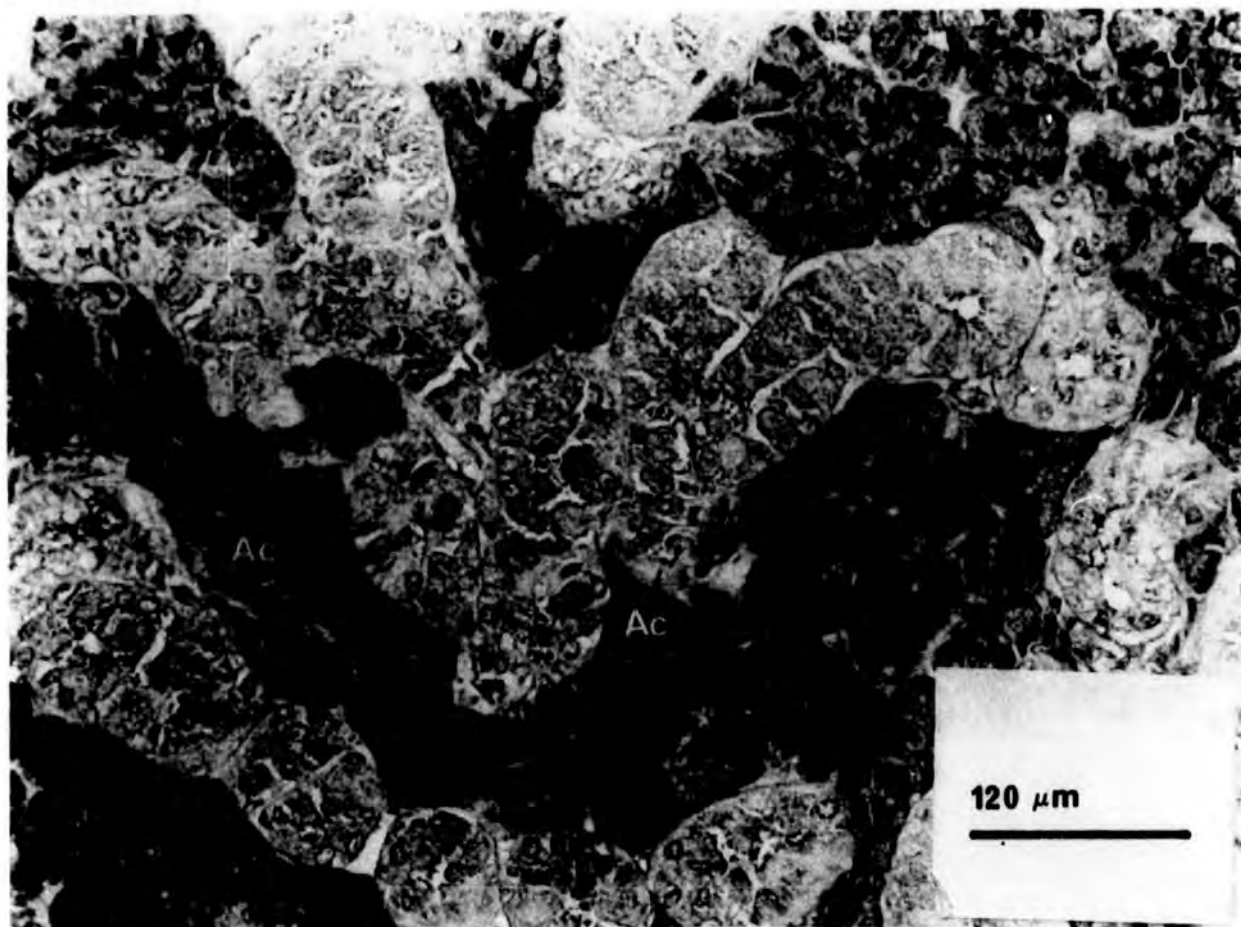


Plate 4.7

Sections from control animals stained with the Alcian Blue / critical electrolyte technique. Once again there is extensive acinar staining (Ac) in the absence of magnesium chloride , but this is abolished by 200 M magnesium chloride . Staining persists in the mast cells.

Plate 4.7



and so the results of this technique suggest that the acidic glycoproteins accumulated by the reserpine-treated rat submandibular gland do not fall into this glycoprotein subclass. The heparin-containing granules in the mast cells again serve as useful "internal controls", demonstrating that the technique has the potential to distinguish between sulphated and carboxylated glycoproteins.

DISCUSSION.

The experiments described in the present chapter indicate that the sympatholytic alkaloid reserpine causes an accumulation of glycoproteins within the rat submandibular gland. This result agrees with the findings of previous authors (Taylor et al. , 1967; Martinez et al. , 1975b). However the present study extends previous observations by indicating that this drug also causes a qualitative change in the glycoprotein population stored within the acinar cells. In the control and untreated animals the acini were found to contain a predominantly neutral population of glycoproteins, although some carboxylated glycoproteins were also present. These findings confirm the results of previous authors (Shackleford and Klapper, 1962; see review Young and van Lennep, 1978). After 7 days of reserpine treatment the acinar cells were found to contain an acidic population of glycoproteins. These acidic glycoproteins were found to be carboxylated rather

than sulphated.

When Martinez et al. (1975b) investigated the carbohydrate histochemistry of the reserpine treated rat submandibular gland they described a simple build-up of PAS positive material, observing none of the qualitative changes in carbohydrate histochemistry described here. The present study employed a wider range of histochemical techniques than did Martinez and his co-workers (Martinez et al. , 1975b), but these authors did employ the Alcian Blue / PAS technique. In the present study this method revealed quite striking differences in glandular histochemistry, whereas in the hands of Martinez et al. (1975b) this was not the case. The reason for this discrepancy is unknown. It may be that the effects described are strain specific as the present study was conducted using Wistar strain rats, whilst Martinez et al. (1975b) employed rats of a Sprague Dawley strain. However, it appears rather improbable that such major differences between strains should exist.

When Taylor et al. (1967) investigated the glycoproteins accumulated by the reserpine-treated rat submandibular gland, they found no significant differences in the ratios of protein / n-acetyl neuraminic acid, and other carbohydrates, in the extracts from either the control or reserpine-treated animals. However these ratios were not identical, and in this paper the authors refer to electrophoretic studies which they had conducted. These experiments apparently demonstrated that qualitative differences between the two

glycoprotein populations do exist. Unfortunately a full account of these experiments does not appear to have been published.

Martinez and his co-workers have proposed that the reserpine-treated rat submandibular gland may represent a useful model of the human genetically transferred disease cystic fibrosis (Martinez et al. ,1975b; Thompson et al. ,1976; Perlmutter and Martinez, 1977). A number of the histological features observed in the present study do indeed resemble features which have been reported to occur in the salivary glands of patients with cystic fibrosis. The deposition of material in the ductal lumina has been observed in the submandibular glands of cystic fibrosis patients (Barbero and Sibinga, 1962; Shackelford and Bentley, 1964), as well as the reserpine-treated rat submandibular gland (Martinez et al. ,1975b; results of the present study). Similarly, the "...synthesis of acidic mucopolysaccharides by cells which normally secrete low levels or none of these substances...." (Shackelford and Bentley, 1964), has been described as a feature characteristic of the submandibular glands from patients with cystic fibrosis. In the present study the acinar cells of the rat submandibular gland certainly accumulated acidic glycoproteins, whilst normally they contain an almost exclusively neutral glycoprotein population. It would appear at first sight that the changes in carbohydrate histochemistry reported here do resemble changes seen in cystic fibrosis. However Shackelford and Bentley (1964)

observed the changes in carbohydrate histochemistry, not in the acinar cells, but in the intercalated ducts which drain the mucous acini. In the present study no such changes were detected in the reserpine-treated rat submandibular gland.

Martinez et al. (1975b) state that the rat submandibular gland resembles its human counterpart in that both may be described as being "seromucous". This statement is technically correct, but the two glands are still very different from one another. The rat submandibular gland contains only one type of acinus, which is of a type intermediate between the typical serous and mucous acini. It should therefore be more correctly described as a homocrine seromucous gland (Shackleford and Klapper, 1962; see review Young and Van Lennep, 1978). The human submandibular gland is seromucous in a different sense as it contains both typical serous and mucous acini (ibid.) and should be more fully referred to as a mixed heterocrine seromucous gland. In the submandibular glands of patients with cystic fibrosis, both types of acinar cells have been reported to retain their normal histology. The accumulation of acidic glycoproteins occurring only in the intercalated ducts draining the mucous acini, (Shackleford and Bentley, 1964). It is therefore difficult to envisage how the histochemical changes caused by reserpine may be said to resemble those caused by cystic fibrosis.

The other major salivary glands are also affected

by cystic fibrosis. In the parotid gland, Shackelford and Bentley (1964) observed degeneration of the secretory cells with, in some cases, deposition of adipose tissue and lymphocytic infiltration. The same authors observed that in the sublingual gland, secretory proteins may leak from the ductal system into the interstitial spaces. The reserpine-treated rat submandibular gland showed none of these changes. This is perhaps not surprising, as Shackelford and Bentley (1964) studied material obtained at post mortem. The changes they describe would represent the end result of several years of degenerative change, it is most unlikely that 7 days of reserpine treatment could produce such effects.

Martinez et al. (1975c) investigated the effects of reserpine upon the composition of the saliva secreted by the rat submandibular gland in response to isoprenaline and pilocarpine. The changes in salivary composition do resemble those reported in cystic fibrosis (see introduction to this chapter). In addition, Martinez and his co-workers found that the saliva from the reserpine-treated rat submandibular gland contained a "ciliotoxic" factor. As such a factor has been reported to exist in the saliva (Dogget and Harrison, 1973) and serum (Spock et al., 1967; Bowman et al., 1969) of patients with cystic fibrosis, this observation seems to support strongly the view that reserpine causes changes which imitate the effects of cystic fibrosis upon the submandibular gland. However it must be remembered that the assay of such factors is a

very subjective procedure. This was amply demonstrated by Wood and di Sant'Agnes (1973) who investigated the reliability of such assays in a true "double blind" study. Coded samples of serum, both from patients with cystic fibrosis and from control subjects (homozygotes), were randomly distributed to 3 separate groups of workers, each of whom had claimed to be able to identify cystic fibrosis serum by such ciliary assays. The results showed that of a total of 5 techniques employed none could in reality achieve this, the negative and positive results being randomly distributed between the control and cystic fibrosis sera. This result suggests that the presence or absence of such a factor in saliva collected from the reserpine-treated rat submandibular gland can have little significance.

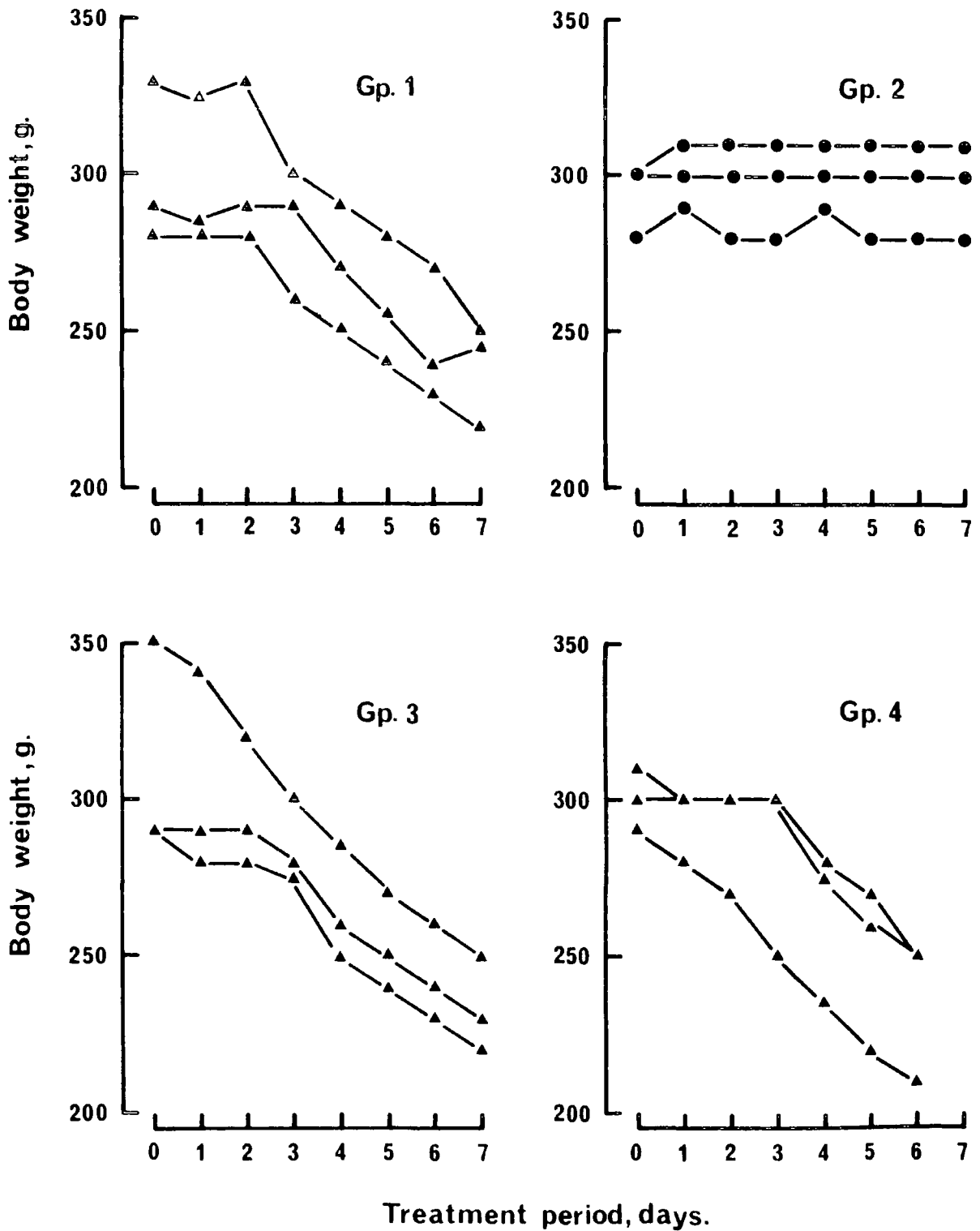
The mechanism by which reserpine exerts its effects upon the carbohydrate histochemistry of the rat submandibular gland has not been investigated in the present study. The results obtained by Taylor et al. (1967) suggest that the accumulation of glycoproteins caused by reserpine is not simply a consequence of sympathectomy. Conversely Martinez et al. (1975b) suggested that the effects of this drug may be due to "...alterations in either the content of physiologic neurotransmitters or, conversely, in the cellular receptors that interact with the transmitter...". The simplest explanation would be that the sympathectomy caused by reserpine prevents the secretion of proteins stored within the secretory cells. The distension of the

acini and accumulation of secretory granules within the granular duct cells are certainly consistent with this view. Why then should this drug cause a qualitative change in the types of proteins stored within the acini? In addition to its sympatholytic properties reserpine is also known to uncouple the synthesis of ATP from the oxidation of metabolic substrates (Schwartz and Lee, 1960; Sun, Sohal, Colcolough and Burch, 1968). This would cause a profound change in the cell's intermediary metabolism which may in some way affect the population of proteins synthesised and stored within the secretory cell. Martinez et al. (1975a), in a separate publication to that cited above (Martinez et al., 1975b), have attributed the effects of reserpine to such a toxic action.

Reserpine caused the experimental animals to lose interest in food and so by the end of the experimental period they had lost weight (see figure 4.2). The control animals did not show this effect. The weight loss was almost certainly due to the loss of appetite, but may be augmented by the action of reserpine upon the mitochondria (Schwartz and Lee, 1960; Sun et al., 1968). This period of starvation may in itself have caused some changes in glandular histochemistry, which would not be reflected in the control groups. In future experiments it would be necessary to undertake appropriate control experiments to test this possibility.

In summary, reserpine was found to cause changes in the carbohydrate histochemistry in the acini of the rat

Fig. 4.2 Changes in the body weight of the control (●) and reserpine-treated (▲) animals during the experimental period.



	Initial weight (mean ± S.E.)	Final weight (mean ± S.E.)	n.	t.	P.
Control.	293 ± 6.6 g	297 ± 8.8 g	3	0.364	N.S.
Reserpine treated.	305 ± 11.4 g	236 ± 5.8 g	6	5.395	<0.01, >0.002

submandibular gland. Similar changes have been observed in the submandibular glands of patients with cystic fibrosis, however, in these glands the changes occurred in the intercalated ducts rather than in the acini. This suggests that the changes caused by reserpine do not parallel those changes which occur in cystic fibrosis, although this result does not preclude the possibility that physiological similarities may exist.

GENERAL DISCUSSION

The experiments described in this thesis fall naturally into 2 groups, those concerned with the proteins secreted by the gland (chapters 1,2 and 3) and those dealing with the effects of reserpine upon the carbohydrate histochemistry of the submandibular gland itself (chapter 4). These will be discussed separately.

i) Protein secretion.

In common with many previous studies (see reviews Young and van Lennep, 1979; Young and Schneyer, 1981) isoprenaline was found to cause the secretion of a considerable amount of protein. However, earlier investigations have indicated that α -adrenoceptors are mainly concerned with fluid secretion (Martinez et al., 1975a; Abe and Dawes, 1978, 1980; Abe et al., 1979, 1980; see Young and van Lennep, 1979). The results of the present study do not support this view, as the protein concentration in the phenylephrine-evoked saliva was similar to that found in the saliva secreted in response to isoprenaline. The discrepancy between this finding and the results of earlier investigations is probably a consequence of the use of different routes of drug administration, the present study employed the intravenous route whilst the earlier investigators have employed intraperitoneal injection. The results of the present study suggest that the role played by α -adrenoceptors in protein secretion may previously have

been underestimated. Muscarinic and "peptidergic" agonists both caused the secretion of saliva which contained relatively little protein approximately 1/80 th of the concentration found after administration of the adrenergic agonists.

The secreted proteins were examined by electrophoresis and by consideration of the protein-specific activities of the proteolytic enzymes present in the saliva. These results indicate that the protein population secreted by the rat submandibular gland is dependent upon the nature of the secretory stimulus. This observation accords with the view that this gland contains two populations of cells capable of secreting protein; the acinar cells and the cells of the granular ducts (Junquera et al. , 1964; Cunningham, 1967; Matthews, 1974; Al-Gailani et al. , 1980). If it is accepted that the proteolytic enzymes within the rat submandibular gland are confined to the granular ducts (Junquera et al. , 1964; Cunningham, 1967; Matthews, 1974; Al-Gailani et al. , 1980; see Young and van Lennep, 1978, 1979; Young and Schneyer, 1981), and the high molecular weight glycoproteins to the acini (Quissell, 1980; Quissell and Barzen, 1980; Quissell et al. , 1981) then the effects of the agents employed upon these cell types may be summarised as shown in table D.1.

Isoprenaline caused the secretion of a fairly large amount of proteolytic activity, but the specific activity of the secreted enzyme was very low, almost identical to that observed after physalaemin. This result may

TABLE D.1

Summary of the secretory effects of various classes of agonist upon the secretory cells within the rat submandibular gland.

	<u>Acinar cells</u>	<u>Granular ducts</u>
α -Adrenergic	+	+++
β -Adrenergic	+++	+/-
Muscarinic	+	+
Peptidergic	+/-	+/-

indicate that the acinar proteins are not totally devoid of proteolytic activity, or that these agents may cause a very small amount of secretion from the granular duct cells.

The experiments described in chapter 2 are the first to investigate the proteins secreted by the rat submandibular gland in response to stimulation of the sympathetic nerves. The results confirm the suggestion that α -adrenoceptors are important in mediating protein secretion. Electrophoretic examination of the secreted proteins indicated that, at a stimulation frequency of 5 Hz, both α -adrenergic and β -adrenergic receptors were involved in protein secretion. However, at the higher frequency of 20 Hz the population of secreted proteins was almost identical to that secreted in response to phenylephrine, suggesting that at this frequency protein secretion was mediated almost exclusively via α -adrenoceptors. The reason why a change in stimulation frequency should cause a change in the population of secreted proteins is unknown. It was ~~was~~ thought that it may reflect a difference in the sensitivities of the glandular α and β -adrenoceptors to the neurotransmitter. However the results of the present study show that, even at 20 Hz β -adrenoceptors may still mediate fluid secretion. Similarly Schneyer (1975) found that at frequencies as high as 25 Hz, sympathetic nerve stimulation could still cause the secretion of a saliva containing high levels of potassium. As the ductal secretion of potassium is evoked via β -adrenoceptors

(Schneyer, 1962; Yoshida et al. , 1967; Young and Martin, 1971a), this observation indicates that β -adrenoceptors are not necessarily inactivated by high stimulation frequencies. Why then the β -adrenoceptors should not cause protein secretion at these high frequencies remains unknown.

The rat submandibular gland was found to secrete considerable quantities of proteolytic enzymes, particularly after activation of α -adrenoceptors. The functional significance of these enzymes remains unknown. As they have alkaline pH optima (Spearman and Pritchard, 1980) they would be rapidly inactivated in the stomach, and this makes a digestive function appear rather unlikely. Esteroproteases (enzymes capable of hydrolysing both peptide and ester bonds) are known to be present in the rat submandibular gland, as well as a number of other salivary glands (Al-Gailani et al. ,1980; see Barka, 1980; Schachter, 1980). However, the salivary esteroprotease kallikrein does not hydrolyse the synthetic substrate used in the present study (Trautschold and Werle, 1961; Habermann and Klett, 1966; Frey, Kraut and Werle, 1968; see Schachter, 1980). The activity recorded would therefore be attributed to "trypsin-like" proteases within the gland. Glandular esteroproteases have been proposed to fulfil regulatory functions by specifically cleaving biological active peptides from certain serum proteins (see Barka, 1980; Schachter, 1980). However, in the rat submandibular gland both classes of proteases are located in the

luminal portion of the secretory cells (Ørstavik and Glenner, 1978; Al-Gailani et al. , 1980), suggesting that they are destined for secretion into saliva. Al-Gailani et al. (1980) proposed that these enzymes may be secreted into the saliva as an adaptation to grooming behaviour. More exotic functions have also been proposed; Hiramatsu, Hatakeyama and Minami (1980) suggested that such enzymes may function as a potent "venom" in the male mouse. This idea does not seem very probable, particularly as these proteins have only a limited toxicity, with an LD 50 of 15 mg/kg (IP) in rats. It is conceivable that the presence of such enzymes may make the consequences of a bite more painful, and in animals such as cats which "spit with rage" the saliva may have a toxic effect in addition to threatening the enemy. Furthermore the fact that several of these enzymes show an androgen dependence in the rat and mouse (see Barka, 1980) strongly suggests some role in sexual behaviour.

In contrast to the function of the glandular proteases it is easier to speculate upon the function of the salivary glycoproteins. These molecules are generally considered to play an important role in protecting and lubricating the oral mucosa, and thus facilitate chewing and swallowing (see Burgen and Emmelin, 1961; Young and Schneyer, 1981). However, these functions will depend upon the structure of the native glycoprotein, and the results of Quissell and Barzen (1980) tend to suggest that this consists of an aggregate

of very high molecular weight.

ii) Effects of reserpine.

Reserpine was found to cause the rat submandibular gland to accumulate carboxylated glycoproteins (sialomucins) within the acini. This result contrasted with the findings of previous authors where the drug was found to cause a simple increase in the quantity of glycoproteins present within the gland, with no qualitative change in the nature of the glycoprotein population itself (Taylor et al. ,1967; Martinez et al. ,1975a).

Martinez and his co-workers have proposed that the reserpine-treated rat may be a useful model of the human disease cystic fibrosis (see chapter 4 for references). They maintain that the abnormal features of the morphology and physiology of the reserpine-treated rat submandibular gland resemble those changes seen in this disease. In some respects the changes in morphology observed in the present study do resemble those seen in cystic fibrosis. The deposition of secretory material in the ductal lumina and the accumulation of acidic glycoproteins by certain cells have been observed in cases of cystic fibrosis (Barbero and Sibinga, 1962; Shackelford and Bentley, 1964). However it must be pointed out that a number of other conditions may adversely affect the salivary glands. In particular the enlargement and vacuolation of the parotid acini seen in diabetes mellitus (Seifert, 1967) very closely resembles

that caused by reserpine in the present study.

A number of other animal models of cystic fibrosis have been proposed (eg the ligated duct of the rabbit submandibular gland, Fraser and Smaje, 1978; Bond, Fraser and Smaje, 1979). Such models certainly reproduce certain features of the disease (in the case of the ligated rabbit submandibular duct decreased sodium transport and goblet cell hyperplasia). However, a true animal model of this disease (ie a spontaneous, generalised exocrine gland dysfunction which is genetically transferred as a simple recessive trait) has yet to be found.

iii) Suggestions for future work.

A major limitation of the electrophoretic studies presented in this thesis is that their results are entirely descriptive. Future studies of protein secretion from this organ ideally should employ a more quantitative method of analysis. It may be possible to raise antibodies to particular proteins by fractionating the saliva electrophoretically and cutting the appropriate region from the gel itself. This portion of the gel could then be homogenised and the slurry used to immunise rabbits. Such antibodies could be employed to localise particular proteins within the gland immunohistochemically (eg are the high molecular weight glycoproteins confined to the acini) and to isolate larger amounts of antigenic proteins from glandular homogenates by affinity chromatography. Eventually it

may be possible to develop an assay system based on "rocket" immunoelectrophoresis in which acinar and ductal protein secretion may be individually quantified.

The process of protein secretion in other glands may also be studied. In particular the cat submandibular gland appears to have a rather unusual relationship between the autonomic nerves and the process of protein secretion. It is now more than a century since Langley (1879) commented that the sympathetic saliva in the cat was much less viscous than that induced parasympathetically, in contrast to the more normal situation in the dog, where the sympathetic saliva is far more viscous. It has since been noted that in the cat submandibular gland sympathetic nerve stimulation does not cause any degranulation of the acini, whereas parasympathetic nervous stimulation does (Barton, Saunders, Schachter and Uddin, 1975). Again this is the reverse of the normal situation seen in other glands (eg Garrett and Thulin, 1975; see Young and van Lennep, 1979; Young and Schneyer, 1981). The secretory response to sympathetic nerve stimulation in the cat submandibular gland is mediated entirely via α -adrenoceptors (Emmelin et al. , 1973; Emmelin and Gjørstrup, 1975), yet this gland has been observed to secrete, often quite vigorously, in response to large doses of isoprenaline (Kaladelfos and Young, 1974). This contradiction was resolved by Emmelin and Gjørstrup (1975) who showed that under physiological conditions the glandular β -adrenoceptors caused secretion only when sympathetic

nerve stimulation was superimposed upon a slow background parasympathetic secretion, a situation which these authors thought to duplicate that seen in the waking state. The role played by these β -adrenoceptors in mediating protein secretion remains unknown.

The experiments concerned with the effects of reserpine have not investigated how this agent exerts its effect upon the glycoprotein population stored within the gland. Although Taylor et al. (1967) maintained that the action of reserpine upon the submandibular gland was independent of its sympatholytic properties, experiments could be undertaken to test this hypothesis. Glands could be studied after sympathetic denervation (removal of the superior cervical ganglion) or decentralisation (section of the cervical sympathetic trunk). As reserpine is known to uncouple oxidative phosphorylation (Schwartz and Lee, 1960; Sun et al., 1968) the effects of chemically unrelated compounds with similar metabolic effects, e.g. 2,4 dinitrophenol, could also be investigated.

APPENDIX:- ATROPINE-RESISTANT PARASYMPATHETIC
VASODILATION IN THE RAT SUBMANDIBULAR
GLAND.

INTRODUCTION

It has long been known that parasympathetic nerve stimulation, in addition to causing a flow of saliva, also causes a profound vasodilation in the submandibular gland (Bernard, 1858; see Burgen and Emmelin, 1961). Shortly after Bernard's original observation, Heidenhain (1872) noted that the vascular response persisted after doses of atropine which were more than sufficient to abolish the secretory response. Several suggestions have been put forward to explain this phenomenon.

Numerous authors have proposed that the hyperaemia may be secondary to the release of metabolites (eg. Henderson and Loewi, 1905; Barcroft, 1914) whilst others have proposed the existence of specific vasodilator fibres (Dale and Gaddum, 1930; Henderson and Roepke, 1933; Beznak, 1934). More recently Hilton and Lewis (1955a, 1955b, 1956) have suggested that the vasodilation is mediated by the esteroprotease kallikrein, which previously had been found in saliva (Ungar and Parrot, 1936). Hilton and his colleagues proposed that kallikrein is released by the active secretory cells, and that this enzyme liberates a vasodilator peptide from a macromolecular plasma substrate (α 2-macroglobulin).

Bhoola, Morley, Schachter and Smaje (1965) favoured the view originally proposed by Dale and Gaddum

(1930) that the parasympathetic vasodilation is mediated by acetyl choline liberated from specific vasodilator fibres. These authors suggest that the atropine resistant nature of this response simply implies that muscarinic receptors show a range of sensitivities to atropine. This view was supported by the observation that in rabbit the vasodilator and secretory responses are equally sensitive to atropine, (Morley, Schachter and Smaje, 1966). Furthermore, Darke and Smaje (1973) showed that the vasodilator response in glands whose kallikrein content had been reduced to very low levels (approximately 0.2% of control) by duct ligation was not specifically reduced.

The aim of the present study was to investigate the nature of the vascular responses which accompany parasympathetic nerve stimulation in the rat submandibular gland, previous studies having concentrated upon the cat, dog and rabbit.

MATERIALS AND METHODS

Male rats of a Wistar strain were anaesthetised with sodium pentobarbitone (May and Baker "Sagatal"; 60 mg/kg IP). The trachea and a femoral vein were cannulated and, in most animals, arterial blood pressure was recorded using a blood pressure transducer (Bell and Howell; 4-327-L221) connected to a cannula ("Portex" green) in the femoral artery.

The submandibular gland was exposed through a midline incision, and cleared of fat and connective

tissue. The venous drainage from the gland was then isolated by ligating all tributaries of the jugular vein other than that draining the gland itself. No attempt was made to separate the major sublingual gland as this procedure may damage the submandibular gland itself. The submandibular duct was cannulated with a short length of polythene tubing ("Portex" PE 50) drawn out to a fine point.

After injection of heparin (Wedal pharmaceuticals; 1,000 iu/kg, IV) the jugular vein was cannulated with a short length of polythene tubing (~~"Portex"~~ "pink" or "white"). Blood flow was recorded by diverting the gland's venous outflow through a photoelectric drop recorder. The drops triggered pulses which were then recorded directly upon a chart recorder. The parasympathetic nerves were stimulated as described in chapter 2.

Atropine sulphate was dissolved in isotonic saline and injected IV in doses of 1 mg/kg.

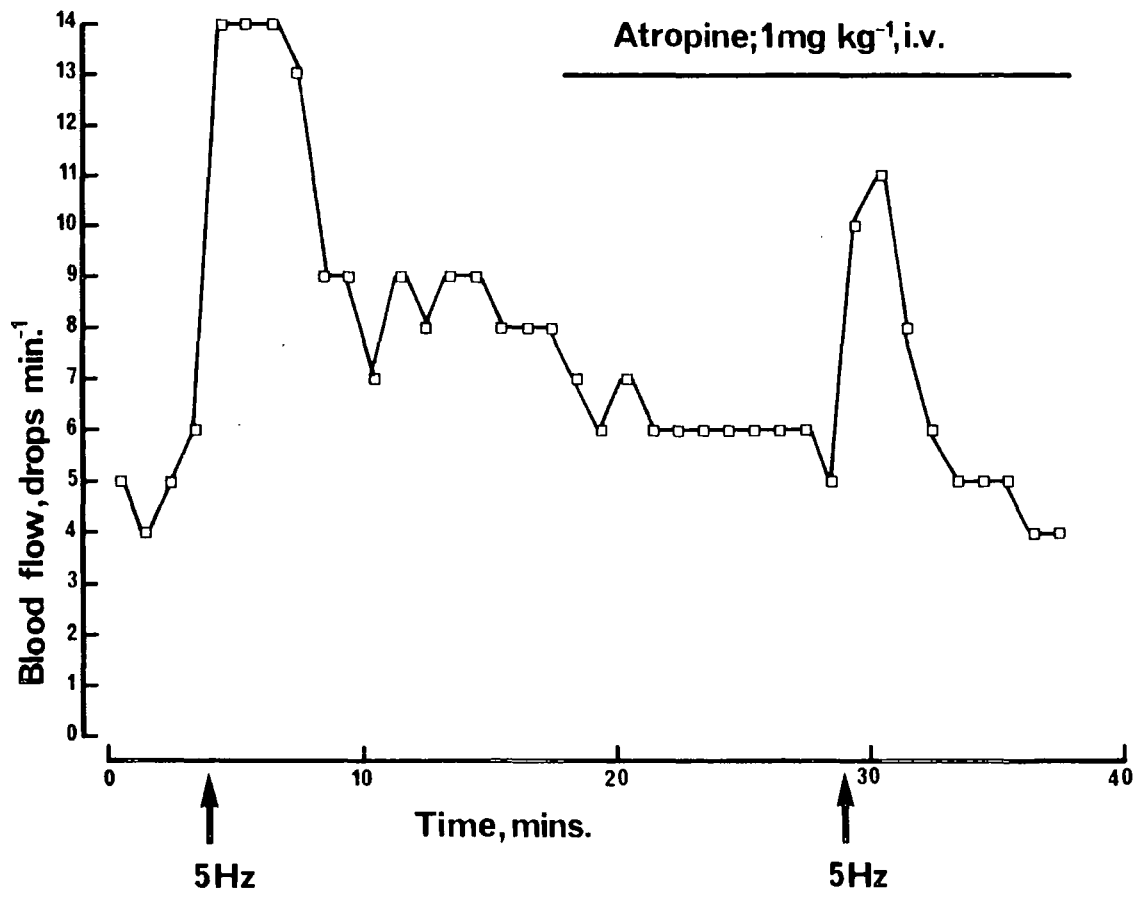
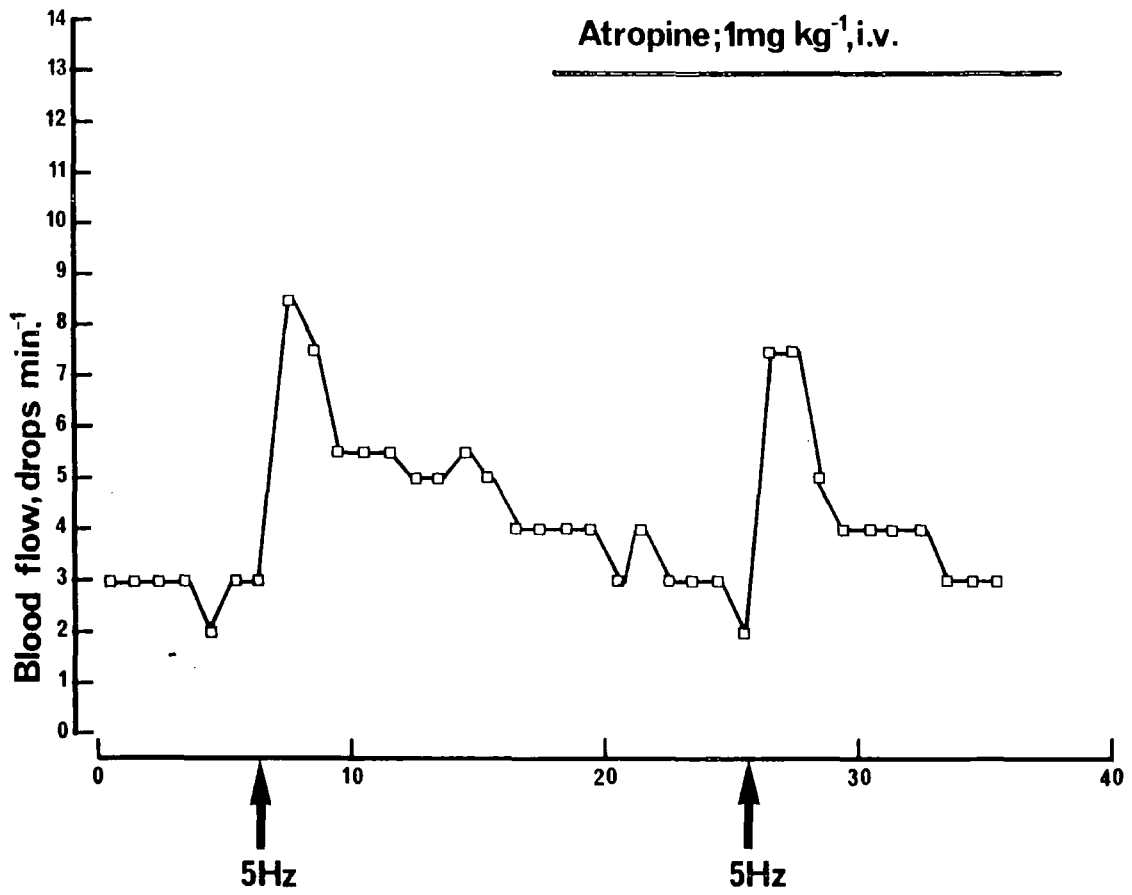
RESULTS AND DISCUSSION

In common with all other glands studied, parasympathetic nervous stimulation caused a brisk increase in the rate of blood flow through the rat submandibular gland. Once the stimulating current was switched off the flow rate took several minutes to decline to its original level, whereas in the other species studied this decline was much more rapid (eg. Darke and Smaje, 1972, 1973; Jones, Mann and Smaje, 1980).

The first experiments in which the effects of atropine were studied followed a very straightforward pattern. Once blood flow was successfully measured the chorda lingual nerve was stimulated for one minute and the vasodilator response recorded. Atropine was then injected and several minutes later the nerve stimulation repeated. Unfortunately the difficulties in establishing a preparation in which blood flow could be satisfactorily measured often prevented even this simple protocol from being followed successfully. The results of two such experiments are presented in figure A.1. As can be seen, stimulation of the chorda lingual nerve caused an increase in the flow rate both before and after administration of atropine. After atropine the magnitude of the increase was smaller, and the flow rate returned to basal levels more rapidly once the current was switched off.

Subsequent experiments were directed towards establishing a frequency response curve for the vasodilator response both before and after administration of atropine. However, the technical difficulties involved in setting up this preparation usually prevented this being achieved. In many animals the glandular blood flow was apparently too low to be measured, whilst in others the flow would cease abruptly after a period of good flow. The results of three partially successful experiments indicated that the vasodilator response was frequency dependant although the nature of this relationship was far from clear. In only one preparation

Fig. a1: Response of the rat submandibular gland vascular bed to parasympathetic nerve stimulation.



was it possible to perform more than one stimulation both before and after atropine. The results agree with the findings of the earlier experiments as they indicate that the vasodilation is atropine resistant, although no frequency-response relationship could be inferred from these observations.

Numerous studies have recently been published in which blood flow through the rat submandibular gland has been successfully measured (Thulin, 1976a; Templeton, 1979; Coroneo et al. ,1979). Templeton and Thulin (1978) have even succeeded in measuring blood flow through the rat major sublingual gland. These studies demonstrate that it is possible to overcome the technical problems encountered by the present author, and that the rat submandibular gland may be a potentially useful organ for such studies.

NOTE ADDED IN PROOF

Since this thesis was prepared an account of a series of experiments essentially similar to those proposed in the general discussion has been published (Fleming, N., Brent, M., Arellano, R. and Forstner, J.F. (1982) "Purification and immunofluorescent localisation of rat submandibular mucin" Biochem. J. 205 225-233). These authors isolated and partially characterised the high molecular weight glycoproteins from the rat submandibular gland. This "mucin" was found to have a molecular weight of between 200,000 and 1,000,000 under native conditions. The amino acid and carbohydrate composition of this molecule was also investigated. Furthermore Fleming et al. (1982) were able to localise this species immunohistochemically, confirming that the high molecular weight glycoproteins present in the rat submandibular gland are indeed confined to the acini.

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