Studies on submandibular salivary gland peroxidase, its cellular localization and release mechanisms

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LOCALIZATION AND RELEASE MECHANISMS

by

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This thesis is based upon the following publications, reference to which will be made by citation of the appropriate Roman numerals.

I.   Carlsöö, B., Kumlien, A. and G.D. Bloom: A comparative histochemical study of peroxidase activity in the submandibular glands of five mammalian species including man. Histochemie 26, 80-88 (1971).

II.   Carlsöö, B.: Histochemical observations on endogenous lactoperoxidase activity of the bovine submandibular salivary gland. Histochemical J. 3, 185-192 (1971).

III. Bloom, G.D. and B. Carlsöö: Fine structure and peroxidase activity of the bovine submandibular gland cells. Zentralblatt für Veterinärmedizin (1974) in press.

IV.   Bloom, G.D., Carlsöö, B., Danielsson, Å., Marklund, S. and T. Stigbrand: Peroxidase and amylase activity of the guinea pig submandibular gland during the secretory cycle. An electronhistochemical and biochemical study. Histochemistry 38, 271-280 (1974).

V.   Carlsöö, B., Danielsson, Å., Marklund, S. and T. Stigbrand: Effects of 3',5'-cyclic adenosine monophosphate, 5-hydroxytryptamine, noradrenaline and theophylline on the simultaneous release of peroxidase and amylase from the guinea pig submandibular gland. Acta physiol. scand. (1974) in press.

VI.   Carlsöö, B., Danielsson, Å., Marklund, S. and T. Stigbrand: Effects of alpha and beta-receptor blocking agents on catecholamine and 5-hydroxy-tryptamine induced peroxidase and amylase secretion from guinea pig submandibular gland. Acta physiol. scand. (1974) in press.

VII. Alm, P., Bloom, G.D. and B. Carlsöö: Adrenergic and cholinergic nerves of bovine, guinea pig and hamster salivary glands. A light and electron microscopic study. Z. Zellforsch. 138, 407-420 (1973).

INTRODUCTION

In 1809, Boulay, by chance, noted that an elixir used against toothache, and which probably contained guaiacum, turned blue intraorally. Shortly thereafter Planche (1810) reported that guaiacum in the presence of horseradish took on a bluish color. These two observations on the bluing of guaiacum by the saliva and in the presence of the fresh root of horse- radish may be the first observations recorded on peroxidase reactions (Carlström, 1968). Guaiacum was widely used during the nineteenth century as a test for oxidizing enzymes. However, no clear distinction was made in those days between peroxidases and true oxidases. The name peroxidase was first introduced by Linossier, who in 1898 isolated an oxidase-free enzyme complex of peroxidases from pus.

Most peroxidases are hemoproteins and they occur in certain animal tissues, bacteria and fungi and are further widely distributed among plants. The en- zymes catalyze the oxidation of certain electron donors by hydroperoxides. In mammals, the myeloperoxidase present in neutrophil leucocytes, the per- oxidase of eosinophils and the lactoperoxidase present in certain glands are the only peroxidases that have been isolated in pure form. Lactoperoxi- dase was first obtained in the crystalline state from milk by Theorell and his co-workers (Theorell and Åkeson, 1943; Theorell and Paul, 1944). The peroxidases present in saliva and in salivary glands, which are the subject of the present study, have been shown to be lactoperoxidases (see e.g. Morri- son and Steele, 1968).

As pointed out above, at a very early stage, "peroxidase activity" was noted intraorally, and numerous later workers have established the presence of per- oxidase activity in the saliva. The source of the peroxidases of the oral cavity is, however, not definitely established, as this activity can be attri- buted to numerous cell types such as eosinophils, neutrophils or even the bac- teria of the mouth (Morrison and Steele, 1968). Moreover, salivary glands have been found to possess peroxidase activity (Bancroft and Elliott, 1934). Thus, in a number of different mammalian species such as man, ox, pig, cat, hamster, dog, guinea pig, rat and mouse, the presence of peroxidase activity in the parotid and/or the submandibular gland has been demonstrated (Alexander, 1959; Thomson and Morell, 1967; Martin and Baserga, 1969; Kumlien, 1972). On the other hand, the salivary glands of the rabbit and sheep do not seem to

contain significant amounts of the enzyme.

Morrison and co-workers (Morrison and Allen, 1963; Morrison et al., 1965)
have established that the peroxidase present in the submandibular gland
of the cow is identical with the lactoperoxidase of milk. This bovine sub-
mandibular gland peroxidase is a water-soluble hemoprotein with 1 heme/mole
and has a molecular weight of 78,000. The enzyme is fairly heat stable and
is not readily inactivated by proteolytic enzymes. The enzyme is further
characterized by a high carbohydrate content (Morrison and Steele, 1968).

Whereas the physico-chemical properties of salivary peroxidase are well-
-studied and documented, its localization at a cellular and subcellular le-
vel in different glands has received less attention. Further, the relation-
ship between salivary peroxidase and the peroxidase present in the salivary
glands has hitherto not been fully clarified although Nickerson et al. (1957)
proposed that the peroxidase of the human saliva is produced mainly by the
salivary glands, and that little of the activity can be attributed to bac-
terial or leucocyte sources.

In exocrine glands, macromolecules synthesized for export are sequestered
intracellularly in membrane-bounded structures. This is e.g. the case with
the digestive enzyme amylase, which is stored within secretory granules of
various salivary glands. As a matter of fact, practically all proteins secre-
ted from digestive glands are hydrolytic enzymes. It has also been demonstra-
ted that catecholamines are the most potent inducers of enzyme secretion in
salivary glands, whereas cholinergic agents such as carbamylcholine and pilo-
carpine, although potent, are much less effective. According to Schramm (1967)
it is quite possible that acetylcholine only indirectly induces enzyme secre-
tion in rat parotid slices by releasing endogenous stores of catecholamines.
Studies on the mechanisms of action of noradrenaline and adrenaline have re-
vealed that 3',5'-cyclic AMP is the intracellular intermediate in the induc-
tion of secretion by these amines in the rat parotid gland.

The aim of the present investigation was to study the cellular and subcellu-
lar localization of peroxidase activity in different submandibular glands,
and to study the mechanisms for release of the enzyme. The following questions
were initially raised as guidelines for this series of investigations: 1) Is
salivary gland peroxidase synthesized and stored within secretory cells as

are other exportable proteins? 2) Are the secretory mechanisms basically
similar to those governing e.g. the release of the hydrolytic enzyme amy-
lase? 3) To what extent are peroxidase-containing glands innervated by the
sympathetic and/or parasympathetic portions of the autonomic nervous system?
In an effort to answer these questions the following lines of approach were
chosen for the study:

1) Histo- and cytochemical studies on the distribution and localization of
   peroxidase activity in the submandibular glands of different species.

2) In vivo and in vitro studies on peroxidase discharge from salivary glands,
   in combination with simultaneous measurements on amylase release.

3) Light and electron microscopic investigations of the autonomic innervation
   of some of the submandibular glands employed in the study.

MATERIAL AND METHODS

Light and electron microscopic studies:

For light and electron microscopical purposes (I - IV) submandibular gland
specimens from man, guinea pig, hamster, cow, dog and rabbit were fixed in
4 per cent glutaraldehyde in either 0.1 M phosphate or cacodylate buffer
(pH 7.3 - 7.4), as well as in the Karnovsky fixative (Karnovsky, 1965) di-
luted 1:1 with 0.1 M cacodylate buffer (pH 7.3 - 7.4). Fixation time was 2
hrs at 4° C. The specimens were rinsed (2 - 12 hrs) at 4° C in 0.1 M phos-
phate or cacodylate buffer (pH 7.4) containing 0.2 M sucrose. For light
microscopy, 6 - 10 μ thick sections were cut in a cryostat prior to incuba-
tion. For electron microscopy sections ($\sim$ 40 μ) were cut on a Sorvall TC-2
sectioner, collected in buffer and transferred to the incubation medium. In-
cubations were carried out in a 3',3'-diaminobenzidine-tetrahydrochloride
(DAB)-hydrogen peroxide medium at pH 7.6 according to Graham and Karnovsky
(1966) or at pH 6.0 - 9.0 in the modified media of Novikoff (Beard and Novi-
koff, 1969; Novikoff et al., 1971). In paper II the benzidine-hydrogen per-
oxide medium of Wachstein and Meisel (1964) was also employed. After incuba-
tion, the cryostat sections were rinsed in buffer and mounted on glass slides.
For ultrastructural studies the sections were rinsed in buffer at pH 7.4, post-
fixed in 1 per cent buffered osmium tetroxide, dehydrated in graded ethanol
solutions and propylene oxide and finally embedded in Epon. Roughly 1 μ thick

Epon sections were cut on an LKB ultrotome and examined unstained in the
light microscope. Thin sections were collected on naked copper grids and
examined after staining with lead citrate or after double-staining with
uranyl acetate and lead citrate. The microscope employed was a Philips
EM 300 electron microscope.

For the histochemical demonstration of biogenic amines (VII), salivary
gland specimens were rapidly frozen in liquid isopentane cooled by liquid
nitrogen and further processed according to the method of Falck and Hillarp
(Falck et al., 1962). For the demonstration of acetylcholinesterase (AChE)
activity the copper thiocholine method of Koelle and Friedenwald (1949),
as modified by Holmstedt (1957), was employed, as was a slightly modified
El Badawi and Schenk (1967) method.

To demonstrate autonomic nerve endings at the ultrastructural level (VII)
specimens were fixed in ice-cold 3 per cent potassium permanganate in Krebs-
-Ringer phosphate buffer (pH 7.0) (Hökfelt, 1968). The specimens were rinsed
in Ringer solution and contrasted en bloc for 60 min at 4° C in 1 per cent
uranyl acetate, also in Ringer´s solution. The specimens were dehydrated and
embedded in Epon as described above.

Studies on secretion and biochemical analyses:

Male guinea pigs were starved overnight prior to the in vitro experiments
(V, VI). They were anesthetized by an intraperitoneal injection of sodium
pentobarbital. The submandibular glands of both sides from two animals were
rapidly excised and extraglandular tissue was removed under a stereomicro-
scope. They were cut into small fragments, which were randomly distributed
among the incubation vessels. All specimens were preincubated for 30 min in
3 ml of bicarbonate buffer (pH 7.4), supplemented with pyruvate, glutamate
and fumarate (Krebs, 1950) and also containing albumin (5 mg/ml) and glucose
(0.6 mg/ml). After preincubation, the medium was replaced by 3 ml of fresh
incubation medium containing different test substances. Control incubations
without added substances were included in each experiment.

Preincubation as well as incubation was carried out at 37° C under continu-
ous gassing with O_2-CO_2 (95:5) in a metabolic shaker (Danielsson, 1974). After
60 min of incubation the specimens were separated from the incubation medium
by filtration through a nylon net and were homogenized in 3 ml of 50 mM phos-

phate buffer (pH 6.9) using an Ultra-Turrax homogenizer, run at a speed of 9,600 r.p.m. for 45 – 60 sec at 4° C. The homogenates were centrifuged at roughly 3,000 g for 5 min. Both incubation media and supernatants were assayed for peroxidase and amylase.

In the in vivo investigation (IV) guinea pigs were injected intraperitoneally with a combination of isoprenaline and pilocarpine. The submandibular glands of both sides were rapidly removed. One gland was used for histochemical purposes and the other for quantitative enzyme analysis. The wet weight of the glands was determined, and each gland was separately homogenized and centrifuged in the phosphate buffer as described above. The supernatants were assayed for peroxidase and amylase.

Peroxidase assays: In the in vivo experiments (IV), samples from the supernatants were transferred to a 17 mM guaiacol solution (pH 7.0) in 50 mM sodium phosphate buffer. The total volume amounted to 3.0 ml. Hydrogen peroxide (0.3 mM) was added and peroxidase activity was calculated from the initial increase in absorbance at 470 nm. The enzyme activities were expressed as Δ A_{470}/g wet weight of tissue per min. In the in vitro studies (V, VI), samples of tissue extract (supernatant) and incubation media were added to 16.7 mM pyrogallol in 200 mM sodium phosphate buffer (pH 6.0) to a total volume of 3.0 ml. Hydrogen peroxide was added to a final concentration of 1.67 mM. Peroxidase activity was calculated from the initial rate of increase in absorbance at 400 nm. The enzyme activities in incubation media and tissue extracts were expressed as Δ A_{400}/g wet weight of tissue per min. Peroxidase release was expressed as percentage of the total peroxidase activity of tissue and medium.

Amylase assays: In both in vivo and in vitro experiments (IV – VI), samples of incubation media and tissue extracts were appropriately diluted with 50 mM phosphate buffer (pH 6.9) and assayed for amylase using a micro-modification of the 3,5-dinitrosalicylate (DNS) method with 2 per cent soluble starch as substrate (Danielsson, 1974). One unit of amylase was defined as the activity liberating reducing groups corresponding to one µmole of maltose monohydrate per min at 25° C. Amylase activities were expressed as units/wet weight of tissue. Enzyme release was expressed as the percentage of the total amylase content of tissue and medium.

RESULTS AND DISCUSSION

1. Light and electron microscopic observations.

In the light microscope the serous cells of the human and guinea pig glands, the granular convoluted tubule cells of the hamster and the seromucous cells of the bovine gland displayed a positive reaction after incubation in a DAB- -H_2O_2 peroxidase medium (I, II). The mucous cells of the human, bovine and guinea pig glands were unreactive. Furthermore the seromucous acinar cells of the rabbit and the mucous cells of the dog were also completely unreactive (II).

At the ultrastructural level, a positive cytochemical staining was observed at particular sites within the serous and seromucous cells of the guinea pig and bovine glands (III, IV). The nuclear envelope consistently displayed a positive reaction, as did the rough endoplasmic reticulum (RER) and the secretory granules, both serous (guinea pig) and seromucous (cow). In the guinea pig gland, (IV) reaction product was also present in transitional elements of the RER and in condensing vacuoles. The Golgi apparatus was generally unreactive, although occasionally reaction product could be observed in the inner Golgi elements. Electron-opaque deposits were frequently observed in acinar lumina of both glands and in intercellular canaliculi of the cow.

In the present study, the Graham and Karnovsky (1966) DAB-H_2O_2 method for demonstrating peroxidase activity was employed. This method is very sensitive and advantageous for electron microscopic studies. DAB forms upon oxidation an insoluble brown polymeric precipitate which is strongly osmiophilic (Seligman et al., 1968). In paper II the classical benzidine technique of Wachstein and Meisel (1964) was also applied.

The DAB technique was originally introduced as a method whereby exogenous peroxidase, used as a tracer protein, could be identified. The technique has proved extremely valuable in studies on transport and uptake, and the enzyme most frequently employed for such studies has been horseradish peroxidase. The technique has also been used to study the uptake of or the capillary permeability to other exogenously introduced hemoproteins such as catalase, myeloperoxidase, lactoperoxidase, hemoglobin and cytochrome c (for ref. see e.g. Herzog and Miller, 1972a). The Graham-Karnovsky technique has also proved invaluable for the fine structural localization of endogenous peroxidase activity in a

variety of cells. Endogenous peroxidase activity has thus been demonstrated
in leucocytes from various species (e.g. Yamada, 1966; Bainton and Farquhar,
1967, 1970; Dunn et al., 1968; Miller and Herzog, 1969; Ackerman and Clark,
1971), the epithelial cells of the uterus (Bröckelman, 1969), acinar cells
of the lacrimal and laryngeal glands of the rat (Essner, 1971; Kataoka, 1971;
Herzog and Miller, 1972a,b), epithelial cells of the rat colon (Venkatachalam
et al., 1970), peritoneal macrophages (Fahimi et al., 1970), Kupffer cells of
the liver (Fahimi, 1970; Novikoff et al., 1971) and thyroid follicular cells
of the adult and fetal rat (Nakai and Fujita, 1970; Strum and Karnovsky,
1970b; Strum et al., 1971). In addition to the findings on salivary gland per-
oxidases reported in this series of investigations, other histochemical stu-
dies have shown peroxidase activity in rat and hamster salivary glands (Bloom
et al., 1970; Herzog and Miller, 1970; Strum and Karnovsky, 1970a; Novikoff
et al., 1971; Strum, 1971; Yamashina and Barka, 1972, 1973). In all above
cited cell types, peroxidase activity, with few exceptions, is localized to
the RER, the nuclear envelope, some components of the Golgi apparatus as well
as to cytoplasmic granules.

It should be stressed that the DAB method is not entirely specific for per-
oxidases; other hemoproteins also give a positive reaction. Endogenous cata-
lase is reactive in liver peroxisomes at high pH and increased concentrations
of DAB (Fahimi, 1969; Novikoff and Goldfischer, 1969). The staining reaction
of mitochondrial cristae is probably due to cytochromes (Beard and Novikoff,
1969) or cytochrome oxidase (Seligman et al., 1968, 1970). However, biochemi-
cal data from experiments on the effects of inhibitors strongly indicate that
the staining of the RER and related structures in peroxidase-synthesizing
cells is due to endogenous peroxidase activity (Novikoff et al., 1971). A
similar localization of peroxidase as that reported in the submandibular gland
of the cow (II, III) has recently been demonstrated in the sublingual gland
of this species using a highly specific immunohistochemical technique for the
demonstration of lactoperoxidase (Harada et al., 1973). No cross-reactivity
between lactoperoxidase and hemoproteins such as catalase, cytochrome c and
hemoglobin was observed with this immunological method.

The results obtained in the present study regarding the intracellular locali-
zation of peroxidase activity in salivary glands (III, IV) are thus in agree-
ment with other reports dealing with peroxidase activity in different enzyme-
-synthesizing glands. The staining of the Golgi apparatus, however, seems to

be somewhat ambiguous, and the role of this organelle in the synthesis of the enzyme is not clear. This organelle has been implicated in the synthesis of carbohydrates in many different cells. Lactoperoxidase has a high content of carbohydrate (Morrison and Steele, 1968), and it is possible that the carbohydrate moiety is incorporated into the enzyme in the Golgi apparatus of salivary gland cells.

It cannot be excluded, however, that peroxidase may play some specific role in certain metabolic functions of the ER. Yamashina and Barka (1972) have suggested the existence of at least two types of peroxidases in salivary glands, a soluble and a membrane bound, not rejecting the interpretation that ascribes the presence of peroxidase in the RER to the synthesis of the enzyme. Further studies are required to clarify whether or not all of the peroxidase present in the RER of salivary glands is destined for secretion.

Current concepts of events in the cell secretory cycle are mainly based on the work by Palade and co-workers (e.g. Jamieson and Palade, 1967a,b) on the exocrine pancreas of the guinea pig. In the cells of this organ secretory proteins are synthesized on ribosomes attached to the membranes of the RER, and are transferred across these membranes to the cisternae of this organelle. They are then transported via small vesicles, pinched off from the cisternae, to a condensing vacuole located in the Golgi region, where condensation of the contents takes place, giving rise to the mature granule.

The results of the present work on peroxidase-synthesizing salivary glands are in accord with the general pathway for intracellular transport of secretory proteins as worked out on exocrine pancreatic cells.

2. Studies on secretion.

In the present investigation experiments were performed to elucidate whether salivary peroxidase is an exportable enzyme and whether the secretory mechanisms involved are similar to those regulating the release of amylase. The studies were carried out on the submandibular gland of the guinea pig. This gland is almost entirely serous and contains large amounts of both peroxidase and amylase (Thomson and Morell, 1967; Bhoola and Dorey, 1971; Kumlien, 1972).

Both peroxidase and amylase were released simultaneously from the submandibular

gland after stimulation in vitro with various secretagogues (V, VI). Catecholamines were the most potent secretagogues tested (V, VI). Cholinergic agents such as carbamylcholine appear to be less effective than the catecholamines in eliciting a secretory response from this gland (Carlsöö et al., 1972). This is in accord with findings on amylase discharge from the rat parotid gland (Schramm, 1967). In a previous report (Carlsöö et al., 1972) 2-4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, was found to abolish the adrenaline-induced secretion of both peroxidase and amylase from the guinea pig submandibular gland. These findings suggest that sustained release of both enzymes requires an adequate provision of ATP.

Bdolah et al. (1964) first showed that adrenaline in vitro instigated amylase secretion from rat parotid slices. Babad et al. (1967) noted that theophylline as well as butyryl derivatives of cyclic AMP also induced amylase secretion from the rat parotid gland. Those authors concluded that cyclic AMP was the most likely mediator of the adrenaline-induced amylase secretion. It is now well established that catecholamines stimulate adenylate cyclase which may be located in the cell membrane. This enzyme catalyzes the formation of cyclic AMP in many cells (Sutherland and Robison, 1967). Robison et al. (1967) have suggested that in most, and perhaps all tissues, the adrenergic beta receptors and adenylate cyclase are identical. Thus, sympathetic stimulation of amylase secretion from salivary glands may be due to stimulation of the adenylate cyclase system; the adenylate cyclase of the salivary gland cell membrane may be identical with the beta receptors (Yamamoto et al., 1968). Recent studies have also clearly established that the enzymatic activity of adenylate cyclase in salivary glands is increased by catecholamines (Schramm and Naim, 1970; Malamud, 1972).

In addition to noradrenaline and adrenaline, dibutyryl cyclic AMP and theophylline stimulated the secretion of peroxidase and amylase from the guinea pig gland (V, VI). Since theophylline is a known inhibitor of the cyclic AMP degrading phosphodiesterase it is conceivable that the theophylline-induced secretion of peroxidase is caused by increased levels of intracellular cAMP.

Sympathetic stimulation of the parotid gland in vivo does not markedly alter the volume of saliva, although it greatly augments the level of amylase. This effect appears to be mediated by adrenergic beta receptors, as isoprenaline is a more potent agonist than either of the naturally occuring catecholamines,

and furthermore, the effects can be blocked by beta- but not by alpha adre-
nergic blocking agents (Pohto, 1968; Katz and Mandel, 1968; Yamamoto et al.,
1968). With respect to peroxidase secretion, the receptors involved also
appear to be beta receptors, as adrenaline- and noradrenaline-induced per-
oxidase secretion was completely inhibited by propranolol (β-blocker) but
not by phenoxybenzamine (α-blocker) (VI). The secretory effect of DBcAMP in
combination with theophylline is not altered by adrenergic blocking agents
(VI).

Dopamine and 5-HT were also found to be potent enzyme secretagogues with re-
spect to both peroxidase and amylase secretion (V, VI). The physiological
significance of the effects of these two amines in salivary gland function
is unclear. A striking degree of synergism between 5-HT, exogenous cyclic
AMP and theophylline on salivary secretion in insects has been reported, and
that the effect of 5-HT is mimicked by theophylline and cyclic AMP stands
as evidence that the effect of 5-HT is mediated via cyclic AMP (Berridge,
1970). However, an effect of 5-HT on adenylate cyclase or on the cellular
levels of cyclic AMP in salivary glands has not yet been demonstrated. In the
dog pancreas, dopamine markedly enhances fluid secretion, and a specific dop-
amine receptor has been postulated to exist in this gland (Furuta et al.,
1973). Further studies are required to elucidate whether specific 5-HT and
dopamine receptors also exist in salivary glands, or whether these amines
only interact nonspecifically with adrenergic receptors.

The present in vivo investigation (IV) has shown that a combined injection of
isoprenaline and pilocarpine causes a virtually total depletion of secretory
granules from the guinea pig submandibular gland. It was also found that after
such an injection roughly 50 per cent peroxidase and 75 per cent amylase was
discharged from the gland. At 24 hrs after the injection the glandular levels
of the enzymes were restored to those of untreated controls. At this stage
the acinar cells were refilled with secretory granules. There was a good corre-
lation between the quantitative analysis of release and subsequent reaccumu-
lation of the enzyme and the sequence of ultrastructural and cytochemical
cellular events.

The morphological changes observed during the secretory cycle in the guinea
pig gland are in agreement with those reported in salivary glands of the rat
(Simson, 1969; Amsterdam et al., 1969). Shortly after induction of secretion,

granules are seen to be fusing with the cell membrane, thus releasing their contents into the duct system. The possibility that cyclic AMP is involved in this process of fusion between granules and plasma membrane has been discussed elsewhere (Amsterdam et al., 1969), but a definite role for the nucleotide in exocytosis processes has not been established.

The present results (IV, V, VI) have clearly shown that peroxidase and amylase are secreted concomitantly from the guinea pig submandibular gland. Furthermore the findings suggest that the cellular release mechanisms governing peroxidase secretion are similar to those controling amylase release. It can also be concluded that cyclic AMP plays an important role in the regulation of peroxidase secretion from salivary glands.

The fact that different exportable enzymes are present in the zymogen granules and are secreted together raises the question whether each individual granule contains the whole complement of enzymes, or a small defined selection (Neurath, 1962). The cytochemical findings seem to indicate that peroxidase and amylase are stored together within the same granule.

3. <u>Light and electron microscopic observations on salivary gland autonomic innervation</u>.

With the aid of histochemical methods the enzyme acetylcholinesterase (AChE) was visualized in nerve terminals around the secretory acini of the submandibular gland of the cow, guinea pig and hamster as well as of the sublingual gland of the two latter species. Furthermore, AChE activity was observed in nerve fibres in close proximity to striated duct cells in all five glands, as well as around the granular convoluted tubules of the hamster submandibular gland. Fluorescence microscopy showed that adrenergic nerves occurred around the secretory acini of the bovine, guinea pig and hamster submandibular gland, as well as around those of the hamster sublingual gland. In the hamster submandibular gland a fluorescent network was also seen surrounding the granular convoluted tubules. The mucous secretory acini of the guinea pig sublingual gland were devoid of adrenergic nerve supply (VII).

The general distribution of nerves in all of the five glands examined when studied in the electron microscope correlated well with the light and fluorescence microscopic observations. In all glands unmyelinated nerve bundles, which usually contained several axons enveloped to some degree by a Schwann

cell, were found in the connective tissue stroma. Axons and varicosities were intimately associated with the acini, the duct system and the glandular vessels but did not penetrate the basement membranes to establish a close contact with the parenchymal cells. The striated duct cells of the hamster sublingual gland displayed a rich supply of axons and varicosities of both adrenergic and cholinergic type. This was in contrast to the same cells of the other glands studied. In this hamster gland an occasional close contact between varicosities and striated duct cells was also found (VII).

Recent developments in histochemical techniques as well as the electron microscope as an instrument have contributed substantial information with respect to the innervation of salivary glands in a wide variety of animals (see e.g. Emmelin, 1967). One aspect which has attracted much attention is the role of adrenergic versus cholinergic nerves in the secretory process of these glands. It is evident from the literature that there is no clear-cut separation of function between the two systems; the response of different glands to nervous stimulation and injected secretion-evoking drugs is extremely varied (Hand, 1972). Catecholamine fluorescence and acetylcholinesterase methods have demontrated that both adrenergic and cholinergic nerves are closely associated with the acinar cells of the parotid and submandibular glands of several animal species (Norberg and Olson, 1965; Fujiwara et al., 1966; Freitag and Engel, 1970). Studying this feature at the electron microscopic level, it is found that certain variations occur with respect to the presence of intraacinar nerve terminals in different salivary glands. Such terminals viz. nerves which penetrate the acinar basement membrane and establish close contact with the effector cells, are frequently encountered in rodent parotid glands (Scott and Pease, 1959) and in the submandibular gland of the cat (Shackleford and Wilborn, 1970). On the other hand, in the submandibular gland of the rat and mouse (Tamarin, 1966; Bogart, 1970; Yohro, 1971) and in the parotid and submandibular glands of man (Norberg et al., 1969) nerve terminals are only found external to the acinar basement membrane. These finer variations in innervation pattern indicate certain differences in the regulation of secretory activity (Scott and Pease, 1959), and as pointed out by those authors this should always be borne in mind when the effect of nervous stimulation of salivary glands is studied.

In the secretory studies on the guinea pig submandibular gland performed in the present investigation (V, VI) as well as in a previous study (Carlsöö

et al., 1972) peroxidase and amylase secretion could be initiated by both
cholinergic agents and catecholamines. The findings are thus consonant with
the observations on the distribution of autonomic nerves in this gland, viz.
the secretory cells are innervated by both sympathetic and parasympathetic
nerves.

The secretory acini of the sublingual gland of the guinea pig were found to
be devoid of adrenergic nerve supply. Similar findings have also been repor-
ted with respect to the sublingual glands of the rat and the mouse (Norberg
and Olson, 1965; Olson, 1967). The only salivary glands studied so far by
histochemical techniques in which the secretory cells apparently lack sympa-
thetic innervation are the sublingual glands of these three mentioned spe-
cies. The hamster sublingual gland, however, with a structure essentially
similar to that of the rat and mouse, is richly innervated by adrenergic
nerves (VII). In the latter gland numerous adrenergic nerves form plexa a-
round the striated ducts. To the author's knowledge a sympathetic innerva-
tion of these ducts has not been previously reported in the literature.

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Recent studies suggest that peroxidase may play a role in at least two pro-
cesses in salivary glands. One concerns iodination reactions and the other
participation in an antibacterial system.

It has been shown experimentally that bovine lactoperoxidase is more active
than most other peroxidases with respect to its ability to catalyze the oxida-
tion of iodide (Morrison and Steele, 1968). Iodide is known to be concentrated
within salivary gland cells of certain species (Myant, 1960). Furthermore, in
19 out of 21 salivary glands from eight different species, a correlation has
been found between peroxidase activity of the gland and its ability to concen-
trate iodide from the blood (Kumlien, 1972). In the thyroid gland, peroxidase
is believed to catalyze the iodination reactions involved in the synthesis
of the thyroid hormones, triiodothyronine and thyroxine. In this gland the
iodine trapping from the blood is followed by a rapid organic binding of
iodine to iodoproteins. In salivary glands, on the other hand, most or possi-
bly all iodine is rapidly excreted into the saliva and no protein-bound iodine
is recovered in salivary gland homogenates (Kumlien, 1972).

Lactoperoxidase, the peroxidase of salivary glands, together with thiocya-
nate and H_2O_2 has also been shown to be very effective in inhibiting the
growth of certain bacteria. Furthermore, lactoperoxidase exerts a strong
viricidal and fungicidal action. The possible role of an antibacterial per-
oxidase system in the saliva has been discussed in papers I, II and III.

GENERAL SUMMARY

1a   The localization of endogenous peroxidase activity in the submandibular
     gland of six mammalian species was studied at the light microscopical
     level. Peroxidase activity was found in the serous acinar cells of the
     human and guinea pig gland; mucous cells were negative. In the hamster,
     only the granular convoluted tubule cells displayed a strong positive
     peroxidase reaction. In the mixed gland of the bovine species the sero-
     mucous demilunar cells revealed a positive staining whereas the mucous
     cells were unreactive. The secretory cells of the rabbit and dog sub-
     mandibular glands showed no staining.

1b   The fine structural localization of peroxidase activity was studied in
     the guinea pig and bovine glands. Reaction product was observed in the
     cisternae of the rough endoplasmic reticulum including the nuclear enve-
     lope, in condensing vacuoles of the guinea pig gland and in secretory
     granules of both animals. The demilunar cells of the cow show a promi-
     nent RER which further appears to be associated with a peculiar peroxi-
     dase-positive labyrinth of interconnecting tubules.

2a   After stimulation of guinea pigs _in vivo_ with a combined injection of
     isoprenaline and pilocarpine, a discharge of peroxidase-positive secre-
     tory granules from the submandibular gland was observed. A few minutes
     after induction of secretion a fusing of granules with the apical cell
     membrane was noticed and a release of peroxidase-positive material into
     acinar lumina took place. At 90 min after injection an almost complete
     depletion of secretory granules was noted. At this stage about 50 per
     cent of the glandular contents of peroxidase was secreted from the
     glands. At 6 and 12 hrs a reaccumulation of peroxidase-positive secre-
     tory granules was noticed. At 24 hrs the acinar cells were refilled with
     peroxidase-positive granules.

2b A concomittant discharge of peroxidase and amylase from the guinea pig
    submandibular gland was recorded _in_ _vitro_. Noradrenaline, adrenaline,
    dopamine, 5-HT and dibutyryl cyclic AMP were all found to be potent
    secretagogues of both enzymes. Theophylline (5 mM) alone had only a
    small effect in eliciting a secretory response, but it markedly poten-
    tiated the effects of noradrenaline, 5-HT and DBcAMP in submaximal con-
    centrations.

2c Catecholamine (noradrenaline and adrenaline)-induced peroxidase secre-
    tion was inhibited by propranolol (a beta adrenergic blocking agent).
    The secretory effects of dopamine and 5-HT could be prevented by both
    propranolol and phenoxybenzamine. The adrenergic blocking compounds
    were, on the other hand, without effect on secretion induced by DBcAMP
    in combination with theophylline.

3   The distribution of adrenergic and cholinergic nerves was studied in
    the submandibular gland of the cow, guinea pig and the hamster. The per-
    oxidase-containing cells of these glands were found to be innervated by
    both parasympathetic and sympathetic nerves, as revealed by fluorescence
    microscopic, enzyme histochemical and electron microscopic studies. Ultra-
    structurally no close membrane-to-membrane contact between autonomic
    nerves and effector cell was observed in these salivary glands.

It is concluded that certain specific secretory cells of the human, guinea
pig, bovine and hamster submandibular glands synthesize and store the oxida-
tive enzyme peroxidase. Judging from experiments on the guinea pig submandi-
bular gland, salivary peroxidase is discharged concomittantly with amylase
both _in_ _vitro_ and _in_ _vivo_. Noradrenaline and adrenaline are potent peroxi-
dase secretagogues and the secretion induced by these two amines seems to
be mediated via beta receptors and cyclic AMP. Peroxidase-synthesizing cells
are innervated by both parasympathetic and sympathetic nerves.

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