TRANSPORT OF IGA IN RAT SALIVARY GLANDS


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DEDICATION

THIS THESIS IS DEDICATED TO MY PARENTS WITH LOVE.
There is, it seems
At best, only a limited value
In the knowledge derived from experience.
The knowledge imposes a pattern,
But the pattern is new in every moment,
And every moment is a new and shocking
Valuation of all we have seen.

T.S. Elliot - Four Quartets, East Coker.
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ABSTRACT

Transport of polymeric immunoglobulin A (pIgA) in rat salivary glands has been investigated by combined morphological and biochemical techniques in vivo and in vitro. The distribution of IgA and its cellular receptor secretory component (SC) was observed by immunoperoxidase staining of cryosections from parotid and submaxillary gland, showing serous acinar cells are the site of IgA transport into saliva. Binding of horse radish peroxidase specific IgA to parotid serous acinar cells in vitro, observed by electron microscopy, shows that only the basolateral domain of acinar cells possesses exposed SC. A combination of new cell fractionation methods and standard western blotting techniques shows that SC present on basolateral plasma membrane of parotid acinar cells has a molecular weight (mwt) >100,000 and shows a high affinity for pIgA in vitro. The existence of a 73,000 mwt SC occurring with pIgA in cellular fractions of parotid gland suggest cleavage of SC occurs prior to secretion.

The kinetics of pIgA trancytosis was studied using isolated parotid acini. Bound pIgA was secreted into the incubation medium as sIgA, within thirty minutes of incubation at 37°C. Secretion of pIgA was initially rapid but slowed over a 2hr period of incubation at 37°C. In addition to facilitating pIgA transport serous acinar cells also synthesise and secrete a diverse range of other salivary proteins which are packaged into secretion granules and secreted directly through the apical plasma membrane. It is improbable that one complex secretory pathway facilitates both bulk secretion of salivary protein and transport of pIgA. Therefore secreted proteins must be selectively segregated during secretion into saliva. Secretion of proteins from
acinar cells in vitro shows proteins are released at two distinct rates.
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1. INTRODUCTION

1:1 Histology

Saliva is produced by three pairs of major salivary glands, the parotid, submandibular (or submaxillary) and sublingual glands, plus numerous minor accessory glands scattered throughout the oral mucosa (Sobotta and Hammerson, 1983).

Two forms of secretory acinar cells are responsible for the production of saliva, serous cells which produce a proteinaceous secretion and mucous cells which produce a highly viscous secretion rich in mucins. The relative proportion of each cell type varies between the major salivary glands. The parotid glands consist totally of serous acini while the sublingual glands contain only mucinous cells, in contrast the submandibular glands are heterogeneous and contain both serous and mucous secretory cells.

Despite variations in the types of acinar cells present the major salivary glands share a common morphology. Each gland is composed of numerous lobules containing many acini or secretory units. In the parotid and sublingual glands the secretory units are "bulb" like structures composed of one single acinar type, whereas in submandibular glands the main part of the acinus is composed of mucous cells with serous cells forming a semilunar cap or demilume (Fig 1). Each secretory unit is enveloped by the processes of myoepithelial cells, which are packed with contractable filaments and assist in salivary secretion, by forcing the contents of the acinar lumen into the salivary ducts.
Figure 1 - Shows the general structural organisation of secretory units found in rat salivary glands. Although the relative proportion of serouse, mucous and mixed acini is dependent upon the gland in question (Section 1:1).
The secretions from several acini first enter intercalated ducts and subsequently drain into larger striated ducts the lining cells of which play a major role in the secondary modification of saliva (Mason and Chisholm, 1975). Finally saliva passes through larger interlobular ducts into the oral cavity.

The major salivary glands differ with respect to the relative proportion of intralobular ducts present. Intercalated ducts are longest in the parotid glands while striated ducts are most prominent in the submandibular glands. Both types of intralobular ducts are inconspicuous in the sublingual gland.

The reticular connective tissue which surrounds the acini contains the major blood vessels which follow the course of the ducts before forming a rich capillary plexus around acini and intralobular ducts. The venous and lymphatic drainage vessels retrace the arterial pathway throughout the gland. Also present in serous cell containing glands are numerous plasma cells responsible for the production of polymeric immunoglobulins (Hurlimann and Darling, 1971; Jeurissen, 1985; Tomasi, 1983; Ham, 1974).

Each major salivary gland is innervated by both parasympathetic and sympathetic divisions of the autonomic nervous system. Innervation is organised in such a way that terminal fibres of both systems are present on the surface of individual acinar cells (Ham, 1974).

1:2 Cytology

The acinar cells of the parotid glands exhibit a very characteristic polarity, frequently compared to that of the pancreatic
acinar cell (Palade 1975; Orci, 1984). A highly organised distribution of organelles is observed in both of these cells (Fig 2). Densely packed stacks of rough endoplasmic reticulum dominate the basolateral portion of the cell while the apical third of the cell contains the Golgi apparatus interdisposed between large numbers of secretion granules. The secretion granules are characteristic of both mucous and protein secretory cells of salivary glands and show considerable variation in size, number and morphology depending upon the state of secretion (Wallach, 1982; Leslie, 1983). The nucleus of salivary acinar cells varies in appearance between serous and mucous cells, nuclei of mucous cells are flattened and situated close to the basolateral plasma membrane while the nuclei of serous cells are rounded and positioned more centrally in the cells.

1:3  Saliva
1:3a Composition of Saliva

Saliva is a viscous secretion containing a complex mixture of water, mucins, proteins and salts. The physiological function of saliva includes primary digestion of food components, protection against environmental bacterial flora and viruses (Section 1:4), and the maintenance of tooth surface integrity (Ellison, 1977). As described in the previous section primary salivary secretions are produced by two distinct sets of cells, the mucinogenic and serous acinar cells. Mucinogenic cells produce a very viscous secretion containing a high proportion of mucopolysaccharides, mucoids and glycoproteins which are all characteristically sulphated and possess multiple oligosaccharide side chains.
Figure 2 shows the characteristic subcellular organisation of rat parotid acinar cells in vivo.

**ABBREVIATIONS.**

N - Nucleus; SG - Secretion Granules; ME - Myoepithelial cell; M - Mitochondria; RER - Rough Endoplasmic Reticulum.
Serous acini produce a non-viscous proteinaceous secretion containing iso-enzymes of amylase (Robinovitch, 1968), DNAase (Sreebny, 1965) and RNAase (Robinovitch, 1968) plus peroxidase (Wallach, 1975)) and immunological components. Other forms of proteins are commonly observed, however, no physiological role has yet been ascribed and nomenclature is therefore determined by the prevalent amino acid component of each, i.e. leucine, proline or cysteine rich proteins. Non-protein components include glycolipid material (Simpson, 1973) and the ions Na\(^+\), K\(^+\), Cl\(^-\), Potassium, phosphate and high levels of Ca\(^{2+}\) (Wallach, 1971).

The primary secretions of acinar cells are subsequently modified by an absorptive transport process in the acinar/intercalated duct region in response to muscarinic cholinergic and adrenergic stimuli. The extent of secondary modification is greatly dependent upon flow rate and involves active reabsorption of Na\(^+\) followed by Cl\(^-\) and active secretion of K\(^+\) and HCO\(_3\)\(^-\). As these ions constitute the major osmolites of saliva the modifications serve to control the final water content of the secretion.

In conclusion, although many salivary components have been identified it is unrealistic to envisage saliva as a regularly composed fluid with constantly present or structured components, as their appearance is strongly influenced by variations in salivary stimuli and post-secretory interactions (Ellison, 1977).

1:3b Production of Saliva

Two dynamically opposed theories exist concerning the relative involvement of hormonal (Wallach, 1982) and neurological (Gallacher,
1985) stimuli in eliciting salivary secretion. In practice secretory mechanisms are complex and varied, as such secretions may be dependent upon both forms of stimuli. As the composition of saliva shows marked variations with different physiological stimuli it is possible that compositional fluctuation in saliva reflects complex biochemical mechanisms not yet elucidated.

1:3c  Cellular Mechanisms of Production

The secretory process of acinar cells is governed by the integration of two distinct transport processes (Fig 3).

Firstly relatively low levels of stimulation result in preferential activation of β-adrenergic receptors yielding rapid protein biosynthesis and secretion. In this mechanism, binding of catecholamines to β-receptors stimulates adenylate cyclase to produce cAMP which subsequently activates a cAMP dependent kinase to phosphorylate proteins involved in regulatory synthesis and secretion. The identities of proteins involved in salivary secretion are unknown but it is possible that phosphorylation of secretion granule membrane proteins could explain the characteristic ‘budding’ of granule membranes and an increased tendency for granule-granule fusion observed immediately prior to secretion.

Secondly, in response to higher concentrations of α-adrenergic and cholinergic effectors a different secretory mechanism is triggered. The response results in only mild stimulation of protein secretion but high levels of K⁺ and water release (Gallacher, 1985). Unlike the β-response, this mechanism operates independently of cAMP and is
Pilocarpine
dependent upon activities of phospholipid cycling and increased cytosolic levels of Ca\textsuperscript{2+}. The exact role of phospholipid cycling is unknown but may be linked to Ca\textsuperscript{2+} mobilisation with phosphatidic acid acting as an endogenous Ca\textsuperscript{2+} ionophore (Ito, 1982).

1:3d **Artificial Stimulation of Salivation**

Pilocarpine whose chemical structure is shown opposite is a naturally occurring alkoloid which serves as a potent, parasympathomimetic agent. Intraperitoneal administration results in secretion from the salivary, lacrimal, gastric and pancreatic glands plus the mucous cells of the respiratory tract. At a subcellular level binding of pilocarpine to muscarinic/cholinergic receptors in secretory cells results in the release of intracellular bound Ca\textsuperscript{2+} and influx of extracellular Ca\textsuperscript{2+}. The resulting increase in cellular Ca\textsuperscript{2+} levels results in solute efflux and release of pre-synthesised proteins as discussed in the previous section (See Fig 3 section 1:3c).

1:4 **Immunoglobulin A**

IgA is the major immunoglobulin component of all seromucous secretions including saliva, tears and colostrum, plus secretions of the lung, genitourinary and gastrointestinal tracts. The function of IgA in these secretions is to protect the surface of exposed mucosal cells against attack by micro-organisms. Specific sequences in the variable region of the IgA molecule, recognise and bind invading micro-organisms, preventing their adherence to the mucosal epithelium. Involvement of the alternative complement pathway in response to IgA aggregation has been reported as a possible method for removal of bound antigen (Roitt, 1980). It has also been suggested that human,
Figure 3 shows a schematic representation of secretory mechanisms occurring within the serous acinar cells of rat parotid gland. Ach-m = muscarinic receptors; α-Adr = α-adrenergic receptors; β-Adr = β-adrenergic receptors.
although not most other species, possess receptors for aggregated monomeric IgA in the surface of neutrophils. The sequence of events responsible for producing IgA molecules with the correct antigenic recognition site is described by Roux (1977), and Mestecky (1978) (Fig 4).

Pre B-cells of adult bone marrow serve as universal B-cell precursors. They possess no immune components but develop into immature B-lymphocytes which express surface IgM and represent the first stage of isotype diversity. Subsequent development results in production of sub-populations of B cells expressing surface IgM+/IgD+ and either surface IgG or IgA isotypes.

At this stage of development B cells migrate from the bone marrow to the gastric, bronchial and conjunctival associated lymphoid tissues, Galt (Cebrá, 1977), Balt (Audzik, 1975) and Calt (Franklin, 1984) respectively. Within the microenvironment of these tissues, antigen passes through membraneous epithelia (M cells) to be presented to underlying IgA or IgG type B cells. The selective proliferation of IgA B cells occurs by the action of isotype-specific "switch" T cells, which recognise both antigen and B-Cells expressing IgA of the correct variable sequence. As a result of T cell binding development of the B-cells is diverted to exclusive production of IgA and proliferation of that B-cell type is promoted. Mature B-cells migrate from the secondary lymphoid tissues to the gut, salivary and other glands producing external secretions, where the final development of IgA secretory plasma cells occurs, possibly via a further T cell mediated process (Elson, 1978; Elson, 1979).
Figure 4 - Shows the sequence of events leading to the localised production of IgA within the interstitium of rat salivary glands.
IgA polymers (pIgA) are produced within the plasma cells prior to secretion (therefore maintaining antigen specificity) by cross-linking of IgA monomers with the polypeptide J-chain. Once released into the interstitium of the mucosal tissue, pIgA binds to secretory component (Sc), is transcytosed through the secretory epithelium and is released into the external secretion (Section 1.5).

Secreted IgA (sIgA) is comprised of two to four IgA monomers (mwt 170,000 each) (Peppard, 1984) joined by the cysteine-rich polypeptide, J-chain (mwt 15,000) and possesses a 60,000 to 70,000 mwt fragment of Sc (Cambier, 1976) (Fig 5).

1:5 Secretory Component

Secretory component (Sc) is present in external secretions of mammals both in association with polymeric immunoglobulins (pIg's) (Bienenslock and Befus, 1980) as bound SC (bSC) and as a single glycoprotein, free SC (fSC) (Marshall, 1974). The proposed functions of bSC include protection of pIgA against attack by proteolytic enzymes (Lindh, 1975) stabilisation of the quaternary structure of sIgA (Mestecky, 1974 and Jerry, 1972) and increased association of sIgA with the mucous coating of secretory epithelium following secretion. In contrast fSC appears to have no specific function in external secretions and may occur primarily as a consequence of sacrificial processing of receptor in the absence of bound ligand.

*FOOTNOTE*: Some IgA committed B-cells which leave secondary lymphoid tissues are retained in lymph nodes as memory cells and are important in initiation of secondary responses.
Figure 5 - Shows the proposed structural organisation of secretory dimeric IgA.
Under normal circumstances SC binds and transports pIgA into external secretions with variable affinity relative to the degree of polymerisation in the sequence dimeric-IgA > trimeric-IgA > tetrameric-IgA, SC has a very low affinity for monomeric IgA or IgG (Vaerman, 1985) but under conditions of IgA deficiency will transport pIgM, suggesting selective transport of pIg's could be a function of J-chain recognition (Eskeland, 1973).

The exact molecule weight of SC is variable, distinct species variations exist (Mostov, 1983 and Cambier, 1976) and slight differences in molecular weight occur in secretions of one individual (Cambier, 1976) possibly due to varying degrees of post-translational glycosylation. Despite such variation, Mostov et al (1983) showed SC is synthesised by epithelial cells as a large transmembrane glycoprotein (mSC) which is proteolytically cleaved prior to secretion as a lower mwt species (fSC and low molecular weight secretory component bound to IgA (sIgA).

The study of SC-mediated IgA transport into bile has revealed the complex post-synthetic pathway of SC (Mullock et al., 1979; 1980a; 1980b) and raised fundamental questions concerning sorting of proteins in endocytosis (Limet, 1985). SC is synthesised in hepatocytes on the rough endoplasmic reticulum as a transmembrane protein. After transport through the Golgi complex, SC travels to the sinusoidal (basolateral) region of the plasma membrane (where IgA may be bound) and is subsequently internalised into the endosomal compartment (Section 1:6, 1:7a). From the endosome, the mSC/pIgA complex is transectosed to the bile canalicular (apical) plasma membrane, where
sIgA is released (the exact stage at which the SC molecule is proteolytically cleaved is unknown).

Evidence for comparable SC-mediated IgA transport has been presented for gut (Nagura, 1979) and to a lesser extent bronchial (Manson, 1980) lachrymal (Sullivan, 1984; Renversev, 1985) and mammary (Solari, 1985) epithelial cells. However, it is crucial to reflect that although SC may transport IgA by comparable mechanisms in all tissues, the process is superimposed on very different cellular functions.

The prominent direction of secretion in hepatocytes is through the basolateral (sinusoidal) plasma membrane, i.e. in the direction which SC travels to collect IgA from serum. In contrast the direction of secretion in salivary acinar cells is through the apical plasma membrane, although IgA is still collected from the basolateral surface of the cell. As such the integration of IgA transport with secretion in salivary acini introduces aspects of intracellular protein sorting which are as yet uninvestigated.

1:6 Protein Transport

Transport of proteins within cells is a diverse and complex area of study, however, almost all transport processes may be catagorised into three basic forms :-

1. **Endocytosis** - in which a protein present in the extracellular medium is internalised and delivered by vesicles to a specific area of the cell. An extension of this class of transport is
transcytosis where internalised proteins are transported through the cell and secreted.

2. **Vesicle Mediated Transport of Endogenous Proteins** - many proteins synthesised by the cell follow a pathway through the endoplasmic reticulum and Golgi apparatus before being segregated into vesicles or granules and directed to a pre-determined area of the cell. This form of transport is common to all integral membrane proteins (e.g. Na⁺/K ATPase), organelle specific enzymes (lysosomal hydrolases) and secretory proteins (amylase).

3. **Non Vesicular Intracellular Protein Transport** - Proteins synthesised on cytosolic ribosomes may remain cytosolic (LDH) or enter relevant organelles, i.e. mitochondria or ER.

It is obvious even from this simple outline that protein sorting must be operative at several different cellular locations. With respect to proteins synthesised by the cell, selective segregation occurs at the point of translocation into ER and in the trans Golgi apparatus (Rindler et al., 1984). In contrast proteins being endocytosed or transcytosed are sorted on the plasma membrane and in an endosomal compartment (Goldstein et al., 1985; Simons and Fuller, 1985).

Considering therefore the proposed cellular route of the IgA receptor secretory component (SC) (Section 1.5), in order to effectively transcytose IgA, Sc must be successfully sorted at every site known to be involved in protein sorting. As such SC provides an attractive model by which we may investigate the specific
characteristics of a protein responsible for its observed behaviour at different cellular locations.

1:7. **Protein Sorting**

Protein sorting is dependent upon two distinct processes, firstly the segregation of proteins in a specific area of a fluid membrane and secondly correct targeting of intermediate transport vesicles to a pre-determined cellular location.

1:7a. **Segregation of Membrane Proteins**

Possibly the best characterised example of protein movement within membranes during protein sorting occurs in endocytosis. Mixed populations of receptors and receptor-ligand complexes are endocytosed from the basolateral plasma membrane of cells into clathrin coated vesicles. The vesicles subsequently lose the clathrin coating prior to fusion with a morphologically defined endocytic sorting compartment, the endosome or C.U.R.L (compartment for uncoupling of receptor and ligand (Geuze, 1983). In this compartment ligand/receptor complexes are specifically segregated before being targeted to different cellular locations.

The process of endosomal sorting can be conveniently divided into four categories based on the different intracellular routes taken by receptors and their ligands after entering the endosome (Fig 6).

**ROUTE 1** - Receptor recycles, ligand is degraded.

This is the pathway by which α-2-macroglobulin, LDL, asialoglycoproteins, insulin and luteinising hormone are endocytosed.
Figure 6 - Schematic representation of events occurring during endocytosis and subsequent processing of different ligand receptor complexes. All ligand receptor complexes appear to share a common pathway into an endosomal compartment but may then follow one of four different routes. I. Receptor is recycled to the basolateral plasma membrane but ligand is degraded. II. Both receptor and ligand are recycled to the plasma membrane, III. Both receptor and ligand are degraded. IV. Both receptor and ligand are transcytosed to the apical plasma membrane through which they are secreted. $\gamma$ receptor, •, Ligand.
Ligands following this route are dissociated from their receptors in the endosomal compartment as a result of the acid environment in the endosome (Brown et al., 1983; Helenius et al., 1983). The ligands are then transported to the lysosome for degradation, while the receptors are packaged into separate vesicles which return to the basolateral plasma membrane to be recycled.

ROUTE 2 - Both Receptor and ligand are recycled.

This pathway was originally demonstrated for the transferrin receptor (Octave 1983). The receptor binds Fe\(^{2+}\)-containing transferrin on the basolateral plasma membrane. The complex is then endocytosed and delivered to the endosomal compartment, where the acidic environment results in removal of the Fe\(^{2+}\) ion from the transferrin to give free Fe\(^{2+}\) and apotransferrin. The apotransferrin is not acid-labile and remains attached to the receptor which then returns to the cell surface. In the neutral pH of the extracellular fluid the apotransferrin molecule dissociates from the receptor, leaving it free to bind a further transferrin molecule.

ROUTE 3 - Both Receptor and ligand are degraded.

This pathway was first characterised for the epidermal growth factor (EGF) receptor. The EGF/receptor complex is endocytosed to an endosomal compartment from where both receptor and ligand are degraded, presumably by subsequent co-translation to lysosomes. However it is unclear whether dissociation of receptor and ligand occurs in the endosome or lysosome.
ROUTE 4 - Receptor and ligand remain associated and are transported from the endosome.

This pathway has been extensively characterised for secretory component (SC) which facilitates cellular transport of polymeric immunoglobulin across epithelial cell surfaces and is discussed in Section 1:5.

In conclusion it is clear that proteins are sorted at several sites within the cell. However understanding of the molecular interactions responsible for guiding proteins in the required directions, or retaining them in one particular domain of a fluid membrane remain unclear.

Although different characteristics of each protein probably influence sorting at each cellular location, some of the general principles involved are demonstrated by the movement of receptors into coated pits during endocytosis.

Certain receptors move into the coated pit regions of plasma membrane in the absence of bound ligand, i.e. the receptors for LDL (Anderson et al., 1982; Basu et al., 1981) transferrin (Hopkins, 1983 and 1985), asialoglycoprotein (Well et al., 1980; Berg et al., 1983), 2-macroglobulin (Via, 1982) and secretory component (Mullock et al., 1980a). Alternatively other receptors are excluded from coated pits until associated with the required ligand as in the case of the EGF receptor and insulin.

The differences observed between the movement of various receptor populations may be due to an inherent or inducible ability to associate
with a specific membrane protein (clathrin in the case of coated pits). Receptors showing no requirement for bound ligand could already possess the correct conformation for association with the required membrane protein, whereas other receptors obtain this conformation as a result of ligand binding. Such a protein could therefore work in reverse in the endosomal compartment, thus dictating which region of the endosome membrane could retain bound or free receptor.

Although plausible this simplistic interpretation must in future be expanded to explain the role of prosthetic groups (Hunter, 1984; Klausner, 1984) and interaction of proteins with specific membrane lipids (Bretscher, 1984), and possibly variations in cellular pH osmolarity.

Targeting of Intermediate Transport Vesicles

This process plays a vital role in protein sorting as the proteins contained within vesicles must be directed to the correct intracellular locations. The exact way in which this process operates is unknown. However several groups have shown that disruption of microtubule organisation by colchicine strongly inhibits previously observed vesicle mediated transport (Mullock et al., 1980b). Rodriguez-Boulan (1983) showed that in viral infected cells treated with colchicine, vesicle transport of viral proteins to plasma membranes was greatly delayed yet still specific with regards to targeting. As such there is indirect evidence to suggest intermediate transport vesicles possess receptors for both microtubules and target organelles. Vesicles would then recognise the correct microtubule 'track' and be transported along the structure (possibly in a similar manner to that observed in axon
preparations) to the correct region of the cell where fusion with the correct organelle occurs.

BRIEF OUTLINE OF AIMS.
2. MATERIALS AND METHODS

2:1 Surgical Procedures

2:1a Cannulation of Parotid Duct

Male hooded rats (University of Surrey strain) weighing more than 250 grams, were used for cannulation of the external parotid duct. The animals were anaesthetised with approximately 0.1ml/100g body weight of Sagatal (May and Baker Ltd, Dagenham, England, UK) administered intraperitoneally. The rats were then placed on a dissection board and secured. An incision was made from the lower jaw to the start of the chest cavity, exposing the salivary glands and cheek area. The salivary duct was then identified (Fig 7) and two ligatures placed around it but not tied. A '30G' dental nerve needle (kind gift from Charles Clifford, Dental Hospital, Sheffield, UK) connected to the required length of capillary tubing (Portex, UK) was then carefully inserted into the lumen of the duct and the ligature tied around it.

2:1b Retrograde Perfusion of Parotid Duct

A perfusion pump was connected to a length of capillary tubing to which the 30G needle had been attached. An appropriate amount of the required perfusate was then drawn up the capillary tubing and the needle inserted into the duct and secured (as described in 2:1a). The perfusion pump was then turned on at a rate of 3ml/hr and allowed to pump for approximately 5 mins. After this the glands were excised and either homogenised or prepared for light microscopy (Section 2:5c).

Foot Note: Specific materials from suppliers are noted in appropriate methods.
Figure 7 - Shows the anatomical localisation of the rat parotid secretory duct.
2:1c Collection of Saliva from Cannulated Parotid Duct

This method of obtaining pure parotid gland secretions was attempted but is very difficult and often yields very small samples of secretion even when the animal is stimulated by intra-peritoneal injection of pilocarpine (Sigma Chemical Co, Poole, Dorset, UK).

2:2 Fractionation Techniques
2:2a Preparation of Microsomes From Parotid Glands

1. Parotid glands were rapidly excised and lymph nodes removed before being placed in homogenisation medium at 4°C.

2. Glands were coarsely minced by hand using a single edged razor blade prior to homogenisation, using a Potter type homogeniser (3-4 strokes) at 2,400 r.p.m, in 0.28M sucrose, 0.5mM MgCl₂, 1mMNaHCO₃ at pH 7.4, to give an approximate 10% (w/v) tissue homogenate.

3. Samples of the homogenate were centrifuged for 15 mins at 12,000 g. av in a High Speed 18 centrifuge (MSE, Scientific Instruments, Crawley, Sussex, UK), using polycarbonate tubes and a 8 x 50ml rotor MSE No. 69182.

4. The supernatant was removed into clean centrifuge tubes and centrifuged for 60 mins at 220,000 g av in a Kontron K-200 ultracentrifuge rotor TFT. 5038, using polycarbonate tubes.

5. Following centrifugation the supernatant (cytosolic fraction) was removed and the microsomal pellet resuspended in 0.28M sucrose,
0.5mM MgCl₂, 0.7mM EDTA, 1mM NaHCO₃ at pH 7.4 using a 1ml hand held homogeniser (Thomas Scientific Apparatus, Phila. P.A).

2:2b Differential Pelleting of Crude Parotid Gland Homogenate

Method :-

1. Parotid glands were rapidly excised and lymph nodes removed before being placed in homogenisation medium at 4°C.

2. Glands were coarsely minced by hand using a single edged razor blade prior to homogenisation, using a potter type homogeniser (3-4 strokes) at 2,400 r.p.m in 0.28M sucrose, 0.5mM MgCl₂, 1mM, NaHCO₃ at pH 7.4, to give an appropriate 10% (w/v) tissue homogenate.

3. The homogenate was centrifuged for 15 mins at 12,000 g av. using an MSE high speed 18 centrifuge (rotor No 69182) and polycarbonate centrifuge tubes.

4. The supernatant was removed, placed in clean centrifuge tubes and subjected to an identical centrifugation as described above.

5. The supernatant was again removed and a third centrifugation carried out as above.

6. Pellets were re-suspended in 0.28M sucrose containing 0.5mM MgCl₂, 0.7mM EDTA, 1mM NaHCO₃ at pH 7.4 using a 1ml hand held homogeniser (Thomas Scientific Apparatus, Phila, P.A).
7. All fractions were kept at 4°C on ice and the activity of the γ-glutamyl transpeptidase activity determined immediately as described in Section 2:4c.

2:2c Preparation of Basolateral Plasma Membrane Vesicles

1. Parotid glands were rapidly excised and lymph nodes removed before placing in homogenisation medium at 4°C.

2. Glands were coarsely minced using a single edged razor blade and homogenised in 0.28M sucrose, 0.5mM MgCl₂, 1mM, NaHCO₃, pH 7.4 using a Potter type homogeniser (3-4 strokes at 2,400 rpm) to give an approximate 10% (w/v) tissue homogenate.

3. The homogenate was centrifuged at 12,000 gav for 40 mins in a High Speed 18 centrifuge (MSE Scientific Instruments, Crawley, Sussex, UK) using a 8 x 50ml angle head rotor (No 69182) and polycarbonate centrifuge tubes.

4. Following centrifugation, the supernatant was removed from the pellet and loaded onto a continuous 0.5M to 1.7M sucrose gradient with a 2ml cushion of 2.0M sucrose, in Kontron polyallomer centrifuge tubes. All sucrose solutions contained 0.5mM MgCl₂, 0.7mM EDTA, 1mM NaHCO₃ at pH 7.4.

5. Tubes were centrifuged at 100,000 gav for 18 hrs in a Kontron K200 ultracentrifuge, using a TST 28.38 swing out rotor.

6. Following centrifugation, fractions were collected by upward displacement with 2.3M sucrose solution (Fig 8).
Figure 8 - Shows a flow diagram of the preparation of basolateral plasma membrane vesicles from homogenised rat parotid glands.
Preparation of Apical Plasma Membrane Sheets

Source:

The technique was developed from the previously published system used by (Issa et al., 1977) to separate bile-canalicular face plasma membrane from hepatocyte homogenates.

Method

1. Parotid glands were rapidly excised, trimmed to remove lymph nodes and placed in homogenisation medium at 4°C.

2. Glands were coarsely minced using a single edged razor blade and homogenised in 0.28M sucrose, 0.5mM MgCl₂, 1mM NaHCO₃, pH 7.4 using a Potter type homogeniser (3-4 strokes) at 2,400 rpm, to give an approximate 10% (wt/vol) tissue homogenate.

3. The molarity of the homogenate was adjusted by the addition of sucrose to give a final molarity of 1.34.

4. Aliquots of the homogenate were then placed above a 2ml cushion of 2.0M sucrose, 0.5mM MgCl₂, 1mM NaHCO₃, 0.7mM EDTA, pH 7.4 and overlayed with 0.25M sucrose 0.5mM MgCl₂,1mM NaHCO₃, 0.7mM EDTA, pH 7.4.

5. Tubes were centrifuged at 220,000 g av. for 30 mins in a Kontron K200 centrifuge using a TFT 50.38 rotor and polycarbonate centrifuge tubes.

6. Following centrifugation the crude apical plasma membrane fraction present at the 0.25M and 1.34M sucrose interface was removed using
a U-bend pipette and the sucrose concentration adjusted to 0.25M by gradual addition of distilled H₂O with mixing.

7. The crude apical plasma membrane fraction was then loaded onto a continuous 0.5M to 1.75M sucrose gradient with a 2ml cushion of 2.0M sucrose. All sucrose solutions contained 0.5mM MgCl₂, 1mM NaHCO₃, 0.7mM EDTA at pH 7.4.

8. Centrifugation was for a period of 40 mins at 5,000 rpm in a Kontron K200 centrifuge using a TST 28.38 swing out rotor and polyalioomer centrifuge tubes.

9. Following centrifugation fractions were collected by upward displacement using a 2.3M sucrose displacement solution (Fig 9).

2:2e Isolation of Secretion Granules

Source :- Adapted from the method of Castle et al (1975)

1. The parotid glands were rapidly excised and coarsely minced by hand in homogenisation medium at 4°C.

2. Homogenisation was in 0.28M sucrose, 40mM potassium phosphate, 0.2mM EDTA, at pH 7.4 using a Potter type homogeniser (3-4 strokes) at 2,4000 rpm to give an approximate 10% (w/v) tissue homogenate.

3. Large debris, unbroken cells and nuclei were pelleted from the total homogenate at 600 g av. for 8 min in polycarbonate centrifuge tubes using a Digifuge centrifuge.
ISOLATION OF APICAL PLASMAMEMBRANE SHEETS

Figure 9 - Shows a flow diagram of the preparation of apical plasma membrane sheets from homogenised rat parotid glands
4. The resulting supernatant was layered over a step gradient consisting of a 3ml layer of 0.42M sucrose, 40mM potassium phosphate, 1mM EDTA at pH 7.2 over a 2ml cushion of 2.0M sucrose, 40mM potassium phosphate, 1mM EDTA, pH 7.2.

5. Tubes were centrifuged at 2,100 g av. for 15 mins in a Kontron K-200 centrifuge using a TFT 50.38 fixed angle rotor and polycarbonate centrifuge tubes, to yield a crude granule fraction located at the 0.42 - 2.0M sucrose interface.

6. The crude granule fraction was collected using a U bend pasteur pipette and the sucrose concentration adjusted to 1.7M using 2.3M sucrose, 40mM potassium phosphate, 1mM EDTA pH 7.4. If an addition of distilled water was necessary to achieve an accurate sucrose concentration, this was done by addition of small drops to rapidly stirred granule fraction in order to prevent localised osmotic shock.

7. After the correct sucrose concentration was achieved the diluted granule fraction was layered over a step sucrose gradient consisting of 3ml of 2.1M and 3ml of 1.9M and covered with an overlay of 3ml of 1.6M sucrose. All sucrose solutions were supplemented with potassium phosphate and EDTA at the concentration indicated above. Polyallomer centrifuge tubes were used.

8. Tubes were then centrifuged at 160,000 g av. for 180 mins in a Kontron K-200 ultracentrifuge using a TST 28.38 swing out rotor.
9. The purified granule fraction sedimented through the 1.9M sucrose to the interface of the 2.1M and 1.9M sucrose interface. The fraction was collected using a U-bend pasteur pipette (Fig 10).

2:2f Preparation of Clumped Acini From Parotid Glands

The isolation technique was adapted from that of Williams et al (1976).

Reagents :-

i. Ca$^{2+}$, Mg$^{2+}$ free Hank's balanced salt solution, pH 7.4, containing phenol red as indicator (Flow laboratories, Irvine, Scotland, UK) as an indicator of pH.

ii. L15 culture medium (Flow Laboratories, Irvine, Scotland, UK).

iii. Soya bean trypsin inhibitor (Sigma, UK).

iv. Collagenase (Worthington type II or Boehringer).

v. Hyaluronidase (Sigma, UK).

Method

Male hooded rats of the University of Surrey strain, weighing approximately 200g, were killed by intraperitoneal injection of pentobarbitone. The parotid glands were rapidly excised, lymph nodes and fatty tissues were trimmed away and the glands minced coarsely with scissors. The tissue was then incubated for 10 min at 37°C in Ca$^{2+}$ and Mg$^{2+}$ free Hanks balanced salt solution containing 64 Units/ml of Collagenase (Worthington Type II) and 500 Units/ml of hyaluronidase.
Figure 10 - Shows a flow diagram of the preparation of secretion granules from homogenised rat parotid gland.
10 ml of medium was used for each gram of tissue. After incubation the medium was removed and replaced by fresh medium followed by a further 40 min incubation. The tissue was then disrupted by passage through a coarse nylon mesh and the acini separated from the filtrate by centrifugation for 6 mins at 50 x g. The acini were washed twice in enzyme-free Hanks medium and placed in Leibowitz L-15 medium (Flow Laboratories, Rickmansworth, Herts) containing 0.01% w/v soya bean trypsin inhibitor.

2.3a Separation of Proteins by SDS Polyacrylamide Gel Electrophoresis


Reagents: --

i. Gel stock: 30g acrylamide (BDH Electra grade) and 0.8g N,N'-methylene bisacrylamide dissolved in 100 ml of distilled water (HIGHLY TOXIC). This was stored at 4°C in the dark (stable for 1 month).

ii. SDS stock solution: -- 10% sodium dodecyl (lauryl) sulphate dissolved in distilled water.

iii. Running buffer: 18.17g Tris (Sigma, UK - Trizma grade) 4ml SDS stock were made up to 100ml (pH 8.8).

iv. Stacking buffer: 6.06g Tris (Sigma UK - Trizma grade), 4ml SDS stock solution were dissolved in distilled water (80ml) and the pH adjusted to 6.8 with HCl before adjusting the final volume to 100ml with distilled water.
v. Electrode buffer: 6.06g Tris (Sigma, UK - Trizma grade), 28.8g glycine were dissolved in 1800ml of distilled water and 20ml of stock SDS solution added. The pH was adjusted to 8.3 with hydrochloric acid and the final volume adjusted to 2 litres with distilled water.

vi. Sample buffer: 3.78g Tris (Sigma, UK - Trizma grade) 25g 2-mercaptoethanol, 75ml glycerol and 5mg Bromophenol blue were dissolved in approximately 300ml distilled water. The pH was adjusted to 6.8 with hydrochloric acid and the final volume adjusted to 500ml with distilled water.

vii. 10% Ammonium persulphate solution was prepared immediately before use.

viii Gel stain - 250ml propan-2-ol, 100ml glacial acetic acid and 650ml of distilled water were mixed and 0.5g of Coomassie blue R-250 added.

ix. Gel destain - 1:1:8 mixture of propan-2-ol, glacial acid and distilled water.

x. Gel storage: 30ml glycerol and 20mg sodium azide were dissolved in 1 litre of distilled water (TOXIC).

Method
i. Running gel was prepared by mixing (for a 10% T gel*) 10ml of running buffer, 13.3ml gel stock and 16.6ml distilled water. Polymerisation was initiated by addition of TEMED (20µl) and ammonium persulphate (240µl).

* See Section 2:3b
ii. Stacking gel was prepared by mixing (for a 3% T gel), 2.5ml of stacking buffer, 1.0ml of gel stock and 6.5ml of distilled water. Polymerisation was initiated by addition of TEMED (20μl) and ammonium persulphate (60μl).

iii. Samples were diluted to an approximate protein content of 1mg protein/ml solution, using sample buffer. Following dilution, samples were placed for 3 mins in a boiling water bath to ensure complete solubilisation and cooled before application to the gel.

iv. Once samples were applied to the gel wells and the buffer systems assembled, a current of 20mA was applied until the bromophenol-blue front reached the stacking gel/running gel interface, when the current was increased to 40mA.

v. When the bromophenol blue front was within 0.5cm of the gel margin the gel was removed and placed in stain overnight.

vi. The gel was then destained until a clear background was obtained.

2:3b. Polyacrylamide gel is formed from acrylamide and the cross linking agent N,N'-methylene-bisacrylamide in the presence of ammonium persulphate as initiator and N,N,N',N'-tetramethyleneethylene diamine (TEMED) as catalyst. The reactions takes place via vinyl polymerisation and gives a random coiled gel structure. For the definition of an acrylamide gel it is necessary to indicate the quantity of monomers, i.e.

\[ T = (\text{acrylamide and bisacrylamide}) \]
\[ \text{i.e. } T = a + b \times 100 \% \]

where a = acrylamide (g)
\[ b = \text{cross-linking monomer (g)} \]
\[ m = \text{volume of buffer (ml)} \]
2:4 Enzyme Assays

2:4a Succinate Dehydrogenase

This enzyme was measured as a marker for mitochondria using a method based on that of Pennington (1961).

In this assay 2-(p-iodophenyl)-3-(p-nitrophenoxy)-5-phenyl tetrazolium chloride (INT) is used as an acceptor. Following reduction of INT, the reaction is stopped with trichloroacetic acid and the red water-soluble formazan is extracted into ethylacetate and read at 490nm.

Reagents :-

0.3M - Sodium succinate, pH 7.4
0.3M - Sodium malonate, pH 7.4
0.3M - Sodium phosphate, (Na2HPO412H2O) buffer, pH 7.4
INT solution (1.5mg/ml) in 0.3M sodium phosphate buffer, pH 7.4
ethylacetate (AR Grade)
6% (w/v) trichloroacetic acid (TCA)

Method :-

Glass test tubes (15ml capacity) were set up as follows :-

Test :-

0.5 ml homogenate or tissue fraction
0.25ml INT in 0.3M phosphate buffer, pH 7.4
0.25ml 0.3M Sodium succinate, pH 7.4
Blank :-

0.5 ml homogenate or tissue fraction
0.25ml INT in 0.3M phosphate buffer, pH 7.4
0.25ml 0.3M Sodium malonate, pH 7.4

The tubes were mixed on a Whirlymix (Fisons Ltd, UK) and incubated at 37°C for between 5 and 20 minutes, until a red colour was visible. The reaction was then stopped by addition of 1.5ml of 6% TCA. 4mls of ethyl acetate was added to each tube, and the tubes were shaken to extract the red colour into the ethyl acetate. The tubes were then centrifuged for 5 minutes at 1500g and the upper layer was carefully transferred into a glass cuvette and the absorbance measured at 490nm.

2:4b Acid α-Glycerophosphatase

Source :- Hinton et al., 1970

Reagents :-

0.2M acetate buffer containing 0.006% digitonin
0.01M p-nitrophenyl phosphate
0.1M Sodium hydroxide

Method :-

0.2ml of suitably diluted sample was mixed with 0.3ml of 0.006% (w/v) digitonin solution and placed on ice before addition of 0.5ml of 0.01M p-nitrophenyl phosphate. The tubes were mixed and incubated for 15 mins at 37°C then rapidly returned to ice before addition of 4.0ml of 0.1M Sodium hydroxide. If necessary tubes were centrifuged (550 g av. for 5 mins) before determination of absorbance values at 400nm.
2:4c γ-Glutamyl Transpeptidase


Reagents:

i. Tris-glycylglycine. 6.06g of tris base and 3.30g of glycyl-glycine were dissolved in 400ml of distilled water adjusted to pH 9.0 using HCl and made up to 500ml (stable for 12 months at -20°C).

ii. Substrate solution. 0.178g of γ-glutamyl-p-nitroanilide were dissolved in 100ml of Tris glycylglycine (may require warming to 37°C, stable at 0°C for 3 days).

iii. 35% (v/v) acetic acid.

iv. Stock standard (20mM p-nitroaniline, diluted 1:100 for use).

Method:

0.5ml of suitably diluted sample was incubated with 2ml of 6.25mM γ-glutamyl-p-nitroanilide dissolved in 0.1M Tris/0.05M glycylglycine pH 9.0 for between 15 and 150 min at 37°C. The reaction was stopped by the addition of 1ml of 35% acetic acid and the tubes cooled on ice prior to centrifugation for 10 mins at 2000 rev/min. The absorbance was determined at 410nm.

2:4d α-Amylase

This enzyme is used as a marker enzyme for the secretion granules of the parotid acinar cells. The method described is a modification of the dinitro-salicylic acid procedure of Bernfeld (1951). The
hydrolysis of starch by amylase is measured by colorimetric determination of the reducing groups produced.

Reagents :-

Substrate solution. A 1% solution of soluble starch (Nordex Standard) in a 0.02M sodium glycerophosphate - HCl buffer, pH 6.9.

Stopping agent. An alkaline solution of dinitro salicylic acid is prepared as follows, 10g of 3,5 dinitro-salicylic acid (BDH, UK) are suspended in approximately 200ml of water, to which a solution of 16g of NaOH in 150ml of water is added dropwise with gentle heating. The total volume is adjusted to 1 litre and the solution filtered through a sintered glass filter. The solution is stable for six months if stored in the dark at room temperature.

Method :-

1ml of substrate solution was added to 1ml of suitably diluted sample for exactly 3 minutes of incubation at 25°C, 2.0ml of stopping reagent was added and the tubes placed in boiling water for 5 minutes. After cooling the reaction mixture was diluted with 20ml of water and the colour read at 550nm.

IMPORTANT : If this assay is being performed on fractions from a sucrose gradient, background values from the gradient material must be determined for each gradient.

2:4e Na/K ATPase Assay

Solution 1 (stable) - 10% w/v ammonium molybdate
Solution II (make fresh) 1g ascorbic acid in 35ml H₂O, 50ml 1M HCl cooled to 0°C, when cold 5ml of solution 1 and 15ml, 20% SDS (w/v) added with rapid stirring. At this stage the solution should be yellow, not blue-green.

Solution III (stable) - 2% sodium arsenite, 2% sodium citrate, 2% acetic acid.

Assay

500 μl of assay medium was added to 100μl of suitably diluted sample to give a final concentration of 170mM NaCl, 25mM KCl, 4mM Na₂ATP, 4mM MgCl₂, 60mM Tris, 1mM EDTA pH 7.5. Assay was performed for 10 min at 37°C and stopped by addition of 1ml solution II and placed on ice. After 10 min at 0°C, 1.5ml of solution III is added and rewarmed to 37°C, after an additional 10 min the absorbance is determined at 705nm.

Nak-ATPase activity is determined from the difference between the phosphate released in control tubes and that released in identical sample containing 10⁻³M ouabain.

2:5 Immunological Techniques

2:5a Crossed Immunelectrophoresis

Source :- Grubb, 1983; also in Axelson (1983)

Reagents :-

(a) Barbitone-acetate buffer, pH 8.6, 11.3g diethylbarbituric acid, 88.8g diethyl-barbiturate sodium and 65g sodium acetate in 10 litres of distilled water.

*Used to avoid latency problems*
(b) Tank buffer. 3 litres of barbitone-acetate buffer were diluted with 2 litres of distilled water.

(c) 1% Agarose in barbitone acetate buffer (Agarose A37, Indubiose Pharmindustrie, Clichy, France).

(d) Bromophenol blue: a small spatula tip of dye was dissolved in 100ml of distilled water.

(e) Coomassie brilliant blue stain, 450ml of 96% ethanol, 450ml of distilled water, 100ml of glacial acetic acid were mixed and 5g of Coomassie brilliant blue R was added, mixed and left to stand overnight prior to filtering.

(f) Destainer: 450ml of industrial methylated spirit (or 96% ethanol) was mixed with 450ml of distilled water and 100ml of glacial acetic acid.

(g) 0.9% NaCl.

Method:

The electrophoresis tank was filled with tank buffer and the plates prepared, 10ml of hot agarose (1% w/v) was spread evenly over each plate and allowed to set. Holes were cut 1.5cm from each of the lower corners of the plate. The plates were then placed on a water cooled platform and the tank set up as shown in the diagram (Fig 11b).

One drop of bromophenol blue was added to the right hand well of the plate and the sample was placed in the left hand well (5 or 15μl of sample was loaded depending if a 2 or 4mm diameter well was used).
Figure 11 b) shows the experimental organisation of apparatus used during crossed immuno-electrophoresis.

Fig 11 b) shows the plate trimming procedure used during crossed immuno-electrophoresis.
Once samples were loaded 200V per plate was applied and electrophoresis was allowed to continue until the bromophenol tracker dye reached the far edge of the plate. The power was then switched OFF, the plates removed and trimmed as shown in Fig 11a.

Antiserum was pipetted into a test tube warmed to 50°C. Sufficient agarose was added to make the volume up to 9ml, mixed by inversion, poured onto the plate and allowed to set. The plate was then connected to the buffer system using clean wicks, the power switched on and adjusted to give a constant voltage of 45 volts. Electrophoresis was continued for at least 16 hours. The power was then switched off and the plates removed. Plates were then blotted with filter paper and pressed using a thick glass plate (approx 500 g/plate) to press (10-15 mins), washed in 0.9% NaCl (1 hr), blotted again (10-15 mins), washed in 0.9% NaCl (2 hrs) and finally washed in distilled water (30 mins) before being dried in a current of hot air. The dried plates were then stained by immersion in Coomassie solution (10 mins) and then destained to achieve a slightly bluish background.

2:5b Western Blotting

Western blotting experiments were performed according to the methods of Burnett (1981) and Towbin (1979) using protein separations on polyacrylamide gels prepared by the method of Laemmli (1970) see section (2:3).
Reagents

Buffer :-

Buffer 1, blot buffer, 1.9M glycine in 0.25M Tris, Buffer 2, wash buffer, phosphate buffered saline (P.B.S) containing 10% (v/v) horse serum, 0.2% (v/v) Triton x 100.

Cellulose nitrate paper (Schleircher and Schull, Dusseldorf, W. Germany) 0.45 m, Whatmann 3mm filter paper, Ponceau S stain (Serva Feinbiochemica, Heidelberg). Diaminobenzidine (BDH/UK).

Method :-

The polyacrylamide gel containing the separated proteins was placed onto cellulose nitrate paper, care being taken to remove any air bubbles, and assembled into the blotting cassette as shown in Fig 12. The cassette was then placed in the tank containing blot buffer and a current of 40V (app 100mA) was applied overnight.

Following transfer the cellulose nitrate paper was removed and stained (5-10 mins) with Ponceau S solution to confirm correct transfer and visualise molecular weight markers. After marking the position of these the stain was removed by rinsing with distilled water. Following three successive 15 minute washes in buffer 2 the cellulose nitrate paper was placed in buffer 2 containing the requisite first antibody at an approximate concentration of 30µg/ml for a period of 1 hr. The cellulose nitrate paper was washed a further three times (15 min each) in buffer 2 and then placed in the second antibody solution for 1 hr using a 1:200 antibody dilution in buffer 2.
Fig. 12 shows the experimental organisation of the cassette used during Western blotting.
In these experiments the first antibody was raised in rats against either pIgA, SC or whole saliva (Anti rat*, and (SC) were a kind gift from Chester Beaty Cancer Research Centre. The second antibody was a goat: anti-rabbit peroxidase) conjugated antibody (Dako Ltd, High Wycombe, Bucks). Following incubation in the second antibody the cellulose nitrate paper was washed (3x15 mins) in buffer 2 and for 15 mins in PBS.

The blot was then developed in 0.1M Tris-HCl pH 7.5 containing 0.5mg/ml of diaminobenzidine chloride and 1:5000 parts H$_2$O$_2$ (30 vols). The diaminobenzidine and H$_2$O$_2$ act as substrates for the conjugated peroxidase enzyme of the secondary antibody and the reaction results in precipitation of a brown pigment at the site of antibody binding. The staining reaction was stopped on development of suitable intensity of colour by immersion in double distilled water.

2.5c Immunohistochemical Staining of Tissues for Light Microscopy

Parotid glands and the submaxillary/sublingual glands were rapidly excised from rats and frozen in hexane cooled to -60°C in cardice-alcohol (Chayen et al., 1973). Sections 10μm thick were cut on a Bright rocking microtome in a cryostat maintained at -30°C. Immunohistochemical staining of the sections was carried out by the peroxidase-antiperoxidase procedure (Sternberger, 1970). The primary antibodies were rabbit anti-rat (α chain) and rabbit anti-rat (secretory component) whose preparations are documented respectively in Orlans et al (1978) and Orlans et al (1979). Specificity was confirmed both by omission of primary antisera and by absorption of primary α chain of Immunoglobulin A
antisera with purified antigen, i.e. rat secretory IgA. This was prepared from crude bile which was first passed through a column of Sephadex G25 in order to separate out the high-molecular weight proteins. The relevant fractions were then applied, firstly to a column of AcA22 Ultrogel and then for a further purification step to a column of AcA34 Ultrogel. The absorbance of the eluants at 280nm was measured and fractions tested for secretory IgA by rocket electrophoresis. The purified secretory IgA was then bound to a cyanogen bromide-activated Sepharose gel. This conjugate was added to the antigen in such proportions as to ensure a 20-fold excess of bound secretory IgA to the total protein present in the antisera and incubated for 18h at 4°C.

2:6 Histology

2:6a Staining with Haematoxylin and Eosin

Sections were taken through xylene and down through alcohol 2x100%, 2x80% (v/v) (30 seconds in each) and stained in Ehrlich’s acid haematoxylin (15 minutes). The sections were then placed in tap water until a blue colour developed (5 mins), differentiated with acid alcohol (5 seconds) and returned to tap water (30 seconds) before being counterstained with 1% (v/v) aqueous eosin (2 mins). The sections were rinsed in tap water (30 seconds) dehydrated in 85% (v/v) ethanol (30 seconds) and 100% ethanol (2x30 seconds), cleared in xylene and mounted in DPX (British Drug Houses Ltd, Poole, Dorset, UK).
2:6b  **Periodic Acid-Schiff (PAS) Reaction**

Sections were stained according to the PAS method described in (Kiernan, 1981) to demonstrate the presence of polysaccharides (glycogen), neutral mucopoly-saccharides, mucoproteins, glycoprotein and glycolipids. Cryosections prepared as described in section 2:5a were brought to water through alcohol as described in 2:6a then oxidised in 1% aqueous periodic acid (5 mins), washed in running water (5 mins), placed in Schiffs reagents (20 mins) washed again in running water (10 mins) and stained in Harris haematoxylin.

2:6c  **Alcian Blue**

At pH 2.5 this dye binds to carboxyl and sulphate ester groups and was thus used in the present investigation to identify mucosubstances which owe their acidity to sulphate or carboxyl groups.

**Source**: Kiernan (1981).

**Alcian Blue Solutions**:–

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcian blue</td>
<td>1.0g</td>
</tr>
<tr>
<td>3% aqueous acetic acid</td>
<td>100ml</td>
</tr>
</tbody>
</table>

(stable for several months at room temperature)

**Method**

1. Immerse cryosections in distilled water (1 min).

2. Stain in Alcian Blue solution for 10-15 minutes. If wax embedded sections are being used this incubation period must be extended to 30 minutes.
3. Wash in running tap water (3 mins).

4. (OPTIONAL) Apply a pink or red counterstain if desired.

5. Dehydrate in graded alcohols. Alcian blue is not removed by alcohol, but counterstains may be differentiated.

6. Clear in xylene and mount in a resinous medium.

**Result:**

All acid mucosubstances are stained with deep blue at pH 2.5.

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**2:7 Electron Microscopy**

**2:7a Preparation of Tissues for Electron Microscopy**

**Method A:**

Based on personal communication from Professor S.J. Holt and J.T.R. Fitzsimons. Material for electron microscopy was fixed for 2-4 hours in 4% (w/v) glutaraldehyde (TAAB Laboratory Equipment Ltd, Reading, Berks, UK) buffered with 0.1M cacodylate : HNO₃ pH 7.4. After fixation the tissue was washed overnight in cacodylate buffer (0.1M) and counterfixed in 2% buffered osmic acid (w/v)/0.1M cacodylate HNO₃ pH 7.4 for 2 hours at room temperature. Following counter fixation, tissues were dehydrated through alcohol solutions 25%, 50%, 75%, 90% (v/v) and absolute (2 x 10 mins each). Dehydrated tissue was then placed in propylene oxide/absolute alcohol (1:1) for 10 mins followed by 2x10 min in propylene oxide and a final 10 min incubation in propylene oxide/Epon 812 (TAAB Laboratory Equipment Ltd, Reading, Berks, UK) 1:1 mix. Tissues were transferred into Epon 812 filled capsules and incubated at 60°C for 48 hrs to polymerise the resin.
Method B:-

This method was used to obtain an increased preservation of secretory component antigenity as compared with that observed using Method A.


Method:-

Tissue was fixed in carbodiimide (cyanalide) (Sigma UK) prepared by dissolving 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide-HCl in phosphate buffered saline (PBS), 20g/litre, containing 0.5% glutaraldehyde. Fixation was for a period of 22hrs at 4°C and was followed by an overnight wash in PBS buffer (pH 7.6). Tissues were then dehydrated through alcohol as described in Method A, before being embedded in LR WHITES resin which was polymerised at 60°C for 48 hrs.

2.7b Histochemical Staining of Tissues for Electron Microscopy

Source: Adapted from the method of Peppard et al. (1984).

Reagents:-

i. Blocking reagent 120ml of 100 vol H_2O_2/220ml of methanol.

ii. Tris buffered saline (TBS) 0.9% (w/v) sodium chloride in 0.1M Tris-HCl (pH 7.4).

iii. TBS containing 10mg/ml Bovine serum albumin

iv. 0.05M Tris-HCl (pH 7.4) containing 2.5mg of 3.3 Diamino benzidine (DAB) (BDH)
Method:

Prior to staining parotid tissue had been incubated with IgA specific for horse radish peroxidase.

Parotid tissue was placed in blocking reagent (10 mins), rinsed (2x 10 mins) in TBS incubated in TBS containing 10mg/ml BSA (10 mins) and placed in TBS containing 1.5mg/ml of horse radish peroxidase (30 mins). The tissue was then removed, washed (2 x 10 mins) in TBS and incubated for a further 90 mins in DAB solution, to which 0.1ml of 1% H₂O₂ was added after 1hr. The tissue was finally washed 2 x 10 mins in PBS and processed for electron microscopy as described in Section 2:7a.
RESULTS

3:1 Histology of Rat Salivary Glands

The structural organisation of each major salivary gland was observed following staining with haematoxylin and eosin (Fig 13). The relative proportion of serous and mucous acinar cells were observed by PAS and Alcian blue histological stains respectively (Fig 14 and 15). The results show that serous acinar cells were present in both parotid and submaxillary glands although the homogeneity of cell type present in parotid glands made this tissue far more attractive with regard to subsequent fractionation work.

3:2 Immunohistochemical Staining of Parotid and Submandibular Glands to Show the Distribution of SC and IgA, IgA.

Salivary glands were excised and 10μm cryosections prepared and stained to show the presence of SC or IgA as described in section 2:5c.

Parotid glands stained using a primary antibody to secretory component or α-chain showed a ubiquitous granular staining of acinar cells plus intense staining within secretory ducts, the lining cells of which were unstained (Fig 16 and 17).

(Fig 18a and 18b)

Staining of the submandibular gland was not uniform; staining of SC and IgA was confined to certain acinar cells and the duct lumen while duct epithelial cells were unstained as in the parotid gland (Fig 17). In sections of both parotid and submandibular glands stained with anti(rat α-chain) small strongly staining cells were apparent in the interstitial tissue which were not present in neighbouring serial sections of IgA.
Figure 13 - Shows the general organisation of rat parotid gland (A) and rat submaxillary and sublingual gland (B). Sections were stained as described in Section 2.6a.

D - Ducts; SM - Submaxillary; SL - Sublingual
Figure 14a - Rat parotid gland stained with PAS (MAG x 10)

Figure 14b - Rat sublingual (SL) and submaxillary (SM) glands stained with PAS (MAG x 40)
**Figure 15a** - Rat parotid gland stained with Alcian blue pH 2.3 (MAG x 40)

**Figure 15b** - Rat sublingual (SL) and submaxillary (SM) glands stained with Alcian blue pH 2.3 (MAG x 40)
Figure 16 - Shows the specificity of staining achieved following immunohistochemical localisation of IgA in rat parotid glands. Brown precipitate shows the presence of IgA.

PA - Parotid acinar cells; F - Fatty tissue
L - Lymphnode (MAG = x 40)
Figure 17a - Shows the relative distribution of IgA within the parotid glands following immunohistochemical staining using a rat (κ chain) primary antibody MAG x 40

Figure 17b - Shows the relative distribution of SC within the parotid glands following immunohistochemical staining using a rat anti SC primary antibody MAG x 40
Figure 18a - Shows the appearance of submaxillary glands stained using a rat anti (α chain) primary antibody. MAG x 40

Figure 18b - Shows the appearance of submaxillary glands stained using a rat anti SC primary antibody. MAG x 40
sections stained using anti(rat secretory component) as primary antibody. These were presumably plasma cells providing a local source of IgA (Fig 19), compare with control (Fig 18).

3:3 Cytology

Electron microscopic examination of normal rat parotid glands show the structural organisation of serous acini with associated myoepithelial cell (Fig 21) and blood vessels (Fig 22). The appearance of serous acini was also examined following artificial stimulation of salivation by intraperitoneal injection of pilocarpine (Fig 23). The results show that tight junctions are clearly visible surrounding lumen of acini which appear 'packed' with electron dense granular material. In addition subcellular organisation appears comparable to normal cells (Fig 24, 25) and the lack of distension in endoplasmic reticulum stacks and integrity of mitochondria suggest that cells remaining viable and 'healthy' following artificial stimulation of secretion.

3:4 Analysis of Whole Rat Saliva

Approximately 0.5ml of whole rat saliva was collected from each 250g rat following intraperitoneal injection of pilocarpine. The protein components of saliva collected were separated by SDS polyacrylamide gel electrophoresis (Fig 24) and by crossed immunoelectrophoresis against anti rat whole saliva (Fig 25). The results show twenty anionic protein components of whole saliva identified by coomassie brilliant blue stained SDS polyacrylamide gels compared to seventeen proteins identified by crossed immunoelectrophoresis (Fig 25).
Figure 19 - Shows the presence of IgA committed plasma cells in the interstitium of salivary glands visualised using a primary antibody to rat \( \alpha \) chain.
Figure 20 - Shows a control section of rat parotid gland stained as described in Section 2:5c but using antisera absorbed against SIGA
Figures 21 and 22 - shows the general cytology of rat parotid acinar cells and the structural organisation of parotid acini. Tissue was stained with osmium and counter stained with lead/uranyl acetate (MAF x 6,000).

N - Nucleus; SG - Secretion Granules; ME - Myoepithelial Cell; B - Basolateral Plasma Membrane; A - Apical plasma membrane; BV - Blood Vessel; RER - Rough Endoplasmic Reticulum.
FIG 21
Figure 23 - Shows the cytology of rat parotid acinar cells following pilocarpine induced salivation. Tissues were stained with osmium tetroxide and counter stained with lead and uranyl acetate (MAG x 7,000).

SG - Secretion Granules; N - Nucleus; RER - Rough Endo-plasmic Reticulum; ME - extensions of Myoepithelial Cells; M - Mitochondria, arrows indicate tight junction complex around lumen of acini.
Figure 24 - Shows the component proteins of whole rat saliva separated by electrophoresis in a 10% polyacryl-amide gel electrophoresis containing SDS and stained with coomassie brilliant blue.

MWT = appropriate molecular weight scale.
Figure 25 - Shows the component proteins of whole rat saliva visualised by crossed immunoelectrophoresis against 3% (v/v) anti rat whole saliva and stained with coomassie brilliant blue.
The presence of serum proteins in whole rat saliva was determined by crossed immunoelectrophoresis (Fig 26). The results show eleven proteins secreted following pilocarpine induced salivation may be serum derived.

3:4a Molecular Weight Determination of Sc and IgA Found In Saliva and Membrane Preparations.

Component proteins of whole rat saliva separated by native polyacrylamide gel electrophoresis were transferred onto nitrocellulose paper by western blotting and stained using primary antibodies to rat secretory component or rat $\alpha$ chain. The results show one prominent secretory component band with a molecular weight of 63,000 plus two more lightly stained bands of 28,200 and 25,100 molecular weights respectively (Fig 27). Staining of neighbouring tracks with anti rat-$\alpha$ chain showed two high molecular weight species of IgA with molecular weights 199,500 and 154,000 respectively and a faint stain around the 63,000 molecular weight region (Fig 28).

In order to determine the molecular weights of cellular secretory component and IgA a crude microsomal preparation was isolated as described in Section 22a. Component proteins were separated by SDS polyacrylamide gel electrophoresis, transferred by western blotting and stained using either anti(rat $\alpha$ chain) or anti(rat secretory component) primary antibodies.

The results show the presence of two secretory component bands with molecular weights 200,000 and 160,000 respectively which were not present in whole rat saliva, plus three additional secretory component
bands of molecular weights 75,800, 66,800, 54,900 respectively (Fig 27).

The high molecular weight species of IgA observed in saliva (molecular weight 200,000 and 114,000) were also present in the crude microsomal preparation. However, in addition a further high molecular weight IgA band (molecular weight of 199,500) and two lower molecular weight proteins (molecular weight 63,000 and 61,000) were present in the microsomal preparation (Fig 28).

3:5 In Vivo Transport of IgA

3:5a Transport of Labelled IgA Into Rat Saliva In Vivo

Initial attempts to introduce $^{125}$I labelled IgA into rat parotid glands or saliva by intravenous injection showed very low levels of incorporation (Fig 29). Therefore an alternative approach was adopted using hybridoma cells which produce IgA specific for horse radish peroxidase (HRP).

Following intraperitoneal injection of $2 \times 10^6$ hybridoma cells per rat, clinical signs of ascites appeared after 7-10 days accompanied by high levels of serum HRP-specific IgA (serum levels of HRP-specific IgA were determined by Dr. B.M. Mullock). Samples of serum and saliva were collected nine days after the administration of hybridoma cells and analysed by E.L.I.S.A to determine the relative levels of HRP specific IgA in each (Fig 30). Microscopic examination of the glands showed that some HRP was also present in the glands (See Section 3:5b) and some were observed within lymph nodes found in close association with
Figure 26 - Shows the proportion of serum proteins present in whole rat saliva observed by crossed immunoelectro-phoresis against 3% (v/v) anti rat serum.
Figure 27 - Shows the molecular weight forms of SC observed in saliva following separation of proteins in native polyacrylamide gel (7.5%) (Lane A) and in crude microsomal preparations (lane B)
Figure 28 - Shows the molecular weight forms of IgA observed in saliva following separations of proteins in native polyacrylamide gel 7.5% (Lane A) and in crude microsomal preparations (Lane B).
Figure 29 - Shows an autoradiograph of rat parotid gland following intravenous injection of $^{125}$I IgA (MAG = x 40).

SA - Serous Acinar cells; S - Septum between lobule of glands. Arrows indicate presence of $^{125}$I IgA.
Figure 30 - Results obtained by E.L.I.S.A method showing levels of horse-radish peroxidase IgA in serum and saliva of male w/a rats nine days after intraperitoneal injections of hybridoma cells. Control animals did not receive hybridoma cells.
the parotid glands (Figs 31 and 32), showing that hybridoma cells not only release large amounts of IgA into the blood stream but also migrate to peripheral lymphoid tissues possibly in a comparable manner to IgA committed B lymphocytes.

3:5b **Immunohistochemical Localisation of HRP Specific IgA in Parotid Glands In Vivo.**

Parotid glands extracted from rats nine days after intraperitoneal injection of $2 \times 10^6$ hybridoma cells were stained to show the distribution of HRP specific IgA (Figs 31 and 32). The results show that the glandular distribution of HRP specific IgA was directly comparable to that previously observed by immunohistological localisation of endogenous IgA (Fig 17). Intense staining was observed in acinar cells, the interstitium, the ductal lumen and IgA producing plasma cells. In addition cells producing HRP-specific IgA were observed within lymph nodes found in close association with parotid glands in vivo. Thus it would appear that hybridoma cells producing HRP specific IgA may show similar migratory properties to endogenous IgA B lymphocytes so providing a 'localised' source of HRP specific IgA.

3:6 **In Vitro Transport of IgA By Rat Parotid Acinar Cells**

3:6a **Cell Viability**

Parotid acinar cells were isolated as described in Section 2.2f. Approximately $6 \times 10^7$ cells were obtained from 2g (wet weight) of excised parotid glands. Cell viability was assessed by the exclusion of trypan blue and remained in excess of 87% in all preparations reported (Fig
Figure 31a - Shows the distribution of horse-radish peroxidase specific IgA (Brown colour) observed in rat parotid glands nine days after intraperitoneal injection of hybridoma cells producing IgA specific for horse-radish peroxidase.

Figure 31b - Shows rat parotid gland from control animals who did not receive hybridoma cells also stained to show the presence of horse-radish peroxidase IgA.
Figure 32 - Shows lymph node situated adjacent to rat parotid glands in vivo stained to show the presence of horse-radish peroxidase IgA (MAG = x10). The arrow shows specifically staining cells.
33). In addition a more critical assessment of cell viability was performed by electron microscopy, following a 1 hour incubation at 4°C and a 2 hour incubation at 37°C comparison of Figs 2 and 35, shows the characteristic cellular polarity of parotid acinar cells in vivo is retained in vitro, also isolated cells appear 'healthy' showing no distention of ER stacks or disruption of mitochondria.

The adopted method of cell isolation and subsequent incubation conditions therefore produces viable cells with a well preserved functional polarity suitable for the investigation of in vitro protein transport experiments.

3:6b Binding of IgA to Parotid Acinar Cells In Vitro

Two experiments were performed to determine the degree to which isolated parotid acinar cells would bind and release \( \text{I}^{125} \) labelled IgA (\( \text{I}^{125} \) IgA). In both experiments cells were isolated as described in Section 2:2f, incubated at 4°C for 1 hour with \( \text{I}^{125} \) IgA washed three times with L15 medium and incubated at 37°C for up to 2 hours. For the purpose of these experiments the zero time point was taken to be immediately prior to incubation at 37°C.

The proportion of \( \text{I}^{125} \) IgA initially bound to acinar cells was defined as the sum total of protein-associated counts (those retained by a Diaflow p10 membrane) released during the full incubation period, plus those counts which remain associated with cells throughout.

**EXPERIMENT 1** - The release of initially bound \( \text{I}^{125} \) IgA from isolated cells at 37°C was assessed during two successive 1 hour incubations (Fig 36). The results show 56% of initially bound \( \text{I}^{125} \) IgA was
Figure 33 - Shows an average cell viability observed throughout the adopted in vitro transport procedure as assessed by the method of trypan blue exclusion.
Figure 34 - Shows the characteristic polarity of serous acinar cells in rat parotid glands in vivo. The sections were stained with osmium tetroxide and counterstained with lead/uranylacetate MAG = (x9,000).

N - Nucleus; SG - Secretion Granules; ME - Myoepithelial cell; M - Mitochondria; RER - Rough Endoplasmic Reticulum.

Figure 35 - Shows parotid acinar cells in vitro following a 1 hr incubation at 4°C and a subsequent 2hr incubation at 37°C. Cells were stained with osmium tetroxide but NOT counterstained with lead/uranyl acetate, MAG = x6,000.

N - Nucleus; SG - Secretion Granules; RER - Rough Endoplasmic Reticulum.

* Fig. 34 not included, it's identical to Fig. 2, page 6.
Figure 36 - Shows the percentage release of bound $^{125}$I IgA from rat parotid acinar cells in vitro during two successive sixty minute periods of incubation at 37°C, plus counts which remained bound to cells throughout. The IgA was initially bound to the cells by incubation at 4°C as described in the text.
released after 1 hour and a further 25% was released during a subsequent 60 min incubation, leaving a residual 21% still associated with cells.

**EXPERIMENT 2** - As the majority of initially bound $^{125}$I IgA was released before the first time point of experiment 1, a further experiment was performed in which the release of $^{125}$I IgA was monitored over three successive 30 min incubation periods. The results show that the release of initially bound $^{125}$I IgA during successive incubations was 75%, 8.7% and 3% respectively (Fig 37) leaving a residual 13.7% of initially bound $^{125}$I IgA still associated with the acinar cells.

Due to the 'clumped' nature of acinar cells used in these preparations, it is possible that unbound $^{125}$I IgA, may be retained within cell clusters to varying degrees. As such the very high percentage release observed during the first 30 minutes incubation period of experiment 2 may partially represent a continued washing effect. However, both experiments 1 and 2 show binding of $^{125}$I IgA during a 1 hour incubation at 4°C in vitro. In addition the majority of IgA bound during this period is subsequently released during a 2 hour incubation at 37°C possibly within the first thirty minutes.

3:6c **Localisation of IgA Bound To Parotid Acinar Cells In Vitro**

Cells were isolated, incubated for 1 hour at 4°C with HRP specific IgA, washed three times with L15 medium and stained as described in Section 2:7b. The results show a distinct 'coating' of IgA around the basolateral domain of the cells with an absence of stain along the apical plasma membrane (Fig 38). This therefore provides evidence that secretory component is present on the basolateral plasma membrane of
Figure 37 - Shows the percentage release of $^{125}$I IgA from rat parotid acinar cells in vitro during three successive thirty minute periods of incubation at 37°C, plus counts which remained bound to cell throughout. The IgA was initially bound to cells by incubation at 4°C as described in the text.
Figure 38 - Shows an isolated rat parotid acinar cell stained to show the localisation of horse-radish peroxidase specific IgA bound to the cell during a 1 hr incubation at 4°C. Arrows indicate IgA.

N - Nucleus; SG - Secretion Granules; M - Mitochondria; RER - Rough Endoplasmic Reticulum; BL - Basolateral plasma membrane domain; AP - Apical Plasma membrane domain. Tissue was stained with osmium tetroxide but not counterstained with lead/uranyl acetate (MAG = 10,000).
isolated acinar cells and that specific membrane domains are preserved during preparation and subsequent incubations.

3:6d Qualitative Analysis of Secretions From Isolated Rat Parotid Acinar Cells In Vitro

The extent of cellular transport and secretion occurring in isolated parotid acinar cells was assessed by monitoring the release of proteins into the incubation medium during four successive 30 minute incubations at 37°C.

Proteins released during each 30 minute incubation were separated by SDS polyacrylamide electrophoresis (Fig 39). Twelve anionic proteins were visible in each 30 minute sample following staining with coomassie brilliant blue (R-250).

Analysis of the proteins released by western blotting using an anti (rat whole saliva) primary antibody showed eight or nine protein bands present in each thirty minute sample (Fig 40). Therefore the proteins released by the acinar cells are salivary proteins.

The 'lens' like patterns which appear in Fig 40 and the intense staining protein band of 44,000 mwt in Fig 39, are due to the high proportion of bovine serum albumin used in this preparation in order to reduce mass aggregation of isolated cells.

3:6e Quantitative Analysis of Salivary Proteins Secreted by Parotid Acinar Cells In Vitro

Samples from each 30 minute time point above were concentrated by
Figure 39 - Shows proteins secreted by rat parotid acinar cells in vitro separated by electrophoresis in a 10% polyacrylamide gel in the absence of denaturing agents. The gel was stained with coomassie brilliant blue R. Tracks A, B, C, and D represent proteins released during four successive thirty minutes incubation periods at 37°C. Track E represents proteins released during one continuous hour of incubation but at 1/2 the concentration of other tracks enabling resolution of intensely staining band marked by arrow into two distinct bands.
Figure 40 - Shows western blot of proteins, secreted by isolated rat parotid acinar cells, stained using a primary antibody to whole rat saliva. Tracks A, B, C and D represent proteins released during four successive thirty minutes incubation periods at $37^\circ$C. Track E represents proteins released during one continuous hour of incubation but at 1/2 the concentration of other tracks enabling resolution of intensely staining band marked by arrow into two distinct bands. L, Lens shaped bands caused by high levels of bovine serum albumin added to incubation medium containing acinar cells in order to reduce mass aggregation during incubation.
a factor of ten (by filtration through a diaflow P10 membrane) and analysed by crossed immunoelectrophoresis using an antibody to whole rat saliva. This method of protein analysis is less sensitive than western blotting, however proteins identified by this method can be more easily quantified.

Five salivary proteins were identified in each 30 minute sample and have been ascribed arbitrary numbers one to five (Fig 41). Proteins 2, 3 and 5 show an increased rate of release starting during the second 30 minute incubation and then show similar increases in rate of secretion over the subsequent 90 minutes of incubation. In contrast salivary proteins 1 and 4 show a constant rate of secretion over the full 2 hour incubation period.

3.6f Identification of SC and IgA in secretions from parotid acinar cells in vitro.

Cells isolated as described in Section were incubated with $^{125}$IgA for 1 hour at 4°C, washed three times in excess L15 medium and incubated for one single 2 hour period at 37°C. Following incubation, the medium was removed from cells and concentrated by a factor of ten using diaflow P10 filter. Secreted proteins were then analysed by split gel crossed immunoelectrophoresis using antibodies to rat SC (Figs 43) and rat alpha chain (Figs 44). Both were used in combination with upper gels containing anti (rat whole saliva).

The results show two proteins are specifically precipitated by both antirat SC and anti (rat α chain). The protein precipitate marked A in Figs 43 and 44 has a comparable shape and location to secretory IgA (SIgA) observed in bile. However the less well defined protein
Figure 41 - Shows the relative levels of 5 individual salivary proteins secreted from parotid acinar cells in vitro. Quantitation was performed by monitoring the levels of each protein secreted during four successive thirty minute incubation periods at 37°C by crossed immuno electrophoresis against anti whole rat saliva.
Figure 42 - Shows western blots of proteins secreted by rat parotid acinar cells in vitro during a continuous 2hr incubation period at 37°C. Track A was stained using a primary antibody to rat secretory component. Track B was stained using a primary antibody to rat α chain. Track C shows a western blot of a secretion granule preparation stained to show the presence of SC for comparison.

MWT = approximate molecular weight scale
precipitate marked B in Figs 43 and 44 appears to have too lower molecular weight to be sIgA and could possibly be a degradation product of the protein in band A. Therefore, parotid acinar cells do secrete sIgA in vitro.

The occurrence of secretory component and IgA in the secretions from isolated cells was also assessed by western blotting of proteins separated by SDS polyacrylamide gel electrophoresis. One single protein of molecular weight 60,000 was stained using an anti (rat-\(\alpha\) chain) primary antibody, while two faint protein bands of molecular weights 56,400 and 75,000 respectively were stained using an anti (rat secretory component) primary antibody (Fig 42). Although the proteins identified by both antibodies are very faintly stained the reactions are specific and show that both secretory components and IgA are released by acinar cells in vitro.

3:7 Cell Fractionation

The fundamental aim of these fractionation procedures was to obtain an optimal separation of the two plasma membrane domains of the parotid acinar cells and also to separate both of these fractions from secretion granules. In all fractionation experiments the marker enzyme \(\text{Na}^+/\text{K}^+\) ATPase (Ouabain sensitive), \(\gamma\)-Glutamyl transpeptidase (\(\gamma\)-GT), \(\alpha\)-amylase, acid phosphatase (AP) and succinate dehydrogenase (SD) were used as marker enzymes for basolateral plasma membrane, apical plasma membrane, secretion granules, lysosomes and mitochondria respectively.

Such preparations could then be used to investigate the subcellular distribution of IgA or sc. In addition analysis of the molecular weight of SC within the various membrane fractions would be necessary to fully characterise the mechanism of IgA transport in rat parotid glands.
Figure 43 - Split gel crossed immuno-electrophoresis of proteins secreted by rat parotid acinar cells in vitro during one continuous 2hr incubations at 37°C. Using anti rat whole saliva in the upper gel and anti rat Sc in the liver gel (A) or no antisera in the lower gel (B). Arrows indicate proteins specifically precipitated by anti rat Sc.

Figure 44 - Split gel crossed immunoelectrophoresis of proteins secreted by rat parotid acinar cells in vitro during one single incubation for 2 hrs at 37°C using anti rat whole saliva in the upper gel and either antibody to α chain (A) or no antibody (B) in the lower gel. Arrow indicates protein specifically precipitated by anti rat α chain.
3:7a Preliminary Separation Following Pelleting

As previously published methods of rat parotid gland fractionation were based on repeated pelleting and resuspension of materials a preliminary experiment was performed simply to determine the degree of aggregation induced by such enforced contacts.

A crude post-nuclear supernatant obtained as described in Section 2:2e (part 3), was centrifuged at 41,250 g av. for 40 mins in a Kontron K200 centrifuge. The resulting pellets were resuspended in 0.25M sucrose, layered over a 0.3-1.7M sucrose gradient and centrifuged as described in Section 2:2d (part 8). Results show that although a high percentage recovery of gamma-GT (82%), Na⁺/K⁺ ATPase (60%) and α-amylase (67%) was achieved, resolution between these fractions along the sucrose gradient was poor and uncharacteristically high density fractions of each marker enzyme was observed (Fig 45).

3:7b Preliminary Isopicnic Separation

Crude separation of component organelles from rat parotid gland homogenate was initially performed using one single isopicnic centifugation along a 0.3-1.7M sucrose gradient at 100,000 g av. for 18 hrs in a Kontron K200 centrifuge using a TST 2838 rotor. The resulting distribution of organelle factions represented in Fig 46, show considerable cross contamination of secretion granules and both plasma membrane domains. As such, a more sophisticated method of fractionation was developed.
Cross-hatched areas represent the proportion of each marker enzyme not present in the initial pellet. See section 3.7a.
Figure 45 - Shows the distribution of marker enzymes following pelleting
(1) alpha-amylase, (2) gamma-glutamyl transpeptidase,
(3) Na /K' ATPase. * see opposite.

Bar means.
Figure 46 - Shows distribution of marker enzyme following isopycnic separation of cellular fractions from crude homogenate. 
3:7c Preparation of Apical Plasma Membrane Sheets Using a Final Isopycnic Centrifugation Step

As separation of component organelles from crude parotid gland homogenate could not be achieved on the criteria of equilibrium density a preparatory rate centrifugation step was employed.

A crude fraction of apical plasma membrane sheets was obtained from parotid gland homogenate by a preliminary flotation procedure as described in section 2:2d. The results show that following flotation the interface band contained the following proportions of marker enzyme activities, expressed as percentage of original homogenate activity. \( \gamma \)-GT (78%), \( \text{Na}^+ / \text{K}^+ \text{ATPase} \) (33%), SD (22%), \( \alpha \)-amylase (33%) plus approximately 36% of total protein. Subsequent isopycnic centrifugation of this fraction again results in co-sedimentation of fractions from both plasma membrane domains and secretion granules (Fig 47).

3:7d Preparation of Apical Plasma Membrane Sheets Using A Final Rate Centrifugation Step.

The results from this preparation show that the percentage of component marker enzymes in the interface fraction following flotation, expressed as percentages of the total homogenate, \( \gamma \) GT (68.9%), AP (28%), \( \alpha \)-amylase (35%), \( \text{Na}^+ / \text{K}^+ \text{ATPase} \) 41% and total protein was 34%. Following a subsequent rate centrifugation at 5,000 rpm for 40 mins the resulting distribution of marker enzyme activity along the 0.3-1.7M sucrose gradient (Fig 48) showed that the resolution of the two plasma membrane domains, both from each other and from secretion
Figure 47 - Show the distribution of marker enzymes following isopicnic separation of cellular fractions taken from the interface band following preparatory flotation. (1) gamma-glutamyl transpeptidase, (2) alpha-amylase, (3) succinate dehydrogenase, (4) Na⁺/K ATPase, (5) Acid phosphatase, (6) protein.

Cross-hatched areas represent the proportion of each marker enzyme which was not present in the interface band following flotation.
Figure 48 - Shows the distribution of marker enzymes following rate separation of cellular fractions taken from the interface band following preparatory flotation. (1) Sodium Na⁺/K⁺ ATPase, (2) acid phosphatase, (3) alpha amylase (4) gamma glutamyl transpeptidase, (5) protein.

Cross-hatched areas represent the proportion of each marker enzyme which were not present in the interface band following flotation.
59%

72%

65%

32%

FIG 48(i)

DENSITY (g/ml) 20°C
% HOMOGENATE ACTIVITY

DENSITY (g/ml) 20°C

FIG 48(ii)
granules has been greatly improved over results obtained by final isopicnic centrifugation (Section 3:7c).

3:7e Preparation of Basolateral Plasma Membrane Fractions

Differential pelleting of crude parotid gland homogenate as described in Section 2:2b shows that centrifugation at 12,000 g av. for 40 minutes would effectively remove 89% of γ-GT present in total homogenate. The results from subsequent isopicnic separation of the residual supernatant along a 0.3-1.7M sucrose gradient (Section 2:2c) show clearly that although approximately 25% of Na⁺/K⁺ ATPase activity is lost by preliminary pelleting the residual basolateral plasma membrane fraction, distributed along the gradient with a median density of 1.16, is relatively clear of contamination from apical plasma membrane or secretion granule fractions (Fig 49b).

3:7f Labelling of Basolateral Plasma Membrane Vesicles With 125I IgA

125I IgA was added to freshly homogenised rat parotid glands and mixed at 4°C for 15 minutes. Samples of homogenate were then fractionated as described in Section 2:2c and the relative distribution of 125I IgA and the basolateral plasma membrane marker enzyme Na⁺/K ATPase observed along the 0.5-1.7M sucrose gradient.

In order to differentiate between the distribution of 'membrane bound' 125I IgA from the excess of free 125I IgA present in the homogenate. Fractions from the 0.5-1.7 sucrose gradient were diluted 1/30 with 0.25M sucrose and centrifuged at 220,000 g av. for 60 mins, using a Kontron K-200 centrifuge (using rotor TST5078) and
Figure 49a  Shows results from differential pelleting of crude parotid gland homogenate after three successive 15 minute centrifugation steps at 12,000g average. P1, P2 and P3 represent proportion of gamma glutamyl transpeptidase activity in pellets, from the three successive centrifugations while S3 represent gamma glutamyl transpeptidase remaining in the supernatant fraction after the final centrifugation. H represents original homogenate gamma glutamyl transpeptidase activity.
FIG 49a

% HOMOGENATE ACTIVITY

FRACTION

H O M O G E N A T E  A C T I V I T Y

P1  P2  P3  S3  H
Figure 49B - shows the distribution of marker enzymes following preparation of basolateral plasma membrane vesicles. 1) α amylase, 2) gamma glutamyl transpeptidase, 3) protein, 4) Na⁺/K⁺ATPase, 5) Acid phosphatase. Crossed hatched areas on each graph represent the percentage of each marker enzyme pelleted during the preparatory differential pelleting.
HOMOGENATE ACTIVITY

1-15 1-2 1-25

80%

1

90%

2

52%

3

195%

23%

4

DENSITY (g/ml) 20°C

FIG 49b
Homogenate Activity

Density (g/ml) 20°C

% Homogenate Activity

1.05 1.1 1.15 1.2 1.25

F1G 49b
polycarbonate tubes.

The distribution of precipitated counts was assumed to reflect the distribution of membrane bound $^{125}$I IgA along the 0.5-1.7M sucrose gradient (Fig 50) and showed a peak of radioactivity with a median density of 1.16, a smaller peak was also present with median density of 1.12. Comparison of this distribution pattern with that of the basolateral plasma membrane marker enzyme Na$^+$/K$^+$ ATPase from the same preparation show a striking similarity. Figs 49-52a. The marker enzyme shows peak activity at a relative density of 1.16 with a minor peak at 1.12. Therefore the results show that addition of $^{125}$I IgA to crude homogenate of rat parotid glands provide a suitable method for specifically labelling basolateral plasma membrane vesicles.

Component proteins of each fraction from the 0.5M-1.7M sucrose gradient were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose paper by western blotting and stained to show the presence of SC (Figure 52a), and autoradiographed to show the distribution of $^{125}$I labelled IgA (Fig 52b & 53). The results show that the distribution of $^{125}$I IgA along the gradient is matched by a similar distribution of secretory component.

With respect to the apparent molecular weight of SC and IgA, it is apparent that, where most prevalent, SC occurs in three different molecular weight forms (63,000, 73,000 and 150,000). The higher molecular weight form of SC would be consistent with the large membrane-associated form of SC possibly following mild degradation in vitro. The appearance of IgA observed by the distribution of $^{125}$I-label showed two differing protein bands with molecular weights of
FIG 50 Shows the distribution of total counts (—) along a 0.5×1.7M sucrose gradient following binding of I^125 IgA to crude parotid gland homogenate in vivo. Broken line (---) represents counts shown by centrifugation to be membrane bound, see section 3.7f.
Figure 51 - Shows results from in vivo labelling of apical surfaces of parotid acinar cells with the fluorescent probe S.I.T.S. prior to glandular fractionation (3:7g)
60,000 and 25,000 corresponding to heavy and light chains of IgA.

3:7g  **A Non- Enzymic Method For the Labelling of Apical Plasma Membrane Sheets**

The interlobular ducts of the rat parotid gland were perfused in a retrograde manner with a solution of the non-specific fluorescent protein stain S.I.T.S as described in Section 2:1b. As only the apical surface of acinar cells are exposed within the lumen of secretory ducts only these surfaces should be coated by the protein stain, therefore providing a morphological label for apical plasma membrane sheets.

Glands were fractionated as described in section 2:2d and the distribution of S.I.T.S label along the 0.5-1.7M sucrose gradient observed by fluorimetry. The results show a peak of activity with a median density of 1.163 (Fig 5t) which reflects the distribution of the apical plasma membrane marker enzyme γGTase shown in Fig 4$. The results therefore show that the marker enzyme γGTase is specific for apical plasma membrane in rat parotid gland and also it is possible to label these portions of acinar cells by retrograde perfusion.

3:7h  **Distribution of SC and IgA Following Preparation of Baso-lateral Plasma Membrane Vesicles**

Crude parotid gland homogenate was incubated at room temperature with $^{14}IgA as described in Section 3:7f. Vesicles were prepared as described in Section 2:2c. Fractions were then collected from the 0.3-1.7M sucrose gradient and the component proteins in each fraction were separated by SDS polyacrylamide gel electrophoresis, transferred to nitro cellulose paper by western blotting and stained to show the
presence of SC (Fig 52a. The western blot was then autoradiographed and the distribution of $^{59m}IgA$ binding in fractions was observed. The results clearly show that a co-distribution of SC and IgA in fractions along the sucrose gradient with a minor peak occurring with a median density of 1.12 and a second more intense distribution with a median density of 1.16 which also showed a direct comparison to the Na$^+$/K$^+$ ATPase distribution observed using the same fractionation procedure (See Section 3:7f).

3:7i Analysis of Protein Composition of Secretion Granule Fraction

The component proteins of a secretion granule fraction obtained as described in Section 2:2e were separated by SDS polyacrylamide gel electrophoresis, transferred onto nitro cellulose paper and stained to show the presence of IgA or secretory component.

Two separate primary antibodies were used to identify IgA, firstly a commercial anti (rat $\alpha$-chain) from Bioyeda revealed one intensely stained protein band (mwt 63,000) plus five faintly staining proteins with molecular weights of 77,500, 60,000, 50,000, 48,000 and 28,000 respectively (Fig 54, Track A) In contrast anti (rat $\alpha$-chain) obtained by the method of Orlans et al (1978) produced a far more specific result, staining only two proteins with molecular weight of 61,000 and 28,000 (Fig 54 Track B).

Secretory component was also identified using a primary anti (rat secretory component) antibody raised by method of Orlans et al (1979). The results show one strongly stained protein (Molecular weight 77,650) plus four additional weekly stained proteins with molecular weights of 63,000, 50,000, 31,000 and 28,000 (Fig 54, Track C).
Figure 52 - Shows a diagramatical representation of the relative distribution of (a) SC (b) IgA and (c) Na⁺/K⁺ ATPase along a 0.5M - 1.7M sucrose gradient following preparation of basolateral plasma membrane fractions.
Figure 53 - Shows the distribution of $^{125}\text{I}$ IgA associated with membrane fractions observed following preparations of basolateral plasma membrane fractions.
Figure 54 - Shows the occurrence of SC in IgA in preparations of purified secretory granules. Lane A shows protein bands visualised using a primary antibody to rat (α chain). Lane B shows protein bands visualised using a primary antibody to rat (α chain) raised according to the method of Orlands 1979. Lane C shows proteins visualised using a primary antibody to rat SC raised according to the method of Orlands 1978.
Assessment of Basolateral Plasma Membrane Contamination of Secretion Granule Fractions

Basolateral plasma membrane vesicles were labelled by addition of $^{125}$I IgA to homogenised rat parotid gland and the distribution of counts observed following preparation of a secretion granule fractions as described in Section 2:2e. The efficiency of this method for the labelling of basolateral plasma membrane vesicles is discussed in Section 2:2c.

The relative distribution of the secretion granule marker enzyme - amylase was measured to assess the efficiency of the preparation and was found to be comparable to previously published results by other workers (Arvan and Castle, 1982). However, the results from this experiment also show that basolateral plasma membrane vesicles are present in the secretion granule fraction. In addition the increase in radioactivity expressed as a function of protein content above that observed in homogenate suggest that basolateral plasma membrane vesicles are not successfully separated from secretion granules by this method of fractionation.
4. DISCUSSION

The purpose of the current investigation was two fold, firstly to characterise the way in which IgA is transported into saliva and secondly to investigate how the process of IgA transport is integrated with the synthesis and secretion of other salivary proteins.

4:1 Transport of IgA into Saliva

Initial investigation of IgA transport into rat saliva was carried out by Hurliman et al (1970) who showed that IgA secreted by submaxillary glands in vitro was antigenically distinct from rat serum IgA and was therefore derived at least in part from an endogenous source within the glands. Subsequent attempts to locate the site of IgA transport into saliva were performed in fixed human tissues and indicated that duct lining cells were involved in IgA transport into saliva. Retrospectively it seems clear that the true location of SC, the receptor for IgA, was masked in these experiments by the reduced antigenicity observed following fixation with almost all standard histological fixatives (Brandtzaeg 1985). For this reason cryosections were used in the present investigation to show the distribution of SC and IgA in rat saliva glands (Section 3.2).

Parotid and submaxillary glands stained, using primary antibodies to either rat SC or α chain, showed intense staining of all parotid acinar cells, certain submaxillary acinar cells and secretion within ducts. In addition, IgA was observed within small plasma cells present within the interstitium of each gland. However, in direct contrast to initial reports, (Kraus and Mestecky, 1971) no staining for SC or IgA
was observed in the duct lining cells of either submandibular or parotid glands.

It is therefore clear that the serous acinar cells of rat salivary glands contain secretory component and facilitate the transport of locally produced IgA through the epithelium into saliva. Subsequent work (Nakamuta et al, 1985) has shown a comparable distribution of S<sup>C</sup> and IgA in human submandibular glands.

Having characterised the cells involved in IgA transport it was possible to investigate the subcellular events responsible in the process. Mostov et al (1983) observed that in rabbit hepatocytes, secretory component was synthesised by cells involved in IgA transport on a large trans-membrane glycoprotein (M SC) which is transported to the sinusoidal (basolateral) plasma membrane to collect polymeric IgA (pIgA) from serum. The receptor ligand complex (i.e. SC/pIgA) was then endocytosed, transported to the bile canalicular surface (apical plasma membrane) and released by proteolytic cleavage of secretory component producing a low molecular weight fraction of SC still associated with pIgA, referred to as secretory IgA (S<sub>IgA</sub>). In order to determine if a comparable sequence of events was responsible for IgA transport through acinar cells, the molecular weight of salivary and membrane associated SC were investigated.

4:1a **Determination of the Molecular Weight of Salivary SC Components**

Following separation of salivary proteins by polyacrylamide gel electrophoresis under non-denaturing conditions, only one form of SC was observed with a molecular weight of 63,000 (Section 3:4a). As the molecular weight of dimeric IgA is approximately 300,000 and it is
known from previous experiments that the antiserum can recognise SC bound to IgA, the 63,000 mw band must be free SC (Peppard et al, 1984). Hence the considerable amounts of IgA (molecular weight > 200,000) were present in the secretion. Therefore either, pilocarpine-induced salivation allows considerable leakage of pIgA through tight junction complexes between neighbouring cells, or sIgA is labile in saliva and a 63,000 molecular weight portion of SC dissociates from pIgA after secretion. With respect to the latter suggestion it is known that covalent binding is not essential for the transfer of IgA into bile (Peppard, J. personal communications).

The number of serum proteins present in pilocarpine induced saliva was determined by crossed immunoelectrophoresis against anti(rat serum) and used as an assessment of protein leakage through tight junction complexes. The results show that ten out of twenty one salivary proteins also cross-react with anti(rat serum), although most of the proteins exhibited high electrophoretic mobility indicating a relatively low molecular weight (Section 3.4). Also structural examination of parotid acinar cells, after inducing salivation with pilocarpine, showed tight junction complexes between neighbouring cells were apparently intact and would thus present a considerable barrier to leakage of high molecular weight proteins such as pIgA, while still allowing low molecular weight proteins to pass through (Section 3.3).

Returning to the possibility that SC is labile in saliva, several groups have reported difficulties in detecting sIgA in rat saliva (Nash et al, 1969; Bistany and Tomasi, 1970). Moreover, Cambier et al 1976 identified a low molecular weight form of SC in rat saliva which showed
a high tendency to dissociate from pIgA. Hence there is considerable evidence that sIgA is labile in saliva. However, in contrast sIgA was detected in secretions from parotid acinar cells in vitro (present investigation, Section 3.6f) and in secretions from submaxillary glands in vitro (Hurliman et al, 1971). Therefore it would appear that sIgA is stable in the artificial environment of culture medium but may dissociate in saliva. Although rat saliva is incompletely characterised with regard to enzyme content, no protease was detected in human saliva (Wallach 1982) as such. It is, however, possible that cleavage of SC from sIgA could result as a consequence of bacterial action within the oral cavity and collected samples of saliva.
4:1b Determination of the Molecular Weight of Cellular SC

Due to the universal distributions of Sc in the parotid acinar cells this tissue was selected for the purpose of determining the molecular weight of cellular SC.

The component proteins of a crude microsomal preparation of parotid gland were separated by SDS polyacrylamide gel electrophoresis and the distribution of SC was detected by western blotting (Section 3.4a). Three major forms of SC were observed with molecular weights of >200,000, 160,000 and 73000 respectively. In order to determine which molecular weight species were present on the basolateral plasma membrane, it was necessary to first achieve adequate resolution of cellular components following homogenisation of rat parotid glands.

4:1c Fractionation of Rat Parotid Glands

Previous experiments performed on fractionation of rat parotid gland (Arvan and Castle (1982) and (1983)) relied heavily on separation of component fractions by repeated pelleting and resuspension of material. A preliminary experiment (Section 3:7a) showed that enforced contact of component fractions which occurs during pelleting produced considerable aggregation of secretion granules and poor resolution between plasma membrane from different domains. As current investigation was concerned with IgA transport through cells and the possible existence of a secretory pathway independent of secretion granules. It was thought to be of primary importance to achieve good separation of basolateral from apical plasma membrane and also from secretion granules. For this reason new cell fractionation techniques
were developed which avoided where possible enforced contact between homogenate components.

It is apparent from the results presented in section 3.7e that fractions of basolateral plasma membrane obtained by the new experimental strategy are relatively uncontaminated by apical plasma membrane and secretion granule fractions (Section 3.7e). With respect to isolation of apical plasma membrane fractions, initial attempts to separate apical and basolateral plasma membrane fractions by flotation methods alone (Section 3.7c) were unsuccessful. Subsequent isopycnic centrifugation of crude apical plasma membrane fractions showed codistribution of basolateral plasma membrane marker Na/K ATPase and the apical plasma membrane marker \( \gamma \) GT. Therefore the procedure was subsequently modified by substituting the final isopycnic separation of fraction by a rate centrifuge step which did provide effective separation of both plasma membrane domains and secretion granules (Section 3.7d). It is important to note however that the distribution of secretion granules in centrifugation may be variable. These structures are not uniform in size or shape and show variable morphology and tendency to aggregate, depending upon the relative state of secretory activity of the gland (Wallach, 1982). Finally the use of continuous sucrose gradient in the current investigation offers a considerable advantage over previous methods of fractionation in which a single fraction or pellet were obtained. The use of continuous gradients allows the distribution of each organelle to be considered when interpreting organelle cross contamination.

The component proteins present in fractions from the 0.3-1.7M sucrose gradient used in preparation of basolateral plasma membrane
were separated by SDS-polyacrylamide gel electrophoresis and stained following western blotting to show the distribution of secretory component along the gradient. The results show that secretory component with a molecular weight >100,000 was present in fractions with a median density of 1.16 showing a distribution pattern directly comparable to that of the basolateral plasma membrane marker enzymes Na/K ATPase (Section 3.7a). Secretory component of this molecular weight was also observed in fractions with a lower median density of 1.12. Although the identification of these low density fractions in unknown, other workers have presented evidence to suggest that this is the median density of Golgi apparatus (Beaufay et al., 1974; Mullock et al., 1980) in liver.

Western blots stained to show the distribution of secretory component were also autoradiographed to show the distribution of $\text{I}^{125}$ IgA bound to fractions in vitro. The results show a bimodal distribution of $\text{I}^{125}$ IgA which corresponds directly to the observed distribution of secretory component (Section 3.7i). These results differ from the results published for liver (Mullock et al., 1980) where only plasma membrane fractions bound $\text{I}^{125}$ IgA in vitro. There are two possible explanations for the discrepancies observed between parotid glands and liver.

Firstly, following previously reported observations in liver (Sips et al., 1982) the assumption is made that organelles and membrane systems broken during homogenisation reform with the correct in vivo orientation, as such only plasma membrane fractions should possess SC of the correct orientation to bind IgA. In practice, however,
corresponding organelles of different tissues may exhibit different tendencies to reform into tight vesicles of the correct orientation following fractionation. From the present results it would appear that fractions with a median density of 1.12 in parotid glands are either derived from Golgi membrane fractions which possess SC but are not as well sealed as equivalent fractions in liver, or alternatively these fractions represent a sub-population of plasma membrane fraction. The latter suggestion is unlikely as under isopycnic conditions plasma membrane of any size would be expected to have a relative density of approximately 1.16 (Section 3:7b,c).

Analysis of the distribution of precipitable counts along the 0.3-1.7M sucrose gradient with the distribution of SC as indicated on the blot shows clearly that basolateral plasma membrane vesicles with a median density of 1.16 possess a higher affinity for pIgA in vivo than fractions with a median density of 1.12 (Section 3:7f). This would be consistent with large quantities of IgA binding to plasma membrane vesicles with the correct 'in vivo' orientation and less IgA binding to some 'leaky' Golgi vesicles with a median density of 1.12. Therefore these results indicate that molecular weight forms of SC (>100,000) are present with basolateral plasma membrane and possibly Golgi apparatus.

4:1d Is Secretory Component Cleaved to Produce sIgA Prior to Exposure on the Apical Surface of Parotid Acinar Cells.

In addition to the high molecular weight form of SC observed in membrane preparations a further lower molecular weight (73,000) form of SC was also observed in crude microsomal preparations and in preparations of secretion granules, where it was present with IgA
The molecular weight of SC in these preparations differs from that observed in basolateral plasma membranes (Section 3:7h), which have been shown to contaminate secretion granular fractions (Section 3:7j). Therefore the evidence is consistent with the Sc not deriving from contaminating basolateral plasma membrane, rather SC and IgA are contained within some membrane structure while the low molecular weight of SC suggests that cleavage of the large SC has occurred producing sIgA. Although the low molecular weight form of SC is present in secretion granule preparation, it is not possible to conclude that Sc or IgA is actually present within the granules. Indeed the in vivo distribution of Sc and IgA observed in human submaxillary serous acinar cells (Nakamura, 1985) show clearly that neither Sc or IgA is present in secretion granules. With the reservation that secretory pathways may not be directly comparable between species and that western blotting techniques used in the current investigation are undoubtedly more sensitive than immuno gold localisation it would nevertheless seem probable that Sc and IgA observed in secretion granule fractions are artificial. Morphological observations of the apical domain of parotid acinar cells (Fig 55) show that secretion granules are surrounded by numerous vesicles comparable in size and location to the SC containing vesicles observed in human submandibular glands (Nakamura, 1985). It is possible that these structures contain sIgA and being in such close proximity to secretion granules in vivo may aggregate with secretion granules during cell fractionation.

To summarise the information obtained from analysis of salivary and cellular secretory component is consistent with the following
Figure 55 - Parotid acinar cells observed in vivo showing the occurrence of small vesicle structures close to secretion granules in the apical domain of cells. See arrows.
speculative sequence of events. Secretory component is synthesised by parotid acinar cells as a large membrane associated protein with a molecular weight of about 200,000 which is present in basolateral and possibly Golgi fractions. This protein would then be equivalent to the membrane secretory component (mSC) described by Mostov et al. (1983). After binding IgA on the basolateral plasma membrane the SC/IgA complex is subsequently internalised and the SC molecule is cleaved at some stage during transcytosis producing sIgA possessing a 73,000 mwt portion of SC. Following secretion of sIgA into saliva the SC fraction appears to be labile and a 60-63,000 mwt fragment may dissociate or be cleaved from sIgA.

4.1e Kinetics of IgA Transport

In order to investigate the kinetics of IgA transport through acinar cells it is necessary to bind labelled IgA to the cells and monitor secretion at repeated time intervals, after establishing a zero time point which IgA is bound but not transported. Despite several attempts these basic experimental criteria could not be satisfied by an in vivo method. Firstly, repeated stimulation and collection of pure parotid gland secretions is very difficult in practice and secondly, as salivary IgA is normally derived from localised plasma cells, it is hard to effectively 'load' the cells with a labelled form of IgA.

Attempts to introduce $^{125}$I IgA into parotid glands by intravenous injection showed only very low levels of $^{125}$I IgA were incorporated into the gland (Section 3:5). The problem of 'loading' parotid glands with labelled IgA in vivo was finally overcome by using hybridoma cells which produced IgA specific for horse radish peroxidase (Section 3:5).
Hybridoma cells were grown in the peritonium of rats over a nine to ten day period after which considerable levels of this specific form of IgA could be detected in serum and also in parotid acinar cells and saliva. However, the exact route by which the horse-radish peroxidase specific IgA reached the parotid glands is not clear. Certainly there were high levels in serum which may have passed into the salivary gland but in addition, there were also cells in lymph nodes near the parotid glands which stained specifically for horse radish peroxidase. Therefore it must be considered possible that hybridoma cells may have migrated from the peritoneum to peripheral sites, thus producing a local source of labelled IgA.

Although successful in demonstrating transcytosis of labelled IgA in vivo, the hybridoma cell system could not be used to investigate the kinetics of IgA transport as no accurate zero time point at which IgA bound to cells could be established. It was therefore, necessary to develop an in vitro system of parotid acinar cells which would allow an accurate zero time point to be established by binding IgA at 4°C and initiate transport when required by shifting to 37°C. Also monitoring of secretion was simply achieved by changing the incubation medium when required.

Using an in vitro system however, it was possible that cell polarity which is crucial for directional transport pathways might have been lost when cells were isolated. For this reason a method of isolating 'clumped' acinar cells was developed in which cells still maintained some association with other cells and incubations periods were limited to 2 hrs at 37°C (Section 3:6). Under these experimental constraints cell polarity was clearly seen to be maintained by electron
microscopy. Also binding of IgA to cells in vitro (Section 3:6c) shows that specific apical and basolateral plasma membrane domains are preserved of a 2 hr incubation at 37°C.

The results obtained using the in vitro acinar cell preparation shows that the majority of 125I IgA bound at 4°C which remained following three consecutive washes in fresh medium was released from cells following a temperature shift to 37°C. However, as IgA was detected in the incubation medium as sIgA (Section 3:6f) it would seem probable that bound IgA is internalised, transcytosed and secreted as in vivo. Also a study of the rate of 125I IgA secretion showed secretion of 125I IgA within thirty minutes of initiating protein transport at 37°C is consistent with previous reports of SC movement within cells in liver (Mullock et al 1980; Hubbard et al 1985), gut (Brown et al 1979), mammary gland cells in vitro (Solari et al 1985) and induced SC, expressed in MDCK cells (Mostov et al 1986). The observed decrease (Section 3:6b) in rats of IgA secretion following the initial 30-60 minute period of incubation at 37°C may be explained as follows.

As secretory component moves into coated pit regions of the plasma membrane even in the absence of ligand (Mullock et al, 1980) it is possible that some 125I IgA will bind to vacant receptors in and around coated pits. Therefore, IgA bound to these receptors would be rapidly internalised when protein transport is initiated at 37°C. In contrast, IgA bound to Sc, which has recently appeared on the plasma membrane will first need to move into coated pit regions of the cell before being internalised. If the movement of receptors into coated pits is
the rate limiting step in transcytosis this would explain the observed
decrease in rate of secretion (Section 3:6b).

4:2 **Integration of Two Secretory Processes**

The universal distribution of SC and IgA, in parotid acinar cells
(Section 3:2), shows quite clearly that IgA is transported through
cells which are also responsible for bulk secretion of other salivary
protein. The integration of the two processes within the same cell
raises the possibility that secretory proteins are specifically
segeregated during exocytosis.

Presumably both secretory component and other salivary proteins
are synthesised within the serous acinar cells and therefore share a
common pathway through the rough endoplasmic reticulum and Golgi
apparatus. Most secretory proteins are then packaged into secretion
granules and transported to the apical domain of the cell, before
secretion through the apical plasma membrane (Wallach 1982). However,
results from fractionation of parotid glands (Section 3:7h) show
clearly that secretory component travels to the basolateral plasma
membrane to collect IgA from the interstitium fluid. It is highly
improbable that one complex secretory pathway could facilitate both
processes, indeed there is strong morphological evidence to the
contrary. Firstly, secretion granules are rarely seen in the basal
region of the cell (Section 3:3) and therefore are unlikely to be
responsible for delivering SC to the basolateral plasma membrane.
Secondly, IgA did not bind to the apical surface of acinar cells in
vitro (Section 3:6c) showing that no SC was exposed on this area of the
plasma membrane. Consequently it is unlikely that SC first travels to
the apical plasma membrane within secretion granules and then to the basolateral plasma membrane as this would probably involve at least transient exposure to the apical surface. Therefore it is clear that movement of secretory component to the basolateral plasma membrane is independent of secretion granules.

Although analysis of secretion granular preparations from parotid gland homogenate shows that SC and IgA are both present (Section 3:7f) it would appear on balance that the two pathways do not converge prior to secretion and this would certainly appear to be the case in human salivary glands (Nakamura et al., 1985. However, further work is necessary to establish this point conclusively, possibly involving ultrastructural immunolocalisation of SC and IgA in rat parotid glands.

Evidence consistent with the idea of two distinct secretory pathways in serous acinar cells was obtained by monitoring the basal release of proteins from parotid acinar cells in vitro. Proteins released from acinar cells into the incubation medium were separated by native polyacrylamide gel electrophoresis revealing eleven proteins of which nine were specifically recognised by antibodies to rat whole saliva following western blotting (Section 3:6d). These results are consistent with previously observed numbers of proteins Sc by parotid glands in vivo (Abe and Dawes, 1978) and therefore suggests proteins are released by normal secretory processes and not simply by cell disruption.

The rates at which individual salivary proteins were released over a 2hr incubation period at 37°C were analysed by crossed immunoelectrophoresis against anti-rat whole saliva (Section 3:6e) and showed
two distinct trends. Proteins possessing a high electrophoretic mobility showed a comparable increase in rate of secretion during successive thirty minute periods of incubation. In contrast, proteins showing a low electrophoric mobility were released at a constant rate throughout. The differential rates of protein release do not strictly confirm the existence of two distinct secretory pathways as it is possible that different proteins sharing a common secretory pathway may not be released at an equal or constant rate. However in the light of morphological evidence to support the existence of two separate secretory pathways (Sections 3:3 and 3:6c) it is assumed that secretory proteins in parotid acinar cells are selectively segregated following synthesis into two distinct transport pathways, are involving 'bulk' secretion of salivary protein, mediated by secretion granules and a second pathway facilitating IgA transport, mediated by secretory component.

The differential rates at which salivary proteins are secreted from acinar cells in vivo (Section 3:6e) are difficult to interpret conclusively. Varying rates of protein secretion in general could either be indicative of two distinct secretory pathways or varying rates of protein synthesis along one common secretory pathway. However, the almost exponential rate of secretion observed for certain proteins from unstimulated cells in vitro was unexpected. It is possible that such a pattern of protein release could arise by two means, either by cell disruption which increases throughout the 2 hour incubation at 37°C or by cell recovery. Firstly, although cell viability is maintained in excess of 87% throughout it is possible that release of all secretion granules from these cells could produce an
artificial increase in observed secretion, especially as secretion under in vivo conditions only result in release of some not all secretory granules. Two points against this argument will be that release of salivary proteins would require the action of lysosomal enzymes and if this were the case a noticeable increase in total numbers of proteins in the incubation medium would be expected and this was not the case. Also on the basis that all cellular contents would be released, some increase in all secretory proteins may be expected, this also did not apply. Alternatively, considering the idea of "recovery period" it is a common observation in tissue culture techniques that cells may undergo a recovery period following isolation. If this is also true for acinar cells gradual return to a normal constitutive rate of secretion may produce the observed pattern. Also proteins normally secreted in lower proportions would achieve a constant rate of release far sooner than other proteins which are secreted in higher concentrations. Both of these postulates are consistent with the observed rate of protein secretion. However, the basis of present data is impossible to say which is a more accurate account of the observed protein release.
5. Conclusions

1. Polymeric IgA is transported into saliva by secretory component which is present in the serous acinar cells of the submandibular and parotid glands.

2. Secretory component is initially synthesised as a large transmembrane protein (mSC-mwt > 160000) which travels to the basolateral plasma membrane to collect pIgA and is then endocytosed as a mSC/IgA complex which is transported to the apical domain of the cell.

3. The mSC portion of the mSC/IgA complex is cleaved internally prior to secretion produced a 73,000 mwt fraction of SC attached to pIgA (sIgA).

4. The sIgA is labile in saliva being degraded to produce a 63,000 mwt form of salivary SC.

5. Transcytosis of IgA through parotid acinar cells in vitro occurs within thirty minutes when incubated at 37°C. Also the decreasing rate of IgA transcytosis observed on prolonged incubation could be due to a rate limiting step involving movement of receptor ligand complex into coated pit regions of the plasma membrane prior to transport.

6. In rat parotid acinar cells secretory proteins are selectively segregated following synthesis into two distinct pathways which show different rates of secretion and may represent the pathways of bulk protein secretion mediated by the secretion granules and IgA transport mediated by secretory components.
7. The in vitro system developed to observe the sorting of salivary proteins offers a rare and convenient experimental model in which sorting mechanisms of endogenous proteins could be further characterised.
6. CONSIDERATION OF FUTURE WORK

Immediate extension of reported work should include a more accurate determination of the rate of IgA transcytosis in vitro using several time points prior to the thirty minute incubation reported. It would also be pertinent to establish if free secretory component was released into incubation medium from cells which had not been previously loaded with IgA at 4°C. With regard to more long-term investigation of IgA transport it would be necessary to determine any endosomal involvement in IgA transcytosis through parotid acinar cells. Use of the in vitro acinar cell system would enable the use of imposed temperature restriction, thus an 18°C block, previously described by Hubbard, 1985 may restrict movement of internalised receptors/receptor ligand complexes following entry into an endosomal compartment. Thus, loading of surface SC with IgA specific for horse radish peroxidase (HRP-IgA) at 4°C, followed by an incubation at 18°C could result in an accumulation of HRP-IgA within an endosomal compartment thus providing a morphological marker for this cellular compartment which could then be either visualised by electron microscopy or isolated possibly by the method of Mullock et al., 1986.

Definitive characterisation of diverging secretory pathways in rat parotid acinar cells is totally dependent upon ultrastructural localisation of SC and salivary proteins such as α amylase. Due to the labile nature of SC during tissue fixation this work is also dependent upon the availability of an ultracryostat to prepare sections for electron microscopic examination. Nevertheless it will be essential to establish whether SC leaves the Golgi or post Golgi apparatus in transport vesicles independently of other salivary proteins.
Availability of ultracyroelectro microscopy would also resolve the question of whether Sc or sIgA are present in secretion granules or transport vesicles in the apical domain of the cell.
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