ELECTROPHORETIC ANALYSIS OF HUMAN PAROTID SALIVARY PROTEINS WITH APPLICATION TO THE STUDY OF RHEUMATOID ARTHRITIS AND SJÖGREN'S SYNDROME

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by

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To my parents and sister

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"He had been eight years upon a project for extracting sunbeams out of cucumbers, which were put into vials hermetically sealed, and let out to warm the air in raw, inclement summers."
(Swift, Voyage to Laputa)

In the course of attempting to extract sunbeams out of cucumbers (or saliva), I have accumulated debts of gratitude. Accordingly, I wish to thank the following persons or committees:

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ABBREVIATIONS

APAAP: alkaline phosphatase/anti-alkaline phosphatase

APS: ammonium persulphate

AUFS: absorbance units full scale

BCA: bicinchoninic acid

BCIP: 5-bromo-4-chloro-3-indolyl phosphate

CAPS: 3-(cyclohexylamino)-1-propane sulphonic acid

CBB: Coomassie Brilliant Blue

ConA: Concanavalin A

DTT: dithiothreitol

EDTA: sodium ethylenediaminetetraacetate

Endo F: Endo-β-N-acetylglucosaminidase F

FCA: Freund's Complete Adjuvant

FIA: Freund's Incomplete Adjuvant

FPLC: Fast Protein Liquid Chromatography

HPLC: High Performance Liquid Chromatography

HRP: horse radish peroxidase

HUK: human urinary kallikrein

IEF: isoelectric focusing

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IPG: immobilised pH gradient

M_r: relative molecular weight

NBT: Nitro Blue Tetrazolium

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCA: perchloric acid

pI: isoelectric point

pK: dissociation constant

PNGase F: Peptide: N-glycosidase F

PRP: proline-rich protein

PVDF: polyvinylidene difluoride

RA: rheumatoid arthritis

SDS: sodium dodecyl sulphate

sIgA: secretory IgA

SS: Sjögren's Syndrome

SLE: Systemic Lupus Erythematosus

TCA: trichloroacetic acid

TBS: tris-buffered saline

TEMED: N',N'-tetramethylethylenediamine

UV: ultra-violet

2-D: two-dimensional

1SS: primary Sjögren's Syndrome

2SS: secondary Sjögren's Syndrome

%T: polyacrylamide gel concentration defined as percentage total monomers (i.e. acrylamide + crosslinking agent, g/100ml)

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SUMMARY

Human parotid saliva contains many proteins with diverse functions. In the course of a number of diseases, especially where the normal function of the salivary gland is affected, changes may occur in the levels of certain of these. Therefore the analysis of some of these proteins may be of diagnostic significance. This study has focused on the development and refinement of electrophoretic and of protein detection techniques in order to allow the fractionation of proteins in small volumes of human saliva with the minimum of sample preparation. In order to give an example of their possible diagnostic significance, the electrophoretic techniques which were developed were applied to the fractionation and partial characterisation of the anionic salivary proteins associated with connective tissue disorders such as rheumatoid arthritis and Sjögren's Syndrome.

The saliva of patients with rheumatoid arthritis and Sjögren's Syndrome contains additional anionic proteins, which are either present in very low levels or below detection limits in the saliva of normal healthy individuals. Research into the identity of these proteins has been largely hindered by the relatively high electrolyte and low protein content of human parotid saliva, making it necessary to desalt and concentrate the saliva samples prior to carrier ampholyte-based isoelectric focusing. Desalting requires relatively large volumes (preferably > 2ml) of saliva, which may be difficult or even impossible to obtain

from diseased glands. Also, one-dimensional isoelectric focusing cannot separate these anionic proteins from the acidic proline-rich proteins of human saliva, as both groups of proteins have overlapping isoelectric points.

In this study, a hybrid carrier ampholyte-immobilised pH gradient isoelectric focusing technique was developed to analyse human salivary proteins. Immobilised pH gradients (IPG's) of 3 pH ranges were prepared: broad-range (pH 4-9) IPG was used for the general study of human salivary proteins; while 2 narrow, acidic range IPG's (pH 2.8-4.5 and pH 3.5-5.0) were used to analyse proteins of low isoelectric points, such as the anionic salivary proteins associated with rheumatoid arthritis and Sjögren's Syndrome. This method allowed the difficulties involved when conventional carrier ampholyte-based isoelectric focusing is used to be circumvented, thus making it possible to fractionate the proteins in small volumes (approximately 50μl) of human saliva without prior treatment except for centrifugation.

Parotid salivary proteins were also analysed by one-dimensional SDS-PAGE. SDS-PAGE gels were subjected to immuno- and lectin affinity-blotting in order to characterise or identify some of the protein bands. Proline-rich proteins were recognised by their characteristic pink-staining with the dye Coommassie Brilliant Blue R250, and some of the bands which were revealed have been correlated with proline-rich proteins which have been isolated and partially characterised by other research groups. SDS-PAGE failed to

reveal any obvious differences between the band patterns of normal subjects and those of patients with rheumatoid arthritis or Sjögren's Syndrome.

Two-dimensional gel electrophoresis was also carried out using hybrid carrier ampholyte-immobilised pH gradient polyacrylamide gels in the first dimension and thin-layer SDS-polyacrylamide gradient gels in the second. By means of a combination of staining and electroblotting of the two-dimensional gels onto nitrocellulose followed by probing with specific antisera, a two-dimensional map of human parotid salivary proteins, in which most of the major components have been identified, has been obtained.

These techniques were applied to the investigation of the nature of the anionic salivary proteins associated with rheumatoid arthritis and Sjögren's Syndrome. Two-dimensional electrophoresis with pH 3.5-5.0 IPG's in the first dimension followed by silver staining revealed these proteins to be heterogeneous (pI's approximately 3.65-4.75) and of a single relative molecular weight (approximately 32,000). In normal healthy controls these silver stained components were less heterogeneous (pI's approximately 3.65-4.25). Incubation with neuraminidase showed that their heterogeneity was largely due to differing contents of sialic acid in their carbohydrate side-chains.

In order to attempt to identify these proteins, the one-dimensional IPG and two-dimensional gels were electroblotted and the blots were probed with a variety of

antisera. The proteins appeared to be immunoreactive with antisera to human tissue kallikrein, a protein the level of which has often been reported to be elevated in the saliva of patients with Sjögren's Syndrome. The proteins also appeared to correspond in terms of their isoelectric point and molecular weight values with salivary kallikrein.

Immunoblotting of IPG's of pH range 3.5-5.0 and 2-D PAGE gels with IPG's of pH range 3.5-5.0 in the first dimension showed that the range of isoelectric points of the proteins which cross-reacted with antiserum to human tissue kallikrein was the same both in normal healthy controls and in the patients (approximately 3.65-4.75). The bands produced were remarkably similar to those of human urinary kallikrein. Elevated levels of kallikrein in rheumatoid arthritis and Sjögren's Syndrome patients was observed by immunoblotting and by enzyme assay.

However, wheareas silver staining of pH 3.5-5.0 IPG's revealed that the anionic proteins being studied appeared to be labile to Endo F and PNGase F digestion, immunoblotting of these gels using antiserum to kallikrein showed the presence of immunoreactive bands whose isoelectric points appeared to be unaltered by such treatment. Two possible explanations for this result have been suggested: The first is that the anionic proteins associated with rheumatoid arthritis and Sjögren's Syndrome and salivary kallikrein may be two different proteins with close isoelectric point and molecular weight values. The second is that deglycosylation

by Endo F or PNGase F is incomplete, as often is the case when attempting to deglycosylate intact native glycoproteins using endoglycosidases; thus there may have been sufficient amounts of the undigested glycoproteins to be detected by immunoblotting.

A partial purification of the anionic salivary proteins associated with rheumatoid arthritis and Sjögren's Syndrome was also obtained by Anion-Exchange High Performance Liquid Chromatography using a Mono-Q column. Separation of these proteins from the acidic proline-rich proteins was achieved despite their overlapping isoelectric points. However, the yield of anionic proteins was too low either to ascertain their purity or for further structural studies. Accordingly, their identity remains to be ascertained, although there is some evidence that indicates that they might be salivary kallikrein. If they are, it is likely that in the normal subjects, the proteins of pI's approximately 3.65-4.25, which were revealed by silver staining of 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension, are the major isozymes of salivary kallikrein, while the minor components of higher pI's (approximately 4.25-4.75) were present at levels which were too low to be revealed by silver staining. However, they were visualised by immunoblotting.

Isoelectric focusing of parotid saliva from these patients in IPG's of acidic pH range, such as pH 3.5-5.0, could be potentially useful for the diagnosis of these disorders as the presence of elevated levels of the anionic

proteins has correlated well with diagnoses of Sjögren's Syndrome (especially in its secondary form) and rheumatoid arthritis.

PUBLICATIONS

The following paper was published during the course of this study:

Khoo, K.S. and Beeley, J.A. (1990), Isoelectric focusing of human parotid salivary proteins in hybrid carrier ampholyte-immobilized pH gradient polyacrylamide gels, Electrophoresis, 11, 489-494.

The following abstracts were presented at conferences and also published during the course of this study:

Sarna, L., Shimmin, C.I.C., Khoo, K.S. and Beeley, J.A. (1988), Further studies on human parotid saliva by SDS-gel electrophoresis, J. Dent. Res., 67, 677.

Khoo, K.S., Lamey, P.-J. and Beeley, J.A. (1988), Two-dimensional electrophoresis of human salivary proteins using immobilized pH gradients, Electrophoresis, 9, 63.

Khoo, K.S. and Beeley, J.A. (1989), Parotid salivary proteins and variants as revealed by 2-D gel electrophoresis, J. Dent. Res., 68, 927.

Khoo, K.S., Lamey, P.-J. and Beeley, J.A. (in press), The salivary kallikreins associated with connective tissue disorders, J. Dent. Res.

1. INTRODUCTION

1.1. Salivary Glands

1.1.1. The Phylogeny of Salivary Glands

The movement of the early amphibians from their aquatic environment to colonise dry land necessitated considerable changes in their anatomy and physiology to allow them to adapt to their new surroundings. For example, certain tissues which could only function when moist had to come into direct contact with the atmosphere. These tissues include the oral cavity which in mammals is bathed by saliva (Dickinson et al., 1987).

The first salivary glands in vertebrates may have evolved from secretory granules which appeared in otherwise unspecialised buccal epithelial cells. This may have led to the development of specialised goblet cells. Salivary glands are absent in fishes, with the exception of some Cyclostomata. These glands are poorly developed in amphibians but are well-formed in reptiles, birds and mammals. This shows the manner in which an increase in the complexity of salivary glands is observed along the evolutionary scale (Young and van Lennep, 1978).

1.1.2. Human Salivary Glands

Salivary glands are present in all mammals. In man and higher mammals, three pairs of such glands are arranged in a bilateral fashion. The mixture of fluids produced and secreted by these glands is referred to as "saliva".

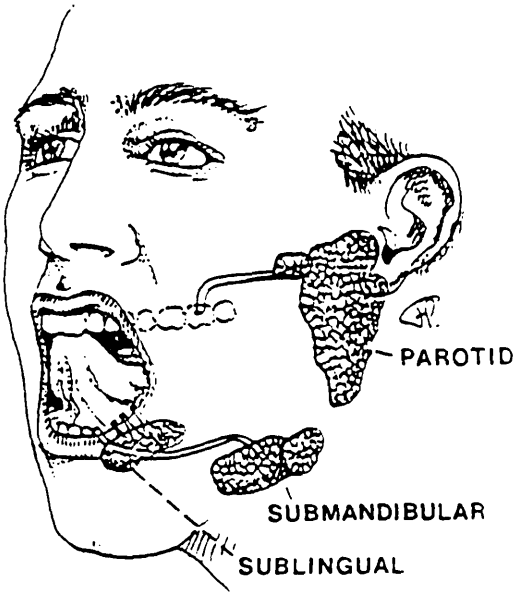
Human salivary glands consist of the following minor glands: labial, lingual, palatal and buccal; and three pairs of major glands; parotid, submandibular and sublingual (Mason and Chisholm, 1975). The locations of the major glands are shown in Fig. 1.1. Their secretions differ in composition, and the relative contributions of the secretions from each pair of glands to mixed saliva vary under different conditions. Mixed saliva may also contain crevicular fluid.

Under "resting" conditions the relative contributions of the three pairs of major salivary glands to the total volume of saliva are approximately: submaxillary, 60%; parotid, 35%; and sublingual, 2%. In acid-stimulated saliva, their relative contributions are 70%, 25% and 4% respectively. The remaining volumes are contributed by the minor salivary glands. The minor glands are widely distributed throughout the mouth but are absent from the anterior region of the hard palate. Their average contribution to whole saliva is less than 10% of the total volume (Jenkins, 1978).

Salivary gland secretion is regulated by the sympathetic and parasympathetic nerves. The main stimulus for inducing the flow of saliva comes from the combined actions of the parasympathetic system and the α-adrenergic component of the sympathetic system while the β-adrenergic component of the sympathetic system modulates the composition of saliva (Ryberg et al., 1989). The neurotransmitters released

Fig. 1.1. Locations of the major salivary glands

(Drawing kindly provided by Miss Fiona Peden)



from these nerve endings act as the first messengers of secretory response in activating the cholinergic and adrenergic receptors on the acinar cell surface. The parasympathetic nerves release acetylcholine which is detected by the muscarinic cholinergic receptors, while the sympathetic nerves release norepinephrine which is detected by α- and β-adrenergic receptors (Baum, 1987).

Activation of the β-adrenergic receptors results mainly in the exocytosis of secretory granules. The activated β-adrenergic receptor interacts with a G protein referred to as G_s, which in turn interacts with adenylate cyclase, causing an increase in the level of cyclic adenosine monophosphate (cAMP). cAMP then activates cAMP-dependent protein-kinase which leads to exocytosis. This system is opposed by the activation of the α_2-adrenergic receptor.

Activation of the α_1-adrenergic and muscarinic receptors results primarily in salivary fluid secretion (Baum, 1987; Putney, 1988). The α_1-adrenergic and muscarinic receptors activate a system which utilises inositol 1,4,5-triphosphate (IP_3), diacylglycerol and ionic calcium. The ionic calcium signal mainly regulates the secretion of electrolytes and water (Baum, 1987; Izutsu, 1989).

Salivary glands contain two types of secretory cells, serous and mucous. Serous secretions are watery and protein-rich while mucous secretions are viscous and carbohydrate-rich. Serous cells are arranged in clusters called acini which are one cell thick and have an extensive

rough endoplasmic recticulum and prominent secretory granules (Tandler, 1987). Mucous cells produce a viscous secretion consisting almost entirely of mucins, which are glycoproteins of high molecular weight (Tabak et al., 1982; De Rossi et al., 1987). Macromolecules are stored in zymogen granules after synthesis, and exocytosis is the major route of protein and glycoprotein secretion into saliva. Their secretion involves the fusion of the granule membrane with the luminal plasma membrane of the acinar cells (Dowd et al., 1983).

Salivary fluid production occurs by an osmotic mechanism in which sodium, potassium and chloride ions are transported across the acinar cell into the acinar lumen by a protein molecule located in the basolateral membrane of the acinar cell, called the $Na^+/K^+/Cl^-$ co-transporter. The movement of sodium chloride results in the formation of an osmotic gradient which draws water from the extracellular space, across the acinar cell, and finally into the acinar lumen (Martinez, 1987).

The most widely accepted model for saliva production is a two-step process (Izutsu, 1987). The first step is the production of an isotonic primary secretion by the acini of the salivary glands, and in the second step this fluid is rendered hypotonic as it flows through the ducts. The latter process involves a ductal reabsorption of sodium chloride and the secretion of potassium and bicarbonate. The sites of secretion and reabsorption of proteins and various ions

during saliva production are summarised in Figure 1.2.

1.2. Saliva

Arglebe (1981) noted that saliva had been "a stepchild of research for a long time". Until 1964, only one laboratory handbook of biochemical data (Kolbe, 1964) provided a list of substances occuring in human saliva. Since then, knowledge regarding the composition and function of saliva has advanced considerably. This reflects the increasing awareness that the chemical composition of saliva may help us to draw conclusions about pathological changes in the salivary glands and may also help to indicate the presence of disease in other parts of the body.

1.2.1. The Composition of Saliva

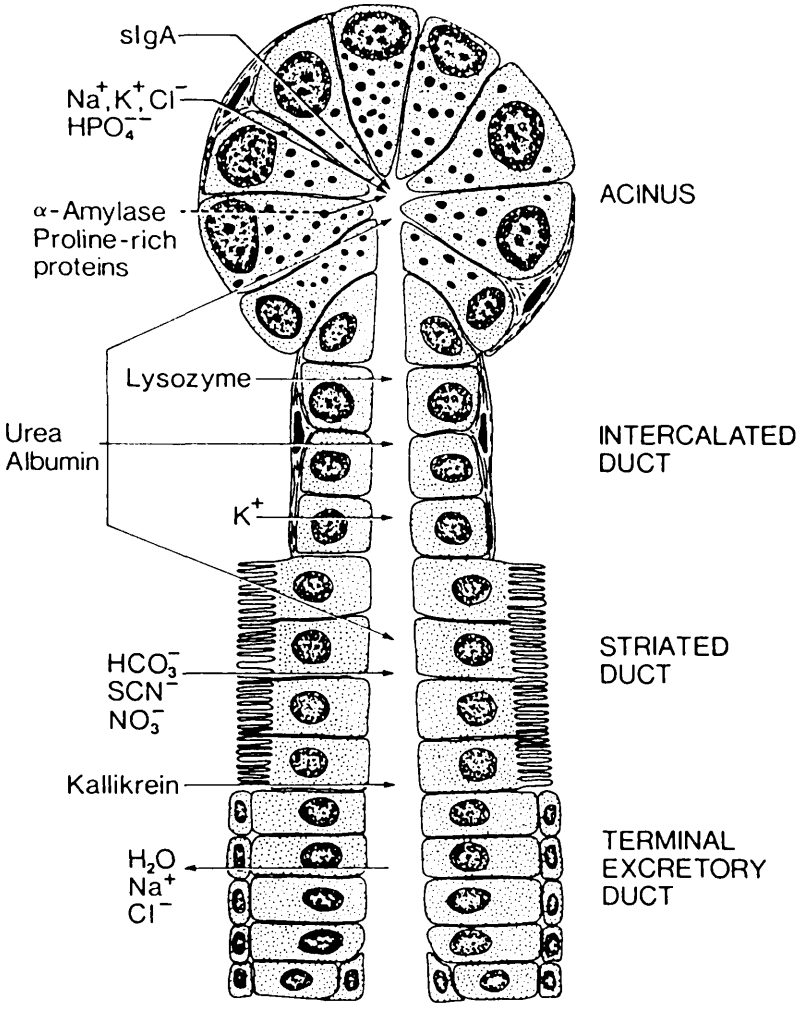
1.2.1.(i) Inorganic Constituents

The function of saliva to a large extent depends on its composition. The concentrations of the most important constituents of parotid saliva are listed in Table 1.1.

The pH and buffering capacity of saliva are largely determined by its inorganic constituents. The major monovalent cations are sodium and potassium while the major divalent cation is calcium which occurs both as free ions and in a protein-bound form in a ratio of approximately 1:1 (Seifert et al., 1986). The major anions are chloride, bicarbonate and phosphate. Salivary phosphate occurs in either the free or protein-bound forms. Bicarbonate and

Fig. 1.2. Sites of secretion within the gland of some constituents of saliva

(Diagram taken from Waterhouse et al., 1990).



The arrows show the sites at which individual salivary components are either secreted or reabsorbed.

Table 1.1. Concentrations of some constituents of parotid saliva (from Mason and Chisholm, 1975)

	Unstimulated	Stimulated
Sodium (mmol/l)	2.6 ± 2.0	54.9 ± 16.9
Potassium (mmol/l)	36.7 ± 12.5	16.0 ± 2.7
Calcium (mmol/l)	0.75 ± 0.25	0.82 ± 0.55
Magnesium (mmol/l)	0.1 ± 0.05	0.02 ± 0.13
Chloride (mmol/l)	24.8 ± 7.6	33.3 ± 13.4
Bicarbonate (mmol/l)	1.1 ± 0.1	29.5 ± 9.6
Phosphate (mmol/l) (inorganic)	3.4 ± 1.4	3.3 ± 2.6
(total)		5.6 ± 2.6
Protein (g/l)	2.35 ± 3.87	1.64 ± 0.51
α-Amylase (g/l)	1.03 ± 0.11	0.95 ± 0.15
sIgA (mg/l)		39.5 ± 13.7
IgG (mg/l)		0.36 ± 0.3
IgM (mg/l)		0.43 ± 0.36
Lysozyme (mg/l)	42 ± 9	23 ± 14
Albumin (mg/l)		2.8
Lactoferrin (mg/l)		5.4 ± 2.6
Urea (mmol/l)	3.0	4.64
Uric acid (mmol/l)	0.57 ± 0.25	0.17 ± 0.05
Glucose (μmol/l)	42 ± 38	8 ± 3

phosphate are the main buffering species in saliva and play a substantial role in the prevention of dental caries (Argus and Schamschula, 1983).

1.2.1.(ii) Organic Constituents

Most of the organic material in saliva is proteinaceous. Proteins in whole saliva may arise from the salivary glands, gingival fluid and bacteria. Most of the salivary proteins are synthesized within the epithelial cells (i.e. acini and ducts) of the salivary glands, e.g. secretory component (Tomasi and Bienenstock, 1968).

A small proportion of the protein is derived by leakage from the serum of the salivary gland capillaries. One example is albumin which is probably transudated passively from the serum into saliva (Strober et al., 1970; Brandtzaeg, 1971).

Non-proteinaceous organic compounds of low relative molecular weight (M_r) in saliva include urea and uric acid, the levels of which are related to serum levels. Very low amounts of cholesterol, free amino acids, free carbohydrates, steroid hormones and lipids are also present (Arglebe, 1981). Of these compounds, the most important for diagnostic purposes are probably the steroid and polypeptide hormones. Corticosteroids in human saliva were first examined by Shannon et al. (1959), and all the major steroid hormones have been found to be present in saliva (Ferguson, 1984). Polypeptide hormones also occur in saliva. An example

is epidermal growth factor (EGF) which has been identified in submandibular saliva (Elder et al., 1978). EGF stimulates cell proliferation and probably has an important role in the repair of injury to the gastrointestinal mucosa.

1.2.2. Salivary Proteins

The total protein content of saliva is normally approximately 1-2 mg/ml, but this value varies considerably. The majority of these proteins belong to one or more of the following categories:

1.2.2.(i) Enzymes

(a) α-Amylase

α-Amylase (E.C. 3.2.1.1) is the major protein in saliva, constituting about 10% of the total protein in human saliva (Bernfield, 1951). It is an α-(1,4)-glucan-4-glucanohydrolase which hydrolyses starch, producing maltose, glucose and limit dextrans. The enzyme is synthesized in the acinar cells and occurs in two forms; glycosylated (M_r 63,000) and non-glycosylated (M_r 59,000). It may be separated into a series of isozymes by isoelectric focusing (Eckersall and Beeley, 1981), the pattern of which is genetically determined. Karn et al. (1973) postulated that a single gene is responsible for the production of an α-amylase polypeptide and that the multiple molecular forms

arise by post-translational deglycosylation and deamidation.

Other than starch digestion, α-amylase may play an antibacterial role as it has been demonstrated that it binds to various strains of Streptococci (Douglas, 1983).

(b) Lysozyme

Lysozyme or muramidase (N-acetylmuramide glycanohydrolase, M_r 15,000) was first discovered in saliva by Fleming (1922). It hydrolyses the β-(1,4)-glucosidic linkages between N-acetylmuraminic acid and N-acetylglucosamine in the cell wall peptidoglycans of certain gram-positive bacteria, resulting in an increase in membrane permeability or lysis. Autoaggregation of some species may also occur (Pollock et al., 1985; 1987).

(c) Lactoperoxidase

Lactoperoxidase (M_r 77,500) is a glycoprotein which is synthesized by the parotid and submandibular acinar cells. It uses the hydrogen peroxide produced by bacteria to oxidise salivary thiocyanate (SCN^-) to hypothiocyanite ($OSCN^-$) and hypothiocyanous acid ($HOSCN^-$) which are oxidising agents which react with the sulphydryl groups (-SH) in key bacterial enzymes, thus inhibiting glycolysis (Tenovuo et al., 1981).

(d) Tissue Kallikrein

Tissue kallikrein (E.C. 3.4.21.35), an acidic glycoprotein which is secreted by the striated salivary duct cells, is a trypsin-like serine protease which cleaves kininogen to form kallidin, a vasodilator kinin (Schachter,

1964; 1980; Skurk et al., 1979). The effects of kinin include vasodilatation and increased vascular permeability which alters local blood flow and pressure, and leucotaxis (Selwyn et al., 1989).

There are two separate kallikrein-kinin systems; one in plasma and the other in the exocrine glands. Plasma and glandular (or tissue) kallikreins are antigenically and enzymatically different (ϕrstavik, 1980). However, both act as kininogenases. Plasma kallikrein acts on a high M_r kininogen, a plasma α_2-globulin, releasing bradykinin (a nonapeptide). Tissue kallikrein acts on both high and low M_r kininogens, with a preference for the latter, releasing the decapeptide lysyl-bradykinin (kallidin) (MacDonald et al., 1988).

Salivary kallikrein belongs to the glandular type, and rabbit anti-human urinary kallikrein serum cross-reacts with both human urinary and salivary kallikreins (Iketika et al., 1983a). Human urinary kallikrein (HUK) has been purified by Hial et al. (1974) who established that it had a M_r of 43,600 and is microheterogeneous with pI values of 3.80, 3.95 and 4.06. Its carbohydrate moiety has been studied by Iketika et al. (1983b) who found that the heterogeneity was largely due to variation not only in the charge of the oligosaccharides (arising from differences in the degree of sialation) but also to the differing M_r's of the oligosaccharide moieties. Examples of other tissue kallikreins are pancreatic, renal and urinary kallikrein

(Schachter, 1980).

Tissue kallikreins isolated from different tissues have been shown to be closely related or even identical in the polypeptide chain to HUK, but to differ in their carbohydrate moieties (Kellermann et al., 1988). Salivary kallikrein has been isolated from human saliva by Hare and Verpoorte (1982), who reported that it had a pI of 4.0 and an M_r (determined by SDS-PAGE) of 27,000. Although its amino acid composition was obtained, it was not sequenced.

Human urinary kallikrein (HUK) has been more extensively studied than salivary kallikrein, and its complete amino acid sequence has been determined (Kellermann et al., 1988; Takahashi et al., 1988; Lu et al., 1989). HUK occurs in both inactive and active forms. The inactive form (prokallikrein) is a proenzyme with 238 amino acid residues and has an additional 7 amino acids at the amino terminus of active kallikrein. Prokallikrein is converted to active kallikrein by the cleavage of the N-terminal peptide consisting of 7 amino acids (Takahashi et al., 1986). HUK displays charge heterogeneity which results from differences in glycosylation. Kellermann et al. (1988) has identified 6 carbohydrate side chains on the polypeptide; 3 of them were N-glycosidically linked to asparagine and the other 3 were O-glycosidically linked to 2 serine and one threonine residues.

(e) Trace Enzymes

Trace enzymes of glandular origin in saliva include

glucose-6-phosphate dehydrogenase, arginase, lactate dehydrogenase, alkaline phosphatase, acid phosphatase, adenosine triphosphatase and ribonuclease (Arglebe, 1981).

1.2.2.(ii) Proline-Rich Proteins

The major group of proteins in human parotid and submandibular saliva are the proline-rich proteins (PRP's). About 75% of their amino acids consist of proline, glycine and dicarboxylic acids. They were first discovered by Oppenheim et al. (1971).

PRP's are a highly complex polymorphic system of proteins, and their synthesis is controlled by a set of genes located on chromosome 12. Differential mRNA-splicing and post-translational modification (Maeda, 1985; Maeda et al., 1985) as well as intragenic unequal crossing over (Lyons et al., 1988) may add to their complexity.

PRP's may be divided into three groups; acidic, basic and glycosylated which account for 30%, 23% and 17% of the total proteins in saliva, respectively (Bennick, 1982; 1987). Four PRP's (designated "A" - "D") have been purified by Bennick and Connell (1971). The amino acid sequence of "A" (M_r 9,900) was found to be identical to the N-terminal 106 residues of "C" (M_r 16,300), suggesting that "A" may be formed by a post-synthetic cleavage of "C" at the arginine-glycine bond in positions 106-107 (Wong and Bennick, 1980). Although prolonged incubation (72 hrs) with tissue kallik-

rein is capable of effecting this cleavage, it is unlikely that this process takes place *in vivo* because the acidic PRP's are synthesized in the acinar cells of the parotid gland while kallikrein is present only in the ductal cells. After their secretion by the acinar cells, the acidic PRP's do not remain in the salivary ducts for a sufficient length of time to be subjected to the action of kallikrein (Wong *et al.*, 1983).

9 non-glycosylated basic PRP's ("IB1"-"IB9", (M_r 6,000-12,000) have been isolated by Kauffman *et al.* (1986) and 7 ("P-C"-"P-I") of similar M_r's and structure have been reported by Saitoh *et al.* (1983). The major glycosylated PRP (M_r 38,900) is basic and contains 30% carbohydrate (Li and Levine, 1980; Levine *et al.*, 1987).

The function of the basic PRP's remains to be elucidated. The acidic PRP's have a strong affinity for calcium, suggesting a possibility that they may play a role in the maintainence of calcium concentration in saliva and in inhibiting the formation of hydroxylapatite crystals on tooth surfaces (Bennick, 1976; Braunlin *et al.*, 1986). Another possible role of the PRP's is the binding of dietary tannins (Hagerman and Butler, 1981). Tannins may prevent the digestion of proteins in tannin-rich foodstuff by precipitating them, thereby reducing their nutritional value. Tannins may also inactivate the digestive enzymes. Tannin-binding by PRP's may help to reduce the occurence of these events (Mehansho *et al.*, 1985; 1987).

1.2.2.(iii) Immunoglobulins

The major immunoglobulin in human parotid saliva is secretory Immunoglobulin A (sIgA, M_r 390,000) (Chodirker and Tomasi, 1963). sIgA consists of an IgA dimer, joined by a J chain and a secretory component (Tomasi et al., 1965). About 95% of the IgA in saliva is synthesized as dimers by the plasma cells within the salivary glands. The IgA dimer, held in the dimer configuration by a J chain, is produced by plasma cells in the insterstitial tissue spaces. Secretory component is produced as a transmembrane protein in the rough endoplasmic recticulum of secretory epithelial cells. Bradtzaeg (1985) has proposed that IgA dimers are transported through the secretory epithelium by secretory component-mediated endocytosis, thus facilitating transport of sIgA into secretions. Secretory component also protects the IgA dimer from proteolysis (Lindh, 1974).

Gingivial exudate is probably the main source of the IgG, IgA, IgE and IgM in whole saliva (Grundbacher, 1988). The IgG content of parotid saliva is approximately 0.4-4 mg/l (Brandtzaeg et al., 1970). 50% of this is transudated from the serum (Ruhwinkel and Münzel, 1975). The IgM content of parotid saliva is between 0.1-0.8 mg/l (Brandtzaeg et al., 1970). IgM is a pentamer of the basic four-chain immunoglobulin unit. Its high molecular weight is due to this and also the fact that IgM μ-chains have one additional domain compared to IgG γ-chains. The transudation of IgM

from serum into saliva is largely blocked because of its large size (Tomasi, 1972).

1.2.2.(iv) Mucins

Mucins are the main organic constituents of mucus which coats all mucosal surfaces. Salivary mucins are secreted by the mucous cells of the submandibular, sublingual and minor salivary glands. About 70% of the total mucins in whole saliva is produced by the minor glands (Tabak et al., 1982). Mucins are glycoproteins which contain 70-80% carbohydrate. The carbodydrate side-chains are O-glycosidically linked. Mucins play an important role in the none-immune protection of the oral cavity. Examples are "MG1" (M_r > 1,000,000) which is a lubricant, binds to hydroxylapaptite, and is a constituent of the pellicle; and "MG2" (M_r 200,000-250,000) which is a poorer lubricant but displays strong agglutinating properties towards oral bacteria (Levine et al., 1987; Cohen et al., 1990; Kawagishi et al., 1990).

1.2.2.(v) Other Proteins

Examples of other proteins found in human saliva are:

(a) Lactoferrin

Lactoferrin (M_r 77,000) is an iron-binding metalloglycoprotein which occurs in exocrine secretions. It has been shown to be present in parotid saliva by Masson and Heremans (1966). In its iron-free (apo-) form, it has a high affinity for ferric cations. As iron is a necessary nutrient

for many bacteria, lactoferrin-mediated iron depletion will lead to the inhibition of their growth. Lactoferrin also exerts a bacteriocidal effect by binding to bacterial cell surfaces (Lassiter et al., 1987).

(b) Statherin

Statherin (M_r 5,380) an acidic phosphoprotein, was isolated from saliva by Hay (1973). It prevents the precipitation of calcium phosphate from supersaturated solutions, thereby stabilising supersaturated saliva. It is also selectively adsorbed onto apatitic surfaces and may therefore be a precursor of the acquired enamel pellicle (Schlesinger and Hay, 1977).

(c) Histatins

Histatins are histidine-rich polypeptides of low M_r. Histatins 1, 3, and 5 which were isolated by Oppenheim et al. (1988) contain 38, 32 and 24 amino acid residues, respectively. Histatins are secreted by the parotid and submandibular glands. In addition to being anti-microbial and anti-fungal, they may also play a role in the maintenance of the enamel pellicle (Troxler et al., 1990; Vanderspek et al., 1990).

(d) Phosphopeptides

Whole saliva contains small phosphopeptides, most of which are N-terminal cleavage products from the acidic PRP's as a result of rapid degradation by proteolytic enzymes of extraglandulary sources, including bacteria and desquamated

host cells. The biological roles of these peptides include calcium binding and binding to hydroxylapatite (Minaguchi et al., 1988).

(e) Blood Group Reactive Substances

The A, B, O(H) and Lewis antigens, which are oligosaccharides found on the surface of erythrocytes are also present in the mucins of the submandibular saliva of 85% of the population, who are termed as "secretors" (Malamud, 1985).

1.2.3. The Functions of Saliva

Saliva fulfils many important roles in the oral cavity These include:

1.2.3.(i) Digestion

Saliva acts as a lubricant, softening food and enabling the formation of a food bolus prior to swallowing. The only important digestive enzyme in saliva is α-amylase which initiates the digestion of starch. As food retention in the mouth is too brief for any significant degree of digestion to occur, it is possible that its main action is digesting starch from food residues remaining in the mouth (Mandel, 1987; Cole and Eastoe, 1988).

1.2.3.(ii) Protection

Adherence to tissue surfaces is a crucial event for many bacteria. The physical flow of saliva removes a large

number of potentially harmful bacteria from teeth and mucosal surfaces which would otherwise adhere to these surfaces (Mandel, 1989).

Mucins provide lubrication, protection against dessication and environmental insult. They also have antimicrobial properties (Tabak et al., 1982; Levine et al., 1987).

Salivary glycoproteins, including sIgA, contribute to the decreased adherence of microorganisms to the teeth and oral mucosal surfaces by aggregating bacteria, which are eventually removed by swallowing (Ericson and Arwin, 1985). Hydroxylapatite-bound acidic PRP's are selective mediators of bacterial adhesion (Gibbons and Hay, 1989).

A number of salivary proteins possess either bacteriostatic or bacteriocidal properties. They include lysozyme (Pollock et al., 1985), lactoperoxidase (Adamson and Pruitt, 1981), histidine-rich polypeptides (MacKay et al., 1984a; 1984b), lactoferrin (Lassiter et al., 1987) and sIgA (Tenovuo et al., 1982).

Salivary histatins have been demonstrated to exhibit fungistatic and fungicidal activity (Pollock et al., 1984). Histatins are rich in histidine which contains imidazole groups which are capable of forming charge-transfer complexes with aromatic residues, giving rise to hydrophobic interactions which play a large part in the antibacterial and antifungal roles of the peptide (Pollock et al., 1985).

1.2.3.(iii) Maintenance of pH

The bicarbonate and phosphate in saliva act as buffers which ensure that a relatively neutral pH is maintained in the oral cavity under resting conditions (Mandel, 1987).

1.2.3.(iv) Pellicle Formation

The pellicle is a selectively adsorbed coating of proteins (including albumin, lysozyme, lactoferrin, 7S IgA, acidic PRP's and mucins) and lipids on the tooth surface, where it provides a protective barrier and lubricating film (Levine et al., 1985; Mandel, 1989).

1.2.3.(v) Remineralisation of Early Carious Lesions

Salivary glycoproteins and proteins rich in calcium and phosphate form precipitates on the dental enamel which are believed to be essential for the effective remineralisation of early carious lesions (Grön and Hay, 1978). A group of phosphoproteins, including acidic PRP's and statherin, bind to calcium, helping to maintain in saliva a state of supersaturation with respect to calcium phosphate. When the plaque pH falls due to acid formation from the fermentation of carbohydrates, these proteins release calcium and phosphate ions which allow early lesions to be remineralised (Hay, 1984).

1.2.3.(vi) Other Functions

Saliva also plays a part in the control of water balance, the excretion of endogenous and extragenous

materials such as iodine, hormones, blood group reactive substances and drugs and also in the control of voice and speech (Cole and Eastoe, 1988).

1.2.4. Factors Affecting the Composition of Saliva

The composition of saliva is affected by a number of factors. In view of this, care should be taken in standardizing the conditions under which the salivary samples are collected.

Factors which may influence the composition of saliva include:

1.2.4.(i) Flow Rate

The concentrations of most of the constituents of saliva are dependent on the salivary flow rate of the individual and the duration of the stimulus applied. The concentrations of proteins, sodium, and bicarbonate ions rise with increased flow rate, while those of magnesium and phosphate fall. The concentration of potassium is little affected by the flow rate (Dawes, 1967; 1969).

At the beginning of stimulation, the protein concentration in parotid saliva falls below that found in the unstimulated secretion, but then increases with the flow rate (Dawes, 1981).

1.2.4.(ii) Circadian Rhythms

Almost all salivary components show variations which

are related to the time of day, and many apparent differences in the composition of saliva from different persons are often a result of this variation. This is called a circadian variation because the rhythms are repeated at approximately 24 hr intervals (Dawes, 1981; Ferguson, 1981).

Daily rhythms in the secretion rate and composition of stimulated parotid saliva (Ferguson et al., 1973), unstimulated parotid saliva (Dawes and Ong, 1973) and stimulated submandibular saliva (Ferguson and Botchway, 1979) have been reported. Although considerable differences between individuals were observed, any one individual showed fairly consistent results. For most of the constituents the daily rhythm showed typical sine curve patterns with well defined maxima and minima.

Unstimulated and stimulated parotid saliva show a circadian rhythm in protein concentration with the acrophase or peak value occuring in the afternoon (Dawes, 1981).

1.2.4.(iii) Diet

Dawes (1970) reviewed the literature dealing with the effects of diet on salivary composition and concluded that there is little evidence that difference in diet can exert systemic effects on salivary flow rate and composition.

Whether the activity of the salivary glands is to some extent determined by the degree of functional stimulation is uncertain. Squires (1953) reported that a diet rich in carbohydrates leads to elevated α-amylase levels. The results

of this experiment, however, could not be confirmed by Bates (1962).

When a group of men were maintained for 7 days on a diet consisting solely of the liquid diet "Metrecal", there was a marked reduction in the total protein content of the parotid gland secretion, which was attributed to a reduction in masticatory and therefore reflex-mediated secretory stimulus of the gland (Hall et al., 1967).

1.2.4.(iv) Drugs

A wide variety of drugs are capable of affecting salivary flow rate, but relatively few have been studied under controlled conditions for their effects on the composition of saliva. Most of the studies have been conducted on animals (Ferguson, 1981).

As the secretion of proteins by the salivary glands is under the control of the sympathetic nerves, sympathetic stimulation of the human parotid glands by sudden body-cooling or by the ingestion of a β-agonist such as isoprenaline causes an increase in the concentration of α-amylase in parotid saliva, while propranolol, a β-adrenergic blocker, has an opposite effect (Speirs et al., 1974).

In the rat parotid gland, it has been shown that stimulation by a β-agonist such as isoproterenol or epinephrine induced the release of secretory proteins from

the acinar cells (Kim et al., 1989) but had little effect on the oligosaccharide structure of a major N-linked parotid glycoprotein (M_r 220,000) (Baum et al., 1990).

Bromhexine, which is often used in the treatment of Sjögren's Syndrome has been shown by Manthorpe et al. (1981) to reduce the level of salivary IgM, but not secretory IgA, IgG or albumin.

Methotrexate is a cytotoxic drug with pronounced immunosupressive and chemotherapeutic effects, and has been widely used for the management of leukemia and several autoimmune diseases. It has in recent years gained an important place in the treatment of rheumatoid arthritis. It probably exerts its effect through an anti-inflammatory mechanism by inhibiting the activity of Interleukin-1 without affecting its production or secretion (Segal et al., 1989). Methotrexate has been reported to have no effect on the function of rat parotid glands (Wolff et al., 1989).

1.2.4.(v) Diseases

A number of diseases may affect the composition of saliva. For example, diseases of the salivary glands such as chronic inflammation may lead to a reduced secretion of salivary-specific substances. If the blood-saliva barrier is breached in the event of acute inflammation, serum constituents such as albumin, IgG and IgM may reach the saliva. Specific defence mechanisms in the salivary glands may also be activated, resulting in an increase in the levels, for

example, of sIgA and lysozyme.

Metabolic diseases such as cystic fibrosis and diabetes mellitus may also affect the salivary glands (Seifert et al., 1986).

1.2.5. Salivary Analysis (Sialochemistry) as a Diagnostic Tool

Alterations in the composition of saliva are known to occur not only in diseases which affect the salivary glands, but also in diseases where other bodily tissues are involved.

Examples of non-tumorous diseases of the salivary glands are sialadenitis, parotitis and Sjögren's Syndrome. Sialadenitis is characterised by increased levels of sIgA, lysozyme and protein (Eichner et al., 1977), and parotitis by decreased protein levels (Skurk et al., 1979).

Cystic fibrosis is a disease of the exocrine glands. The changes in the composition of saliva in cystic fibrosis patients include alterations in the levels of calcium and sodium (Marmar et al., 1966), protein (Allars et al., 1976) and α-amylase (Chernick et al., 1964).

HIV-1 antibody-positive patients exhibit elevated levels of salivary albumin, lysozyme, lactoferrin, sIgA and histatins, which may reflect alterations in the salivary gland function following HIV-1 infection (Atkinson et al., 1989b).

The saliva of patients with juvenile chronic arthritis has been reported to have decreased levels of calcium, phosphate, potassium, lysozyme and sIgA (Siamopoulou et al., 1989).

During pregnancy there is a decrease in the levels of salivary proteins and sialic acid, which is probably related to hormonal changes (D'Alessandro et al., 1989).

Membrane damage as a result of salivary gland tumours, may facilitate the leakage of serum proteins into saliva (Eichner, 1976).

With increasing knowledge of the properties of saliva and how its composition may be altered under different conditions, it has in recent years become more widely used for diagnostic purposes (Mandel, 1980; Ferguson, 1987; Baum, 1989). Sialochemistry has been applied to studying diseases of the salivary glands per se with no evidence of systemic involvement; for example, in acute viral or bacterial sialadenitis. It has also been applied to assist in the study of systemic diseases in which the salivary glands are involved; for example, Sjögren's Syndrome and cystic fibrosis; and in clinical situations in which salivary flow rate and chemistry are helpful in diagnosis or monitoring patient progress.

Other than the endogenous substances such as the proteins produced by the acinar cells of the salivary glands, saliva may also contain exogenous substances such as drugs and pharmaceutical agents which may be utilised for

diagnostic purposes. Examples of exogenous substances that may reach the saliva are steroid hormones (Ferguson, 1984), pharmaceutical agents such as phenytoin and phenobarbital (Seifert et al., 1986) and ethanol (McColl et al., 1979).

Although it is not yet possible to diagnose any disease on the basis of biochemical data obtained from saliva alone, nevertheless the information derived from salivary analysis may be useful in furthering the knowledge of disease pathogenesis, and may also serve as an indicator of how patients are responding to treatment.

1.3. Sjögren's Syndrome and Rheumatoid Arthritis

Sjögren'Syndrome (SS) is an autoimmune exocrinopathy which predominantly affects females, who constitute approximately 90% of all patients (Talal, 1985). The three main components of SS are keratoconjunctivitis sicca (dry eyes), xerostomia (dry mouth) and one of a number of well-defined chronic inflammatory connective tissue disorders. Primary SS occurs when keratoconjunctivitis sicca and xerostomia are present, and secondary SS occurs when another connective tissue disease is present together with either keratoconjunctivitis sicca or xerostomia, or both (Manthorpe et al., 1986). Secondary SS with RA accounts for approximately 50% of all patients with SS. Other connective tissue diseases commonly associated with SS are systemic lupus erythematosus (SLE), osteoarthritis (OA), progressive systemic sclerosis (PSS) and mixed connective tissue disease

(MCTD) (Manthorpe et al., 1981b).

The classic lesion of SS is lymphocytic and plasma cell infiltration of salivary, lachrymal and often other exocrine glands resulting in symptoms such as xerostomia and keratoconjunctivitis sicca.

The immunological features of SS include hypergammaglobulinaemia, which is a diffuse elevation of all immunoglobulin classes; the presence of multiple serum autoantibodies including antinuclear antibodies, anti-salivary duct antibodies and precipitating antibodies to tissue extracts (Alspaugh et al., 1976).

The serum of RA patients contains all of the classes of anti-gammaglobulin antibodies, called rheumatoid factors (Heimer and Levin, 1966).

Parekh et al. (1985; 1988) showed that the oligosaccharide structure of serum IgG from patients with RA differs from that of serum IgG from normal individuals in the degree of galactosylation of the outer arms. This results in a shift in the population of IgG molecules towards those in which one or more arms of the carbohydrate moieties terminate with N-acetylglucosamine.

In studying the circulating B cells of RA patients, Axford et al. (1987) found a reduction in the activity of galactosyltransferase, the enzyme which catalyses the addition of galactose to the oligosaccharide side-chains of glycoproteins. This finding helped to explain the occurence of reduced galactosylation of IgG in RA patients, and indi-

cated that the glycosylation defect that leads to reduced galactosylation of IgG is synthetic in nature, rather than due to a post-secretory cleavage.

Tomana et al. (1988) has hypothesized that this defect in glycosylation may be responsible for the induction of rheumatoid factor, possibly by exposing new antigenic determinants in the IgG molecule, or by creating lectin-like activity which results in autoaggregation.

1.3.1. Salivary Gland Dysfunction in Sjögren's Syndrome and Rheumatoid Arthritis

1.3.1.(i) Salivary Gland Morphology

SS is characterised by lymphocytic infiltration of salivary and lachrymal glands which leads to symptomatic dry eyes and mouth (Block et al., 1965). The majority of patients have benign infiltrates with minimal glandular enlargement, but a small number develop massive swelling of the salivary glands (Fox et al., 1983a; 1983b). The affected parotid glands show diffuse infiltrations of lymphocytes, histiocytes and plasma cells (Kitamura et al., 1970). Most acinar cells are collapsed and the content of their secretory granules is decreased.

The spectrum of lymphoproliferation in SS ranges from prelymphoma to malignant lymphomas (Schmid et al., 1989).The majority of lymphocytes infiltrating the salivary glands are T cells which account for more than 70%, while B cells account for 5-15% of the infiltrating cells (Fox et al.,

1982). Monoclonal antibodies to cell surface determinants, such as antibody OKT4 ,which is reactive with T helper/inducer cells, and antibody OKT8, which is reactive with T cytotoxic/supressor cells, allows recognition of T lymphocyte subsets. This revealed the predominance of OKT4-positive (T helper/inducer cells) in salivary gland and lip biopsies (Takaya et al., 1985; Fox et al., 1982). As local rheumatoid factor production occurs in the salivary glands of SS patients (Anderson et al., 1972) and rheumatoid factors have been detected in their salivary secretions (Dunne et al., 1979; Mairs and Beeley, 1985), it seems likely that the synthesis of rheumatoid factor is dependent on OKT4-positive T cells, and the infiltration of the salivary glands by T helper and B cells may lead to local autoantibody production and subsequent tissue damage by complement or antibody-dependent cytotoxicity. Large amounts of IgG and IgM are synthesized by lymphoid cells infiltrating the labial salivary glands (Talal et al., 1970; Matthews et al., 1986). The majority of IgA-producing cells were restricted to histologically normal areas of the infiltrated glands and there was no significant difference in the number of these cells in patients and controls.

Class II major histocompatability antigens, which are encoded for by the HLA region of chromosome 6 in man, have been associated with autoimmune diseases including SS (Moutsopoulos et al., 1979). The HLA-DR region of chromosome 6 encodes 2 non-identical polypeptides (α and β)

that were initially recognised on macrophages and B-cells (Unanue, 1984). Salivary gland epithelial cells of patients with SS express high levels of HLA-DR antigens (Fox et al., 1986a; Lindahl et al., 1985). HLA-DR-positive cells were seen in close proximity to the periphery of dense lymphocytic infiltrates. The HLA-DR molecules on the epithelial cells may allow them to present antigens to immune T cells, thus initiating or perpetuating the immune responses.

Freimark et al. (1989) studied the immunoglobulin and T cell antigen receptor (TCAR) gene rearrangements in DNA obtained from salivary gland lymphocytes from SS patients and reported that there were oligoclonal rearrangements of the κ,λ and TCAR β-chain genes. This suggested that the B or T cells may clonally expand in the salivary gland tissues of SS patients with pseudolymphoma.

Salivary gland abnormalities may also occur in patients with RA alone. Waterhouse and Doniach (1966) demonstrated that there was an increased prevalence of focal lymphocytic submandibular adenitis in patients with RA, and suggested that this focal lesion represented a subclinical form of SS. Janin-Mercier et al. (1982) reported the occurence of impaired salivary scintigraphy and mild lymphocytic infiltrates in RA patients and proposed that RA produced a frequent, although limited, salivary gland dysfunction. Andonopoulos et al. (1989) reported the occurence of parotid gland enlargement without severe extraglandular manifestations in 20% of RA patients studied. Abnormal scintiscans

have also been reported in patients with the autoimmune disease systemic lupus erythematosus (SLE) (Katz et al., 1980).

1.3.1.(ii) Alterations in the Composition of Saliva

By quantifying the constituents of saliva, valuable information regarding salivary gland function which may be useful in the diagnosis and study of the pathogenesis of SS may be obtained (Mandel, 1980; Stuchell et al., 1984; Baum, 1989; Thorn et al., 1989).

The levels of sodium and potassium in unstimulated saliva of SS patients were significantly elevated (Nahir et al., 1987). Upon stimulation there was a marked decrease of 40% in sodium concentration and a significant decrease in potassium concentration. By contrast,the normal response of healthy salivary glands to stimulation includes a significant increase in sodium concentration and little change in that of potassium (Shannon et al, 1974).

The levels of a number of proteins are elevated, including albumin and IgA (Stuchell et al., 1984), lactoferrin (Jezequel et al., 1989), IgG (Bluestone et al, 1972), β_2-microglobulin (Swaak et al., 1988) and kallikrein (Friberg et al., 1988).

Michalski et al., (1975) found a correlation between the concentration of salivary β_2-microglobulin and the degree of local lymphoid infiltration.

The salivary IgA of SS patients consists of 11S IgA (sIgA) and a small quantity of 7S IgA (Ben-Aryeh et al.,

1983). This shows that the elevation in IgA levels is due to an increase in local production of the immunoglobulin rather than transudation from serum, as serum IgA is composed predominantly of the 7S type.

In SS patients, the total salivary protein levels are increased (Anderson et al., 1972; Benedek-Spät et al., 1975). A possible explanation for this has been proposed by Friberg et al. (1988): during an acute stage of inflammation there might be disintegration of glandular tissue which also includes damage to striated duct cells. This leads to the release of salivary kallikrein, the level of which is elevated, not only into the normal secretion, but also into the interstitial tissues of the gland. In these tissues, kallikrein could exert its activity on its natural substrate, kininogen, with resulting liberation of the vasoactive kinin, kallidin. Kallidin then acts upon the vessels causing increased vasodilation and increased vascular permeability, leading to the transudation of plasma proteins and inflammatory cells.

Rheumatoid factor of IgA, IgG and IgM classes is also present in the saliva of patients with RA and SS (Dunne et al., 1979; Mairs and Beeley, 1985).

Increased amounts of anionic proteins have been observed in the saliva of RA and SS patients. Fischer et al. (1968) used paper electrophoresis to study the saliva of SS patients, and reported that whole and parotid saliva from patients with SS contained a greater quantity of proteins

which migrated to the anode. This finding was later confirmed by Herzberg et al. (1973) using disc electrophoresis. Chisholm et al. (1973) fractionated salivary proteins from RA and SS patients by isoelectric focusing in carrier ampholyte-containing polyacrylamide rod gels and noted the presence of proteins with low isoelectric points. Benedak-Spät et al. (1980) performed electrophoresis in polyacrylamide gels to study the band patterns of saliva from SS patients and found that the concentration of protein fractions with a low R_f (Relative Mobility) showed a considerable increase, while one or two additional protein bands with high R_f values could be detected.

Mairs and Beeley (1985) subjected parotid saliva from RA patients to isoelectric focusing in carrier ampholyte-containing polyacrylamide slab gels and found multiple protein bands of pI's 3.95-4.25, which did not cross-react with antisera to IgG, IgA or albumin.

Most recently, utilising isoelectric focusing followed by immunoblotting, Greaves et al. (1989) reported that some of these anionic salivary proteins correspond to multiple forms of tissue kallikreins and their low pI values are due to their high sialic acid content, the latter being inferred from the shift in the pI's of the proteins after incubation with neuraminidase. However, this study had the following limitations:

(1) Only the most anodal proteins (in the pH range 3.0 to 4.0) were investigated, possibly because one-dimensional

IEF gels are unable to resolve the kallikreins from the acidic PRP's which dominate the IEF pattern above pH 4.0. Thus most of the bands identified as tissue kallikreins have lower pI's than those earlier described by Mairs and Beeley (1985), which have pI's of 3.95-4.25. Urinary tissue kallikrein has been resolved by isoelectric focusing into at least 8 different pI (isoelectric point) forms (pI's approximately 3.75-4.25) by Lu et al. (1989).

(2) The study failed to show conclusively whether the sialated forms of kallikrein occur in healthy subjects who do not have high concentrations of the anionic proteins. Thus, whether the increase in the levels of tissue kallikreins is accompanied by unusual glycosylation remains questionable.

(3) The saliva samples were concentrated by lyophilisation and reconstituted with water, but they were not desalted prior to isoelectric focusing. The high levels of salts present in saliva resulted in the loss of resolution of the protein bands and distortion of the pH gradient of carrier ampholyte-based isoelectric focusing gels.

(4) No attempts were made to improve the resolution of the protein bands, nor were any further studies on their nature carried out (e.g. determination of their molecular weights).

1.3.2. Methods of Monitoring the Oral Component of Sjögren's Syndrome

Salivary gland function in SS may be monitored by several methods which vary in sensitivity and specificity.

Opinions differ concerning the value of these methods. The most commonly used criteria for evaluating salivary gland function in SS are:

1.3.2.(i) Biopsy

The presence of focal lymphocytic infiltration in the labial salivary glands of SS patients is well documented (Chisholm and Mason, 1973; Daniels et al., 1975). The quantitation of the infiltrate has been largely made by the method of Chisholm and Mason (1968) or the "Focus-Score" method (Daniels et al., 1975; Daniels, 1984).

The clinical usefulness of biopsy techniques is limited by their lack of specificity. For example, Hedfors and Lindahl (1989) demonstrated the presence of lymphocytic infiltration in the labial salivary glands of patients who had undergone bone marrow transplantation, patients with myaesthenia gravis (an organ-specific autoimmune disease) and also patients with various connective tissue disorders.

A further weakness of this method is that labial salivary gland biopsy is carried out on the assumption that it also reflects changes in the major salivary glands (Schiødt and Thorn, 1989). A study by Marx et al. (1988) indicated that parotid gland biopsy has a higher sensitivity for SS than labial gland biopsy. Biopsy of the parotid glands is considered to carry more risk than labial gland biopsy (Wise et al., 1988).

1.3.2.(ii) Parotid Sialography

In this method, a water-soluble contrast medium is injected into the ducts of the parotid gland after the catheterisation of the Stensen's duct (Vitali et al., 1988). Following this, radiographs are taken. The disadvantage of this technique include the difficulty involved in cannulating the Stensen's duct. The procedure is painful and there is also a possibility that the contrast medium may be retained in the salivary glands for long periods afterwards (Shiødt and Thorn, 1989). Sialography gives a static picture of the status of the salivary gland ductal system as it emphasizes the morphological abnormalities of the gland, but not its functional state (Arrago et al., 1987).

1.3.2.(iii) Scintigraphy

In contrast to sialography, scintigraphy shows the functional abnormalities of the salivary glands because it is based on the ability of the major salivary glands to trap the radioisotope technetium-sodium pertechnetate ($^{99}Tc^{m}$) (Sugihara and Yoshimura, 1988). After intravenous injection, the radioisotope becomes rapidly concentrated in the salivary glands. Scanning by a gamma scintillation camera provides functional information about the glands. In SS, the production of saliva is impaired due to the destruction of glandular parenchyma. This loss of active acinar cells is indicated by a reduced uptake of the radioisotope.

1.3.2.(iv) Ultrasonography

Ultrasonography is the most recent technique for monitoring salivary gland function in SS (Kawamura et al., 1990). It has the distinct advantage of being non-invasive. Using this method, de Clerck et al. (1988) detected a decreased parotid gland echogenicity in SS patients which could be due to the lymphocytic infiltrates in the gland.

1.3.2.(v) Sialometry

Sialometry, or the measurement of the flow-rate of saliva, although not specific for SS, is a simple, non-invasive assessment for the evaluation of xerostomia in SS. There has been difficulty in establishing a range of normal values. Fox et al. (1986b) measured parotid flow and established a lower normal limit of 0.5 ml/min, but Skopouli et al. (1986) established a lower limit of 0.2 ml/min.

1.3.2.(vi) Sialochemistry

Sialochemistry, or the chemical analysis of salivary composition, has at present a limited role in monitoring the oral component of SS because it is often non-specific (Scully, 1989). There have been few attempts to correlate salivary changes with data obtained from histopathology. As such, the available data is as yet not sufficiently proven to be of diagnostic use (Schiødt and Thorn, 1989).

In view of its non-invasive nature, the analysis of sialochemical changes could be of potential value in

monitoring the disease process. Sialochemistry also has the advantage of being considerably less costly and less time-consuming than the other methods of monitoring salivary gland involvement in RA and SS. Saliva samples may be easily collected from the patients, causing them little or no discomfort. Indeed, in recent years there has been an increasing interest in sialochemistry as a diagnostic aid which may be of use in the evaluation of pathogenic events and prognosis (Thorn et al., 1989).

1.4. Electrophoretic Techniques and their Application to Sialochemistry

1.4.1. Isoelectric Focusing

1.4.1.(i) Historical Background

Isoelectric focusing (IEF) is presently one of the most successful techniques in separating ampholytes such as proteins. It has wide applications in research. Its beginnings may be traced back to Ikeda and Suzuki (1912) who described a method for producing sodium glutamate in an electrolysis apparatus consisting of compartments separated by membranes permeable to ions. This method was later adapated to separate amino acids and other ampholytes (Williams and Waterman, 1929). An important contribution to the understanding of the phenomenon of isoelectric fractionations was made by Tiselius (1941), who noted that if an ampholyte is added to a multimembrane electrolyser, it will migrate until it reaches a compartment with a pH equal to

its isoelectric point. Thus, in the stationary state all anions are found in the compartment adjoining the anode while all cations in that adjoining the cathode with the ampholytes in intermediate compartments.

Kolin (1954; 1955a; 1955b) made a significant contribution to IEF methodology by developing an artificial pH gradient in a column by placing the substance to be separated at the interface between an acidic and basic buffer. After an appropriate diffusion time, an electric field was applied. However, Kolin's pH gradients are "artificial gradients" as their slope was continuously changed by migration and diffusion of the buffer ions. This made the recovery of separated proteins difficult.

Svensson (1961; 1962a; 1962b) developed the concept of a "natural" pH gradient in which the buffers used in the system had two fundamental properties: firstly, in order to reach a steady state position along the separation column, they were amphoteric. Secondly, they were carriers, or good conductors of electricity. Stable pH gradients could be obtained by the isoelectric stacking of a large series of carrier ampholytes which when subjected to an electric current become arranged in order of increasing isoelectric points from the anode to the cathode. In effect, the pH in every part of the gradient is defined by the buffering capacity and conductivity of the isoelectric carrier ampholyte located in that region.

A list of good ampholytes was compiled by Svensson

(1961; 1962a; 1962b), but it became clear that between pH 5 and pH 7 there were no species which satisfied this requirement.

This problem was solved by Vesterberg (1969a; 1969b) who successfully synthesized carrier ampholytes over the pH range 3-10. These ampholytes were polyprotic aminocarboxylic acids synthesized by coupling α,β-unsaturated acids to oligoamines. They were made commercially available by LKB Produkter AB, Bromma, Sweden, as "Ampholine".

Initially, isoelectric focusing was carried out in liquid columns. For analytical purposes, these were superceded firstly by polyacrylamide rod gels and subsequently by flat-bed gels.

1.4.1.(ii) Limitations of Isoelectric Focusing in Carrier Ampholyte-Based Systems

(a) Cathode Drift

Although the pH gradient generated by carrier ampholytes was sufficiently stable in most cases for the optimum resolution of proteins, it soon became evident that the pH gradient had a tendency to migrate slowly towards the cathode (Bates and Degoe, 1972). This phenomenon, called "cathode drift" or the "plateau phenomenon", progresses with time and voltage.

Various hypotheses for the cause of cathode drift have been put forward. Miles et al. (1972) proposed that am-

pholytes at their isoelectric points can reach a state of "transient charge" which causes a temporary migration. Before such a transient ion has been discharged it will meet another molecule of different isoelectric point with which it forms a complex. The formation of such complexes will impoverish the neutral zone as molecules gradually migrate away and complex with other ampholytes towards the ends of the gradient.

Another explanation (Haglund, 1975), is that the pH drift may be the result of hydrolytic or chemical changes at the basic and acidic ends of the pH gradient. Thus the isoelectric points of the carrier ampholyte molecules would gradually change during IEF, decreasing the pH of the acidic end of the gradient and increasing the pH of the basic end.

It seems more likely, however, that the reason for the pH drift is electroosmosis. The water production within a pH gradient has a maximum near pH 7.1, inducing lateral water flow which may wash out the mobile ampholytes present in this region (Hagedorn and Fuhr, 1990a; 1990b).

Although cathode drift is not normally detrimental to the resolution achieved, difficulties may arise when basic proteins are fractionated, especially those with pI's > 9. Thus in practice, use of this procedure above pH 8 is limited.

(b) Sensitivity of the pH Gradient to Electrolytes

If a sample containing electrolytes is fractionated by carrier ampholyte-based IEF, the application of an electric

field will dissociate the electrolytes into their component cations and anions. This results in the formation of acidic and alkaline boundaries which migrate towards the cathode and anode respectively. These boundaries will distort the pH gradient and impair the resolution of protein bands (Jonsson, 1980). Therefore prior to focusing all samples must be desalted to ensure that their ionic strengths are extremely low.

(c) Chemical Interactions with Proteins

There are few reports of carrier ampholytes causing chemical changes in proteins although there are known cases where IEF has caused proteins to be denatured or lose activity. Possible explanations of this phenomenon have included protein insolubility at their isoelectric points leading to denaturation (Haglund, 1975).

Changes in proteins resulting from the splitting of protein complexes during IEF have been known to occur. An example is albumin-fatty acid complexes which are gradually dissociated after prolonged focusing (Valmet, 1970).

Oxidation of proteins in the course of IEF has been noted. Wadström and Vesterberg (1971) demonstrated that the activity of an enzyme was higher when an antioxidant such as dithiothreitol (DTT) was present in the IEF gel. Changes in the amino acid composition of the enzyme bovine ribonuclease (a basic protein) during IEF include the partial oxidation of the sulphur-containing amino acids, cysteine and methionine (Jacobs, 1973).

Reactions occuring in the electrode compartments are the most common cause of oxidation. In particular, if the samples are applied at the extreme ends of the gel, chemical changes may occur due to exposure of the sample to extremes of pH. Thus Haglund (1975) recommended that in the case of flat-bed IEF the samples should be applied to several different parts on the gel.

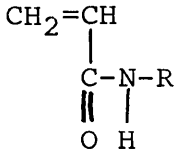
Carrier ampholytes may chelate metals. The chelation maxima occur in the pH region 8.5-10.5 where most of the nitrogen functions are deprotonated (Davies, 1970; Galante et al., 1975). Carrier ampholytes are powerful chelators of copper, thus copper-containing proteins may be rapidly inactivated (Christensen et al., 1971). The chelating power of carrier ampholytes towards other metals is generally weak. The loss of activity by metal chelation is seldom irreversible and full activity may usually be restored by incubation in appropriate amounts of the sequestered cofactor.

1.4.1.(iii) Immobilised pH Gradients

In 1982, a new and innovative method of IEF, called immobilised pH gradients (IPG's) was introduced by Bjellqvist et al. These were subsequently made commercially available by LKB Produkter AB, Bromma, Sweden, as "Immobiline".

In this method, the pH gradient is grafted, or immobilised, to the matrix of the polyacrylamide gel. This represented a departure from amphoteric buffers in favour of non-amphoteric ones. Immobilines are derivatives of

acrylamide with the general structure:



where the R is either a carboxylic acid or a tertiary amino group. The molecular formulae of Immobilines, which are not disclosed by LKB, have been established by Chiari et al. (1989a; 1989b), and are listed in Table 1.2.

The Immobilines are copolymerised into the gel matrix with acrylamide and bisacrylamide, where they effectively determine the ionic strength, buffering capacity and the pH range of the IEF gel. Recipes for the preparation of gels spanning the pH range 4 to 10 are supplied by LKB. In addition, Altland (1990) and Altland & Altland (1990) have formulated computer programs for creating recipes for IPG's.

IPG's offer the following advantages over IEF in carrier ampholyte-based systems:

(1) Since the pH gradients are covalently bound to the gel matrix, "cathode drift" is abolished. There is therefore no risk of losing basic proteins by this phenomenon. This means that long running times may be used, which gives proteins with low mobilities close to their isoelectric points time to reach their equilibrium positions.

(2) There is no longer any need to use samples of extremely low ionic strengths as electrolytes will not distort the pH gradient.

Table 1.2. Chemical formulae of Immobilines

(from Chiari et al., 1989a; 1989b)

pK	Formula	Name	M_r
3.6	$CH_2=CH-CO-NH-CH_2-COOH$	N-acryloyl glycine	129
4.6	$CH_2=CH-CO-NH-(CH_2)_3-COOH$	4-acrylamido butyric acid	157
6.2	$CH_2=CH-CO-NH-(CH_2)_2-N$ 	2-morpholino ethylacrylamide	184
7.0	$CH_2=CH-CO-NH-(CH_2)_3-N$ 	3-morpholino propylacrylamide	198
8.5	$CH_2=CH-CO-NH-(CH_2)_2-N(CH_3)_2$	N,N-dimethyl aminoethyl acrylamide	142
9.3	$CH_2=CH-CO-NH-(CH_2)_3-N(CH_3)_2$	N,N-dimethyl aminopropyl acrylamide	156

(3) IPG's offer the possibility of performing pH gradient engineering, i.e. tailoring the limits and widths of the gradient to suit the needs of any possible separation problem. It is usual to make use of narrow gradients of 1 pH unit, or even 0.1 pH unit. Broader gradients of 2-6 units have been described by Gianazza et al. (1985a; 1985b).

(4) The pH gradient of IPG's is more reproducible than that of carrier ampholyte gels, as the desired pH interval is mathematically formulated and fixed before the run (Righetti, 1984).

(5) IPG's are well suited for preparative work as Immobilines, unlike carrier ampholytes, are bound to the gel and will not contaminate extracted materials (Ek et al., 1983).

(6) Greater loads of samples may be applied to IPG's than carrier ampholyte-based gels. This may be useful in detecting minor components (Bjellqvist et al., 1982).

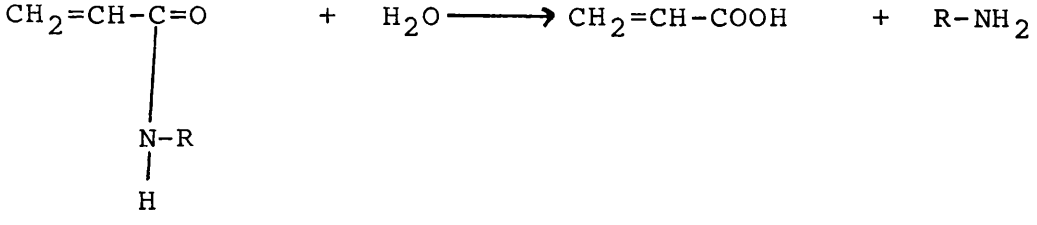
1.4.1.(iv) Problems Generated by the IPG Technique and Their Solutions

The IPG technique, however, was soon discovered to give rise to new problems, most of which are a result of the inherent low conductivity of the gels or the instability of the alkaline Immobilines. The problems include:

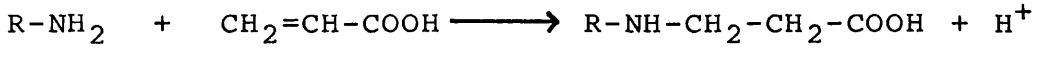
(a) The low conductivity of IPG's, which is a hundredth of that of carrier ampholyte gels, results in difficulties such as partial failure of samples to enter the gel, lateral zone spreading and the formation of "salt fronts" which halt

protein migration (Righetti, 1984; Fawcett and Chrambach, 1986a).

(b) The amide bonds of Immobilines, especially the alkaline ones, are susceptible to hydrolysis which results in the formation of acrylic acid and a primary amine (Pietta et al., 1985). The reaction proceeds as follows:



In a subsequent step, the primary amine released undergoes nucleophilic addition to the double bond of acrylic acid:



(c) The alkaline Immobilines are susceptible to autopolymerisation (Righetti et al., 1987). This phenomenon is autocatalytic and is accelerated by deprotonated amino groups. This process results in the formation of oligomers which may be incorporated into the gel. These oligomers may cause proteins to precipitate out of solution because they are able to bridge them by two unlike binding surfaces (ionic on one side and hydrophobic on the other) (Rabilloud et al., 1987).

(d) The amino groups of the alkaline Immobilines may be oxidised by ammonium persulphate during the polymerisation process, leading to the formation of tertiary amine oxide

($R_3N^+O^-$), which in turn may oxidise cysteine residues of proteins, leading to the formation of disulphide bridges. In exceptional cases, the disulphide bridges may even be further oxidised into cysteic acid (Righetti et al., 1989).

Solutions to these problems have been proposed. The difficulties brought about by the low conductivity of the IPG's may be remedied by "hybrid isoelectric focusing" or "mixed-bed carrier ampholyte-immobilised pH gradient gels" (Fawcett and Chrambach, 1986a; Altland et al., 1987). In these gels a primary insolubilised pH gradient coexists with a secondary, soluble, carrier ampholyte-driven pH gradient. This procedure overcomes most of the problems related to conductivity. Also, hydrophobic interactions between proteins and the autopolymerisation products of the alkaline Immobilines are minimised because the carrier ampholytes may act as shielding agents between the proteins and the oligomers produced by autopolymerisation (Rabilloud et al., 1987).

The first generation of Immobilines was supplied in liquid or powder form. Each bottle had to be reconstituted with 25ml of distilled water to make 0.2M solutions. However, when the alkaline Immobilines are stored as aqueous solutions, they are prone to undergo hydrolysis of the amide bond. This occurs even when they are stored frozen (Astrua-Testori et al., 1986). Following the discovery by Gåveby et al. (1988) that reconstituting the alkaline Immobilines in n-propanol abolishes the occurence of deamidation and

autopolymerisation, "Immobiline II" was launched late in 1988. These were supplied directly as 0.2M solutions. The acidic species (pK 3.6 and 4.6) were supplied in inhibitor-containing water, and the basic species (pK 6.2, 7.0, 8.5 and 9.3) in n-propanol.

The problem of the formation of tertiary amine oxide ($R_3N^+O^-$) during polymerisation is solved in the following manner: as the tertiary amine oxide is unreactive in the protonated state, it was recommended by Righetti et al. (1989) that following polymerisation, the gels should be subjected to a short washing step in 0.1M ascorbic acid solution.

1.4.1.(v) Measurement of the pH Gradient

The pH gradient of carrier ampholyte-containing gels may be measured by an antimony microelectrode with a calomel reference electrode (Beeley et al., 1972), but it is not possible to measure the pH gradient of IPG's by such a method because the buffering ions and titrants are grafted to the gel matrix (Righetti et al., 1986).

The pH gradient of IPG's may be measured by focusing mixed-type gels containing Immobilines and 1% (w/v) carrier ampholytes, after which gel slices were cut from the anode to the cathode and the carrier ampholytes contained in each slice were eluted with a solution of potassium chloride. The pH of each eluate was measured (Righetti et al., 1986). This method, however, is laborious, and may suffer from errors

due to the absorption of atmospheric carbon dioxide by the gel slices and by dilution of the eluted carrier ampholytes in potassium chloride solution. Furthermore, in order to ensure that there are sufficient amounts of carrier ampholytes to be eluted from the gel slices for pH measurement, it was recommended that 1mm thick mixed-type gels be prepared, rather than the standard 0.5mm thick gels, a procedure that would entail increased cost.

Another means of determining the pH gradient is by using a calibrated mixture of pH markers covering the range of interest. Ideally these markers should be coloured proteins or dyes of known pI's which allow the progress of focusing to be monitored visually (Righetti and Caravaggio, 1976).

1.4.1.(vi) Urea as a Gel Additive

Urea is frequently used as a solubilising agent in electrophoresis (Andrews, 1986). At high concentrations, it destabilises the native configuration of a protein sufficiently to produce its unfolded state. This is possibly brought about by increasing the aqueous solubility of the hydrophobic portions of the protein while maintaining the hydrogen-bonding capability of the aqueous solvent (Nozaki and Tanford, 1963; Roseman and Jencks, 1975).

Creighton (1979) studied the behaviour of proteins which were separated by electrophoresis in polyacrylamide gels containing urea concentration gradients perpendicular

to the direction of protein migration and found that the unfolding of proteins was accompanied by a reduction of mobility because the expanded polypeptide chains experiences greater molecular sieving by the gel matrix.

Urea concentration gradients perpendicular to the pH axis in IEF gels (Hobart (1975) , Altland et al. (1981), Altland et al. (1984) and Görg et al. (1985)) may yield important clues to the structure of proteins. For example, proteins which consist of non-covalently associated subunits are often dissociated by high urea concentrations, and in the process a single protein band may give rise to a number of subunit bands as the urea concentration is increased (Hobart, 1975). Alterations to the net charge of proteins will occur if their unfolding results in the exposure of charged groups (Creighton, 1979).

1.4.1.(vii) Isoelectric Focusing of Human Salivary Proteins

The first attempt at isoelectric focusing of human salivary proteins was made by Beeley (1969) using carrier ampholyte-containing polyacrylamide rod gels, and by Arneberg (1969; 1972) using sucrose density gradient columns. Unlike many biological fluids (e.g. serum) saliva contains relatively few proteins (20-30 bands were visualised upon staining of the IEF gels), and is therefore well suited to this technique. Parotid, mixed and submandibular salivary proteins have been fractionated in thin-layer polyacrylamide gels (Beeley, 1975). Using pH 3-10 IEF gels, Eckersall et

al. (1981) compared the band patterns of several individuals and concluded that they were essentially similar with minor individual variations.

Parotid saliva from patients with RA and SS were studied by focusing in rod gels (Chisholm et al., 1973) and the anionic proteins associated with these disorders were easily visualised and detected by densitometry as they precipitated out at or near their pI's. Mairs and Beeley (1985) analysed parotid saliva from RA patients in thin-layer gels and found multiple protein bands of pI's 3.95-4.25.

Carrier ampholyte-based IEF depends on the presence of samples of extremely low ionic strengths. As saliva has a low protein content of approximately 0.5-1.5 mg/ml but a high content of electrolytes, concentration and desalting prior to IEF is necessary. These procedures could be accomplished simultaneously by ultrafiltration (Beeley, 1975). Alternatively, the saliva samples could be dialysed, and then concentrated by means of Aquacide (Eckersall et al., 1981). Such procedures, apart from being time-consuming, require relatively large volumes of saliva (preferably > 2ml). While such volumes are easily obtainable from healthy glands with normal flow rates, it is often difficult, or even impossible, to obtain similar volumes from diseased glands such as those of patients with xerostomia. Continued research into the nature of the additional anionic proteins associated with RA and SS has been largely hindered by this

factor.

Desalting may also result in the loss of some minor components of saliva (Beeley, 1975), especially those of low M_r (< 10,000) due to passage through the dialysis membrane.

The IPG technique has not hitherto been applied to the analysis of human salivary proteins. The advantages that IPG's offer, such as higher tolerance to the electrolyte levels of samples, thus allowing the desalting step to be bypassed, high resolution and the possibility of designing the pH gradient around the pI's of the proteins of interest, make their application to the study of salivary proteins highly desirable. In addition, because the basic salivary PRP's have pI's > 8, they are especially well-suited to analysis by this technique as they are probably lost from conventional carrier ampholyte gels through cathode drift.

1.4.2. SDS-Polyacrylamide Gel Electrophoresis

1.4.2.(i) Introduction

Polyacrylamide gel electrophoresis (PAGE) is one of the most widely-used procedures for the estimation of the relative molecular weights (M_r's) of polypeptides, which is of great importance in their characterisation.

At any pH other than their pI's, proteins will carry a net charge and will migrate when placed in an electric field. The rate of migration is proportional to the charge density (ratio of charge to mass) of the protein. In zone electrophoresis, proteins of different mobilities travel as

discrete zones which gradually separate from one another (Hames, 1981). Zone electrophoresis of proteins in polyacrylamide gels is most commonly carried out in a buffer system which dissociates proteins into their individual polypeptide units. The most commonly used reagents for this purpose are the anionic detergent sodium dodecyl sulphate (SDS) and the thiol reagent dithiothreitol (DTT) which cleaves disulphide bonds. These reagents are usually used together, and protein mixtures are denatured by heating in an excess of SDS and DTT. Large amounts of SDS may be bound to the proteins, which is sufficient to overwhelm the intrinsic charge of the polypeptides, thus making the net charge per unit mass approximately constant. Viscosity analysis suggests that the reduced polypeptide-SDS complexes form rod-like particles with lengths proportional to their M_r's (See and Jackowski, 1989).

1.4.2.(ii) Gradient Gels

Polyacrylamide gels with a gradient of increasing acrylamide concentration produce greater sharpening of the protein bands as the migrating proteins are continually entering areas of decreasing pore size, the advancing edge of the migrating zone is retarded more than the trailing zone. On approaching the "pore-limit" when further progress is impeded by the pore-size, the protein banding pattern does not change appreciably with time. This results in a sharpening of the protein bands and therefore increases their resolu-

tion. In addition, the polyacrylamide gradient increases the range of M_r's of proteins which may be fractionated on a single gel (Hames, 1981).

1.4.2.(iii) SDS-PAGE of Human Salivary Proteins

SDS-PAGE has been used to study human salivary proteins (Smith et al., 1974; Oberg et al., 1982; Shiba et al., 1986; Lindsay and Beeley, 1986; Marshall and Williams, 1987; Marshall et al., 1989). Oberg et al. (1982) reported that the protein patterns of different individuals varied markedly, while the pattern for each individual was constant and free from circadian and feeding effects.

Human salivary proteins contain a group of phosphoproteins referred to as "lumicarmines" which exhibit unusual staining properties with the dye Comassie Brilliant Blue R250. These are stained pink-violet instead of the usual blue (Shatzman and Henkin, 1983). This phenomenon, known as metachromasia, was first noted in collagen (Duhamel, 1983) and the salivary proteins of rats (Shatzman and Henkin, 1983) before it was reported in human salivary proteins (Humphreys-Beher and Wells, 1984). It is most clearly observed when the gels are destained in a solution of 10% (v/v) acetic acid in the absence of alcohol (Lindsay and Beeley, 1986). Metachromasia has been hypothetically attributed to the presence of closely-spaced proline and hydroxyproline residues in sequences from triple-helical domains (Duhamel, 1983).

1.4.3. Two-Dimensional Electrophoresis

1.4.3.(i) Introduction

The underlying concept of two-dimensional (2-D) electrophoresis is the coupling of a charge fractionation method with a size fractionation method. O'Farrell (1975) successfully combined IEF with SDS-PAGE, thereby fractionating proteins according to their pI's in the first dimension and according to their M_r's in the second. Using this technique, over a thousand proteins from lysed Eschericia coli could be visualised on a single 2-D map.

1.4.3.(ii) 2-D PAGE with IPG's in the First Dimension

IPG's (Dunn and Patel, 1986; Görg et al., 1987) and hybrid carrier ampholyte-immobilised pH gradient gels (Görg et al., 1985) have been used in the first dimension of 2-D PAGE. The most significant advantage of using IPG's rather than carrier ampholytes is the abolition of cathode drift which has been the cause of much inter-experiment pattern-variability (Fawcett and Chrambach, 1986b). By combining hybrid carrier ampholyte-IPG polyacrylamide gels with polyacrylamide gradient electrophoresis, highly reproducible 2-D patterns could be obtained. In each case, the steady state is defined as a gel pattern which remains constant with time. IEF in IPG's is truly an equilibrium method while pore limit, or gradient-pore electrophoresis, is a quasi-equilibrium method as the protein zones are embedded in a

maximally restrictive pore and do not migrate appreciably with time (Fawcett and Chrambach, 1986b).

1.4.3.(iii) Thin-layer Polyacrylamide Gradient Gels as the Second Dimension of 2-D PAGE

The use of thin-layer (0.5mm thick) polyacrylamide gradient gels (Görg et al., 1986) for second-dimension separation in 2-D PAGE has the following advantages:

(a) Thin-layer gels are more efficiently cooled than conventional thick gels, so the vertical thermal gradient is abolished, thus the zones migrate perpendicular to the applied voltage.

(b) The improved heat dissipation also allows the application of higher field strengths, which improves the resolution of protein zones and shortens the separation time.

(c) Staining and destaining are achieved in much shorter times than for thicker gels.

(d) The gels may be cast on a plastic backing, and thus do not alter in size during staining and destaining. This facilitates more accurate comparisons of the protein spots between different gels.

1.4.3.(iv) 2-D PAGE of Human Salivary Proteins

There are few reports of 2-D PAGE of human salivary proteins in the literature. Giometti and Anderson (1980) and Marshall (1984) analysed the proteins of whole human saliva using conventional O'Farrell (1975) 2-D

electrophoresis. Bustos and Fung (1981) obtained 2-D maps of the abnormal salivary proteins associated with cystic fibrosis. Mogi et al. (1986) fractionated protein mixtures of sublingual and submandibular saliva by microscale 2-D electrophoresis. All these studies have utilised carrier ampholyte-based IEF for first dimensional separation. The prolonged focusing time (usually overnight) necessary for IEF rod gels make cathode drift unavoidable, thereby resulting in some loss of basic proteins through cathode drift. Thus previous 2-D maps of human salivary proteins displayed very few protein spots of pI's > 8.

The most recent methods of 2-D PAGE, including the use of hybrid carrier ampholyte-IPG polyacrylamide gels in the first dimension and thin-layer SDS-polyacrylamide gradient gels in the second, have hitherto not been applied to the fractionation of salivary proteins. The abolition of cathode drift by using IPG's would enable basic proteins to be located on 2-D maps.

2-D PAGE would provide invaluable information regarding the anionic salivary proteins associated with RA and SS. Although the pI's of these proteins are known, their M_r's have not yet been established.

1.5. Protein Detection Methods

1.5.1. Staining Procedures

Following gel electrophoresis, proteins were detected by one or more of the following methods:

Three general protein-staining methods, i.e. Coomassie Brilliant Blue R250 (CBB R250), Coomassie Brilliant Blue G250 (CBB G250) and silver stain; as well as a glycoprotein-specific stain (PAS) were used. Coomassie Brilliant Blue G250 is a dimethyl substitute of Coomassie Brilliant Blue R250. They differ markedly in their staining properties towards salivary proline-rich proteins (PRP's). The phenomenon of metachromasia which results in PRP's staining with a pink-violet colour (Lindsay and Beeley, 1986) is observed when CBB R250, but not CBB G250, is used. Other salivary proteins, such as α-amylase, are stained a blue colour by CBB R250. This provides a means of distinguishing PRP's from other salivary proteins in SDS-PAGE gels.

A colloidal suspension of CBB G250 in trichloroacetic acid (Dietzel et al., 1975; Neuhoff, 1988; Neuhoff et al., 1990) is a convenient means of staining proteins because very little destaining is necessary. The bands, therefore, may be visualised within an hour.

Silver stain has proven to be one of the most sensitive staining developments. It was first reported by Switzler et al. (1979), and has since undergone a number of modifications with varying degrees of sensitivity. Examples of various modifications of the silver stain technique are those of Oakley et al. (1980), Sammons et al. (1981), Morrisey (1981) and Heukeshoven and Dernick (1985).

Friedman (1982) reported the lack of silver-staining

sensitivity in salivary PRP's, this being especially so with the acidic PRPs which have a high content of glycine, proline and dicarboxylic acids but are lacking in sulphur-containing amino-acids such as tryptophan, tyrosine and threonine. Basic and sulphur-containing amino-acids are believed to be responsible for the binding of silver ions (Heukeshoven and Dernick, 1985; Hochstrasser et al., 1988) and the low levels, or absence, of these amino acids in most salivary PRP's is the most likely explanation for their poor staining properties with silver stain.

1.5.2. Protein Blotting

1.5.2.(i) Principles of Protein Blotting and Detection

A method of electrophoretically transferring fractionated DNA to nitrocellulose was described by Southern (1975). Subsequently, Towbin et al. (1979) devised a method for the transfer of proteins from polyacrylamide gels to nitrocellulose membranes. The mechanism of protein binding to nitrocellulose is not well understood but hydrophobic interactions are believed to be largely responsible (Tovey and Baldo, 1989).

Most of the advantages of protein blotting are due to the greater accessibility of macromolecules bound to the surface of a thin sheet compared to those buried within the matrix of a gel. This makes the processing times for the staining, destaining, and incubation of the membranes very much shorter than for gels (Gershoni and Palade, 1983).

The immobilised proteins may be detected by various staining, immunochemical and lectin-binding procedures. The use of secondary antibodies is common. In this method, the additional binding sites on the nitrocellulose membrane are blocked by excess protein or a non-ionic detergent such as Tween 20. A specific antibody to the protein of interest is then added to it, and finally a secondary antibody, usually an anti-immunoglobulin directed against the first antibody, is added as the detecting antibody. This antibody may be coupled to fluorophores, radioisotopes or enzymes.

Following gel electrophoresis, proteins may also be electroblotted to polyvinylidene difluoride (PVDF) membranes. PVDF, being inert to most solvents, is ideally suited for use in automated gas-phase sequencers. In this procedure, the proteins electroblotted to PVDF may be detected by staining with Coomassie Blue, after which the protein bands of interest are cut out to be analysed by protein micro-sequencing (Matsudaira, 1987; Findlay and Geisow, 1989).

1.5.2.(ii) Detection by Antibodies

Most proteins retain their antigenicity after electrophoresis and electroblotting, and therefore may be detected by immunological methods using specific antibodies. Proteins which have been immobilised on blots may be detected using the following systems:

(a) Detection of antigens by alkaline phophatase-conjugated anti-antibody

Following electroblotting, the remaining sites of attachment on the membrane are blocked by washing with a solution of non-ionic detergent such as Tween 20 dissolved in Phosphate-Buffered Saline (PBS). The blot is incubated in a specific antiserum-containing solution, washed, and then incubated with an alkaline phosphatase-conjugated anti-antibody-containing solution. The blot is finally incubated in a solution containing 5-bromo-4-chloro-3-indoxyl phosphate (BCIP), a substrate for alkaline phosphatase. This is cleaved by the enzyme to form to form an intense blue precipitate of diformazan (Blake et al., 1984). Fig. 1.3 illustrates the detection of antigens by this method.

(b) Detection of antigens using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes)

The previously mentioned method occasionally results in weak staining reactions, particularly if the antigen is present in small amounts (< 1 μg). In order to intensify staining, an immune complex of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complex) may be used (Cordell et al., 1984). The APAAP complex consists of 4 molecules of alkaline phosphatase enzyme complexed to an equal number of mouse monoclonal antibodies (Fig. 1.4). The rabbit antibody against mouse IgG is used as a "bridge" between two APAAP complexes. Two repetitions of stages 3 and 4

Fig. 1.3. Detection of antigens by the alkaline phosphatase-conjugated second anti-antibody method

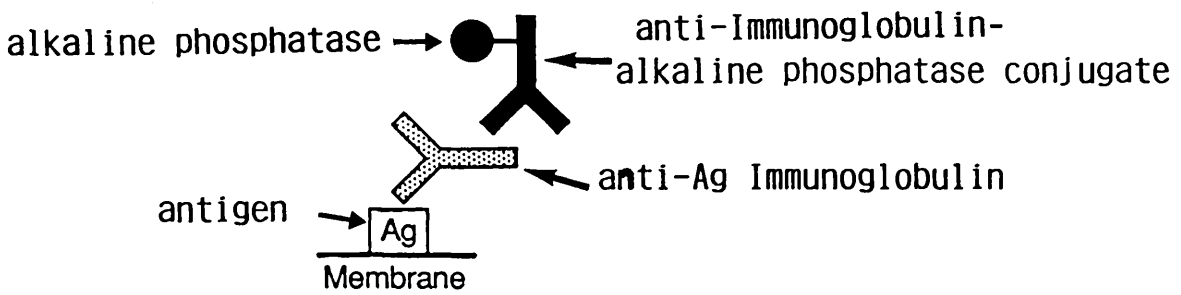
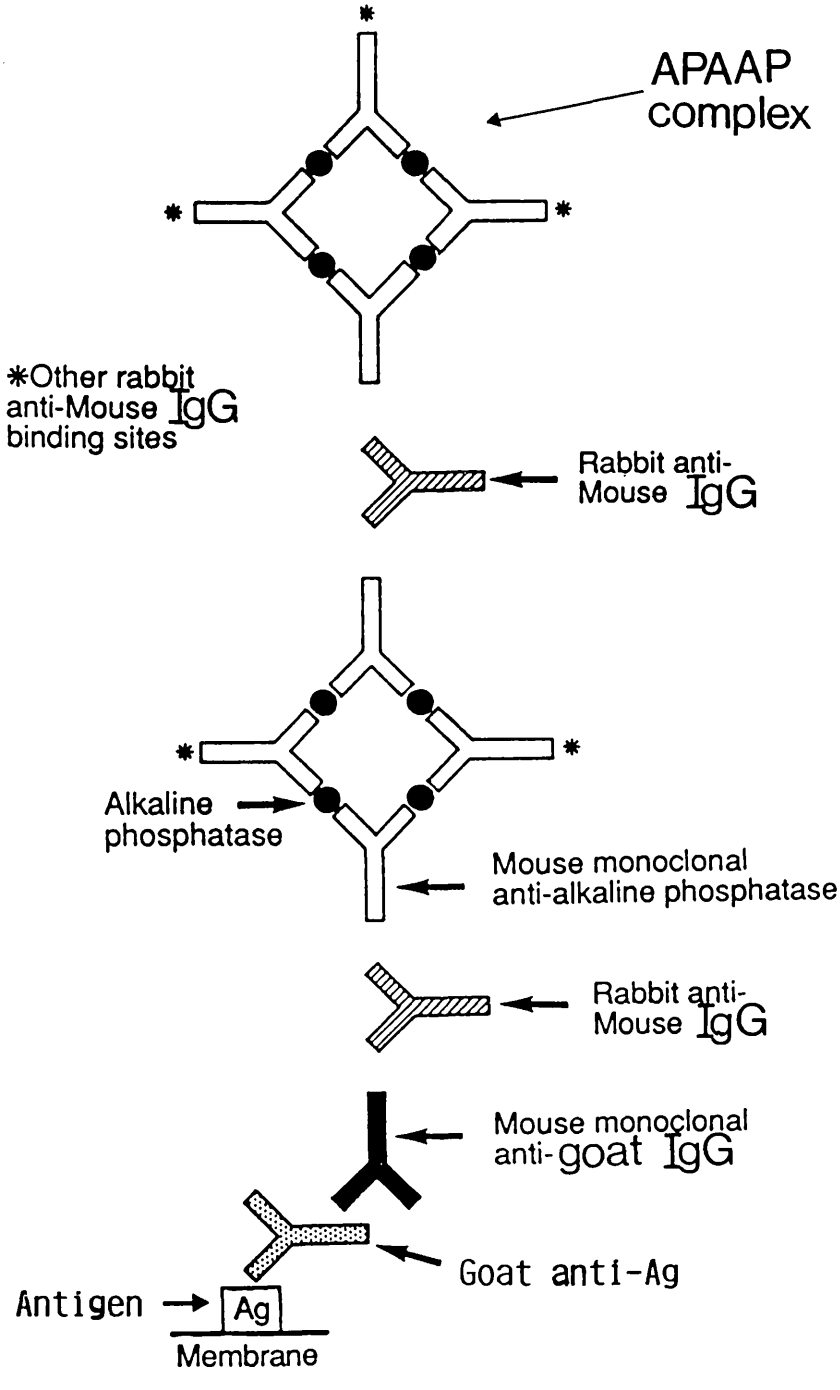


Fig. 1.4. Detection of antigens using the APAAP method



in Fig. 1.4 results in amplification of the signal.

1.6. Aims of the Study

The aim of this study is the development and refinement of electrophoretic techniques with the view of enabling the analysis of proteins in small volumes (e.g. < 100 µl) of human saliva. Such sensitive and high-resolution techniques could prove invaluable when the volumes of saliva samples available are relatively small, as is often the case in RA and SS as many of these patients suffer from xerostomia. These techniques were then applied towards the characterisation of the anionic parotid salivary proteins associated with RA and SS. The analysis of these anionic proteins may form the basis of a non-invasive diagnostic procedure and also further the understanding of salivary gland involvement in connective tissue disorders.

Research into the nature of the anionic proteins has hitherto been hindered by the relatively high electrolyte and low protein contents of human parotid saliva, which necessitates desalting and concentrating saliva samples prior to analysis by isoelectric focusing. However, this requires relatively large (preferably > 2ml) volumes of parotid saliva, which may be difficult or even impossible to obtain from the patients, many of whom suffer from xerostomia. The IPG technique is ideal for the analysis of human salivary proteins as its pH gradient is unaffected by electrolytes present in the samples, thus allowing applica-

tion of samples without prior desalting. Another significant advantage of IPG's is that they allow pH gradient-engineering. Therefore, an acidic range (e.g. pH 3.5-5.0) pH gradient could be tailored in order to fractionate the anionic salivary proteins.

Further information regarding the anionic salivary proteins could be obtained from 2-D PAGE using IPG's in the first dimension and SDS-PAGE in the second. By electroblotting the 2-D gels onto nitrocellulose membranes and incubating the blots with specific antisera, a 2-D map of human salivary proteins could be constructed.

The identity of the anionic proteins could be established by protein blotting followed by probing the blots with antisera to specific proteins, especially proteins the levels of which in either serum or saliva are known to be elevated during the course of inflammation. Further characterisation of the anionic proteins could be achieved by incubation with exo- and endoglycosidases in order to elucidate the nature of their pI heterogeneity.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sources

Materials were obtained from the following sources:

Amersham Laboratories, Amersham, Bucks, U.K.: "Hybond-C" nitrocellulose membrane and "Rainbow" protein molecular weight markers.

BDH, Poole, Dorset, U.K.: Arginine, acetic acid, acrylamide, ammonium persulphate (APS), bromophenol blue, sulphuric acid, hydrochloric acid, phosphoric acid, coloured isoelectric point markers (pH 2.4-5.65 and pH 4.7-10.6), ethanol, formaldehyde, glutamic acid, glycine, glutaraldehyde, glycerol, HPLC-grade water, hydrogen peroxide, methanol, 2-mercaptoethanol, N,N'-methylenebisacrylamide (bisacrylamide), potassium hexacyanoferrate III, potassium hydroxide pellets, perchloric acid (PCA), periodic acid, sodium dodecyl sulphate (SDS), sodium azide, sodium carbonate, sodium thiosulphate, sodium hydroxide pellets, sodium chloride, sodium acetate, sodium ethylenediaminetetraacetate (EDTA), sodium sulphate, sodium thiosulphate, toluene and trichloroacetic acid (TCA) were of "Analar" or " Electran" grades.

Activated charcoal, dimethyldichlorosilane and Triton X-100 were of "Laboratory Reagent" grade. Polyethyelene glycol 10,000 was of "Organics" grade and urea of "Aristar" grade.

The Binding Site Ltd., Edgbaston, Birmingham, U.K: Sheep an-

tiserum to human α_1-acid glycoprotein.

Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, U.K.: Bio-Rad Protein Assay Reagent.

bioMérieux, Marcy l'Etoile, France: "Arthri-Slidex" latex slide test kit for the detection of rheumatoid factors.

Boehringer Mannheim, Lewes, East Sussex, U.K: Dithiothreitol (DTT), Endo-β-N-acetylglucosaminidase F (Endo F), Peptide: N-Glycosidase F (PNGase F), Trasylol (lyophilised aprotinin), and tris(hydroxymethyl)aminomethane (Tris).

Calbiochem, La Jolla, CA, U.S.A.: "Aquacide", Concanavalin A (ConA), goat antiserum to human serum albumin, rabbit antiserum to human plasma kallikrein and protease-free neuraminidase purified from Vibrio Cholerae.

Cymbus Bioscience Ltd., Southhampton, Hampshire, U.K: Mouse monoclonal antibody to human J-chain, rabbit antiserum to mouse IgG and the immune complex of alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (APAAP complex).

Dako Ltd., High Wycombe, Bucks, U.K.: Rabbit antiserum to human secretory component, rabbit antiserum to human C-reactive protein and rabbit antiserum to human lysozyme.

Davis Gelatin Ltd., Leamington Spa, U.K.: Agar.

Difco Laboratories, Surrey, U.K.: Freund's Complete Adjuvant and Freund's Incomplete Adjuvant.

Formachem Ltd., Strathaven, Scotland, U.K.: Disodium hydrogen phosphate and potassium dihydrogen phosphate.

Gibco-BRL, Paisley, Scotland, U.K.: Microdialysis membrane with cut-off of M_r 6,000-8,000.

ICN Biochemicals, Bucks, U.K.:GelBond PAG film.

Johnson Matthey Chemicals Ltd., Herts, U.K.: Silver nitrate.

KabiVitrum, Uxbridge, Middlesex, U.K.:
H-D-Val-Leu-Arg-NH-pNA (S-2266, a chromogenic substrate for glandular kallikreins).

Millipore, London, U.K.: Polyvinylidene difluoride (PVDF) membrane and filtration membranes of pore-size 20μm.

Nordic Immunological Laboratories, Berkshire, U.K.: Sheep antiserum to human salivary α-amylase and rabbit antiserum to human lactoferrin.

Pelikan, Hanover, West Germany: India ink.

Pharmacia-LKB Biotechnology, Milton Keynes, U.K.: Immobilines (pK's 3.6, 4.6, 6.2, 7.0, 8.5 and 9.3), Ampholines (pH 3.5-10.0, pH 5-7 and pH 3.5-5.0) and Electrophoresis Calibration Kit (M_r's 14,400 to 94,000).

Pierce and Warriner (U.K.) Ltd., Chester,Cheshire,U.K.: Bicinchoninic acid (BCA) protein assay reagent.

Protogen AG, Läufelfingen, Switzerland: Human urinary kallikrein and goat antiserum to human urinary kallikrein.

Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland, U.K.: Mouse monoclonal antibody against human HLA-DR Class II antigen, sheep antiserum to human IgG (γ-chain specific) and sheep antiserum to human IgM (μ-chain specific).

Sigma Chemical Co., Poole, Dorset, U.K.: Anti-mouse immunoglobulin-alkaline phosphatase conjugate, anti-rabbit immunoglobulin-alkaline phosphatase conjugate, anti-sheep

immunoglobulin-alkaline phosphatase conjugate, anti-goat immunoglobulin-alkaline phosphatase conjugate, bovine serum albumin, bovine α-chymotrypsinogen, 1,3-bis[tris(hydroxymethyl)-methylamino]propane (bis-tris propane), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 3-(cyclohexylamino)-1-propane sulphonic acid (CAPS), Coomassie Brilliant Blue R250 (CBB R250), Comassie Brilliant Blue G250 (CBB G250), 4-chloro-1-naphthol, Folin-Ciocalteau reagent, gelatin, goat antiserum to human IgA (α-chain specific), human colostral sIgA, human serum IgG, horse radish peroxidase, iodoacetamide, α-D-methylmannoside, mouse monoclonal antibody to goat IgG, mouse monoclonal antibody to human β_2-microglobulin, Nitro Blue Tetrazolium (NBT), N,N,N',N'-tetramethylethylenediamine (TEMED), pararosaniline hydrochloride, polyoxyethylenesorbitan monolaureate (Tween 20), rabbit antiserum to human κ-light chain and rabbit antiserum to human λ-light chain.

Whatman Biosystems Ltd., Maidstone, Kent, U.K.: DE32 anion exchanger and No. 1 filter paper.

Gifts

These items were kindly donated:

(a) The acidic acrylamido derivative (pK 0.8) was a gift from Prof. P.G. Righetti, University of Milano, Italy.

(b) Acidic proline-rich human salivary proteins "A" and "C" were a gift from Prof. A. Bennick, University of Toronto, Canada.

(c) Basic proline-rich human salivary proteins "IB1", "IB3",

"IB4", "IB5", "IB6", "IB7", "IB8", "IB9"" and "Basic proline-rich glycoprotein" (BPR glycoprotein) were a gift from Dr. D. Kauffman, University of Washington, Seattle, U.S.A..

2.1.2. Buffers

Phosphate-Buffered Saline (PBS), pH 7.2, contained 170mM sodium chloride, 3.4mM potassium chloride, 10mM disodium hydrogen phosphate and 1.8mM potassium dihydrogen phosphate. Phosphate-Buffered Saline containing sodium azide (PBS-azide) consisted of 0.01% (w/v) sodium azide in PBS.

All other buffers, unless otherwise indicated, were prepared as recommended by Dawson et al. (1986).

2.2. Collection and Subsequent Treatment of Human Parotid Saliva

2.2.(i) Subjects

Parotid saliva was collected from normal healthy individuals, from patients with primary and secondary SS, and from RA patients. The RA patients were seropositive for rheumatoid factor. The samples were collected from patients at either the Department of Oral Medicine and Pathology in the Glasgow Dental Hospital or the Rheumatology Clinic in the Glasgow Royal Infirmary.

2.2.(ii) Collection and Treatment of Samples

Lemon juice was used as the stimulant and the saliva

samples were collected using a modified Carlsson-Crittenden cup (Stephen and Spiers, 1976) (Fig. 2.1).

When it was necessary to concentrate the samples, this was accomplished by one of the following methods:

(a) the samples were lyophilised and subsequently reconstituted to a fifth of their original volume with distilled water.

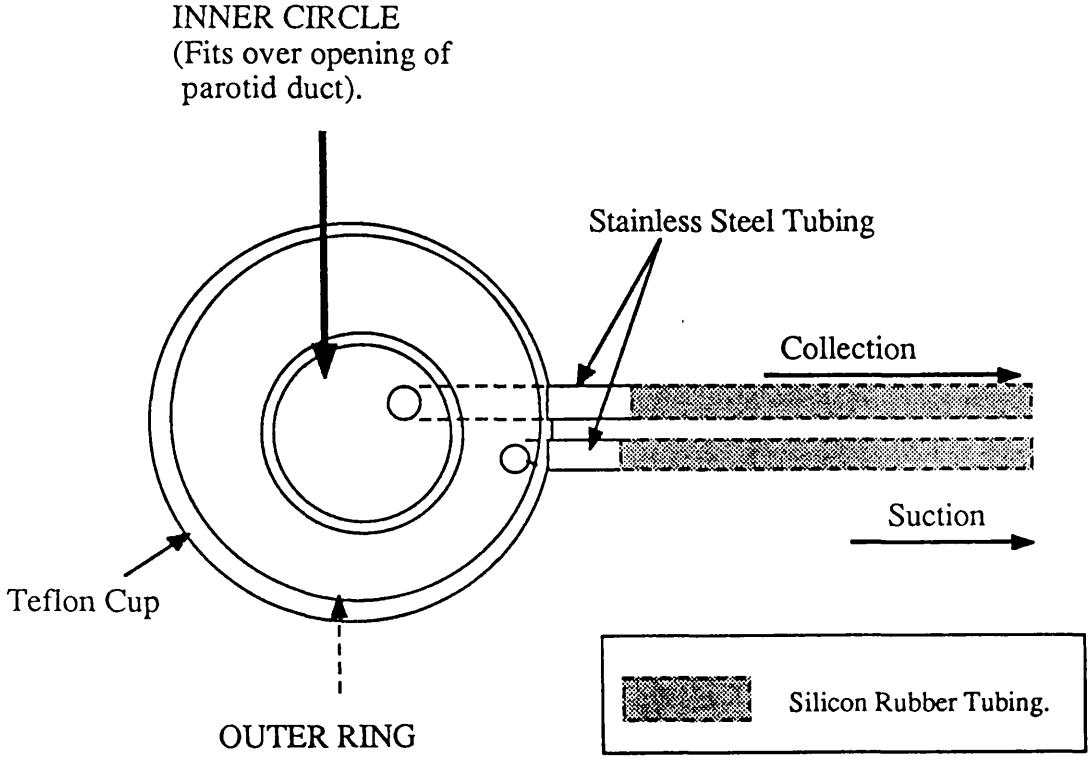
(b) the samples were dialysed overnight against 30% (w/v) polyethylene glycol 10,000 using a BRL 1200MA Microdialysis Unit fitted with a membrane with a cut-off of M_r 6,000-8,000. This procedure reduced the samples to 20-30% of their original volumes.

The samples were stored at -20°C before use. After thawing, they were centrifuged at 10,000g for 20 minutes at 4°C to remove insoluble material. All subsequent operations were performed at 4°C.

2.3. Protein Assay

The protein content of saliva was determined by the BCA micro-protocol (Smith et al., 1985). Bovine α-chymotrypsinogen, standardised by its extinction coefficient, was used as the protein standard (Wilcox et al., 1947). This procedure allows the determination of protein concentrations in saliva volumes as small as 5μl. After incubation with the BCA working reagent for 30 minutes at 60°C, the absorbance was read at 562nm using a Pye Unicam SP8-100 uv/visible Spectrophotometer.

Figure 2.1. Modified Carlsson-Crittenden Cup for Collection of Human Parotid Saliva.



In order to compare the sensitivities of different assay methods when used on saliva and to obtain absorbance curves using different protein standards, the Lowry assay (Lowry et al., 1951) and the Bio-Rad assay (Bradford, 1976) were also studied, although these two methods were not used in the routine analysis of saliva samples. Standard curves were also obtained for the BCA standard procedure, in which the samples were incubated at 37^{0}C for 30 minutes. Bovine serum albumin (BSA) was also standardised by its extinction coefficient was used in order to compare the sensitivities of the methods.

2.4. SDS-PAGE

2.4.1. Stock Solutions

Stock solutions were prepared as follows:

(A) Acrylamide ("30:08") solution containing 30% (w/v) acrylamide and 0.8%(w/v) N,N'-methylenebisacrylamide.

(B) 10% (w/v) ammonium persulphate in water.

(C) 20% (w/v) SDS in water.

(D) Resolving gel buffer for 12.5 %T (%T=Total acrylamide, or the polyacrylamide gel concentration defined as percentage total monomers, i.e. acrylamide + bisacrylamide g/100ml) gels was 750mM Tris-HCl, pH 8.8.

(E) Resolving gel buffer for 20 %T acrylamide gels was 3M Tris-HCl, pH 8.8.

(F) Stacking gel buffer was 170mM Tris-HCl buffer solution, pH 6.8.

(G) The reservoir buffer contained 25mM Tris, 192mM glycine, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide, pH 8.3.

(H) The solution used to denature the saliva samples contained 20% (w/v) SDS. 250mM DTT and 0.01% (w/v) bromophenol blue in aqueuous solution.

2.4.2. Gel Preparation

A Bio-Rad Protean II apparatus was used for casting and running the gels. The gels were prepared as follows:

2.4.2.(i) Homogeneous Gels

SDS-polyacrylamide gels were prepared using a modification of the method of Laemmli (1970). Gels with a stacking portion of 5 %T and resolving portion of 12.5 % or 20 %T were prepared using the following volumes of solutions:

Solutions	5 %T gel	12.5 %T gel	20 %T gel
(A)	2.0ml	12.5ml	20.0ml
(B)	40µl	100µl	100µl
(C)	60µl	150µl	150µl
(D)	---	15.0ml	---
(E)	---	---	3.75ml
(F)	8.8ml	---	---
distilled water	1.16ml	2.25ml	6.0ml
TEMED	5µl	10µl	10µl

2.4.2.(ii) Gradient Gels

A gradient former (Bio-Rad Model 385) was used to prepare 5-20 %T linear gradient acrylamide gels (Hames, 1981). The apparatus for forming gradient gels is shown in Fig. 2.2. Thirty ml each of light and dense solutions, sufficient for making 2 gels, were prepared as follows:

Light solution (5 %T)

5.0ml stock acrylamide solution (A)

3.75ml 3M Tris-HCl, pH 8.8 (E)

0.3ml 10% (w/v) SDS solution

0.7ml 1.5% (w/v) ammonium persulphate solution

20.25ml distilled water

Dense solution (20 %T)

20.0ml stock acrylamide solution (A)

3.75ml 3 M Tris-HCl, pH 8.8 (E)

0.3ml 10% (w/v) SDS solution

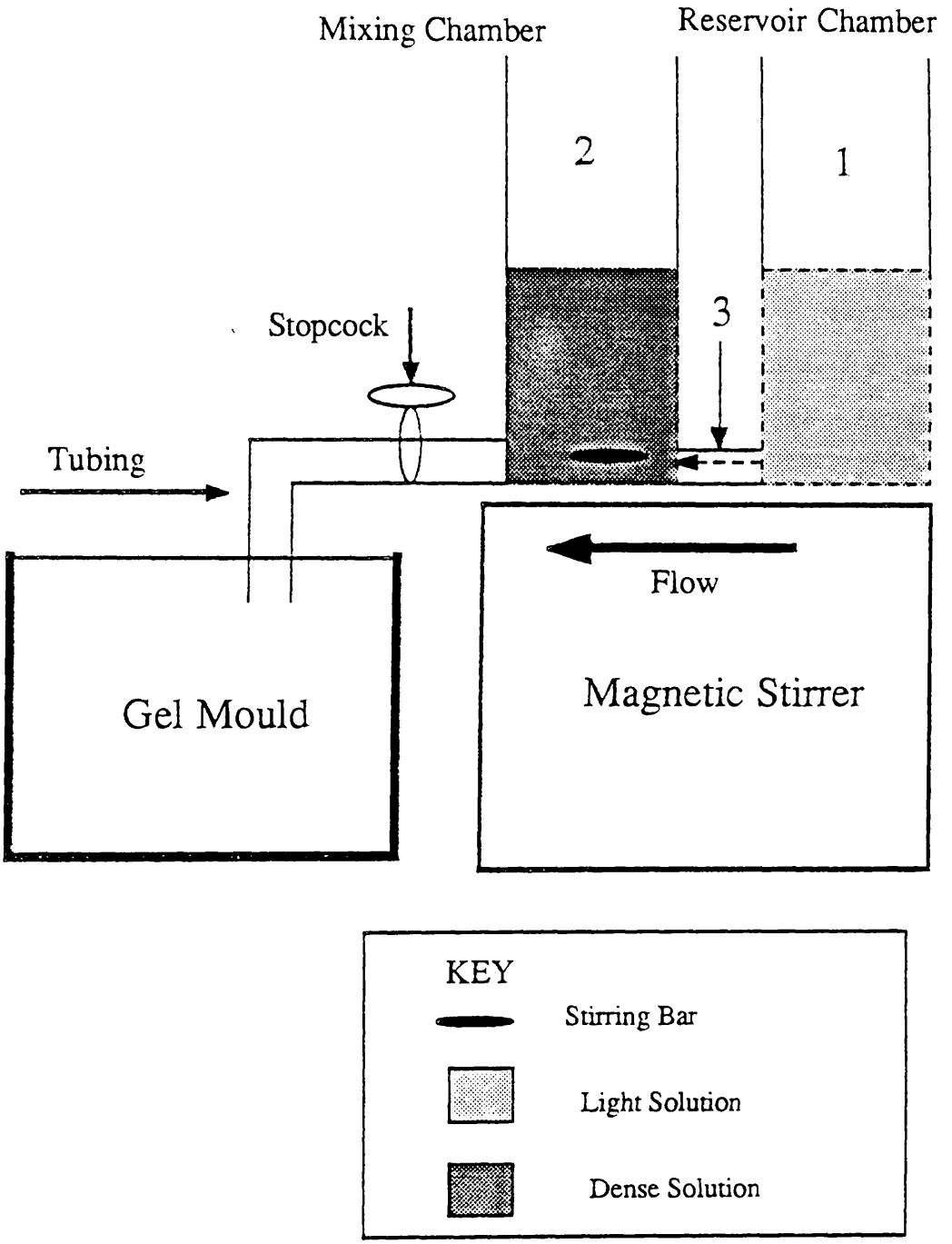
0.7ml 1.5% (w/v) ammonium persulphate solution

4.5g sucrose (equivalent volume to 2.5ml)

2.75ml distilled water

Fifteen ml each of the light and dense solutions were pipetted into the reservoir and mixing chambers, respectively, of the gradient former. Ten μl of TEMED was pipetted into each chamber before the gel was poured.

Figure 2.2 Diagram of a Gradient Former.



The flow of liquid is from chamber 1 to chamber 2 after the connecting channel (3) has been opened.

2.4.3. Running Conditions

All gels were run overnight at 4^0C and continued the following day until the dye front reached the bottom of the gel, a total running time of approximately 17 hours. A constant voltage of 60V was applied using a Shandon Vokam 2621 power supply system.

2.4.4. Treatment of Saliva Samples

Ten μl of solution H and 10 μl of 95% (w/w) glycerol were added to 100 μl of parotid saliva and the mixture was placed in a boiling water bath for 2 minutes before loading to the gel.

In order to determine the optimum protein load for different stains such as Coomassie Brilliant Blue R250 (Lindsay and Beeley, 1986), Coomassie Brilliant Blue G250 (Mairs and Beeley, 1985) and silver stain (Damerval et al., 1987), various protein loads were applied to the gel.

2.4.5. Determination of Molecular Weights

Gels which were to be stained after electrophoresis were loaded with Pharmacia Electrophoresis Calibration Kit standard M_r markers. Gels which were to be electroblotted onto immobilising membranes such as nitrocellulose were loaded with Amersham "Rainbow" protein molecular weight markers which being coloured are easily visualised on the membrane.

The Pharmacia low M_r markers consisted of the following proteins:

Protein	Subunit M_r
Phosphorylase B	94,000
Bovine Serum Albumin	67,000
Ovalbumin	43,000
Carbonic Anhydrase	30,000
Soybean Trypsin Inhibitor	20,100
α-Lactalbumin	14,400

Five μl of these markers were loaded onto each gel.

The Amersham coloured M_r markers consisted of the following proteins:

Proteins	Subunit M_r
Myosin	200,000
Phosphorylase B	97,400
Bovine Serum Albumin	69,000
Ovalbumin	46,000
Carbonic Anhydrase	30,000
Trypsin Inhibitor	21,500
Lysozyme	14,300

Ten μl of these markers were loaded onto each gel.

2.4.5.(i) Homogeneous Gels

Standard protein markers were treated and loaded according to the manufacturers' instructions. Relative mobility (R_f) values were calculated and the M_r's deter-

mined by using a plot of $\log_{10} M_r$ versus R_f, which gives a straight line relationship (Hames, 1981).

2.4.5.(ii) Gradient Gels

The molecular weights of protein spots were estimated as recommended by Lambin (1980). In the presence of SDS, there is a linear relationship between the $\log_{10}$ of the molecular weight and the $\log_{10}$ of the acrylamide concentration reached by the protein after electrophoresis.

Using protein standards, a linear relationship of M_r to %T as defined by the following equation is obtained:

$$\log_{10} M_r = a \log_{10}(\%T) + b$$

where a and b are constants, %T the total acrylamide and M_r the relative molecular weight of the polypeptide.

2.4.6. Running Conditions

Electrophoresis was carried out overnight at a contant voltage of 60V and a cooling temperature of 4^0C. This was continued until the dye front reached the bottom of the gel. A Shandon SAE 2621 power supply unit was used.

2.5. Isoelectric Focusing in Immobilised pH Gradients (IPG's)

2.5.1. Preparation of the Gel Mould

Glass plates with U-frames of dimensions 0.5 X 120 X

250mm and 1.0 X 120 X 250mm, supplied by Pharmacia-LKB, were used to prepare gels of 0.5mm and 1.0mm thicknesses, respectively. In order to prevent gels from sticking to the glass plates, the plates were coated with dimethyldichlorosilane solution which makes the glass surface hydrophobic.

Prior to use, the GelBond PAG film upon which the gels were cast were washed for 3 X 20 minutes in distilled water in order to minimise the occurence of spot streaking when the IPG's were used for 2-D PAGE (Görg et al., 1985).

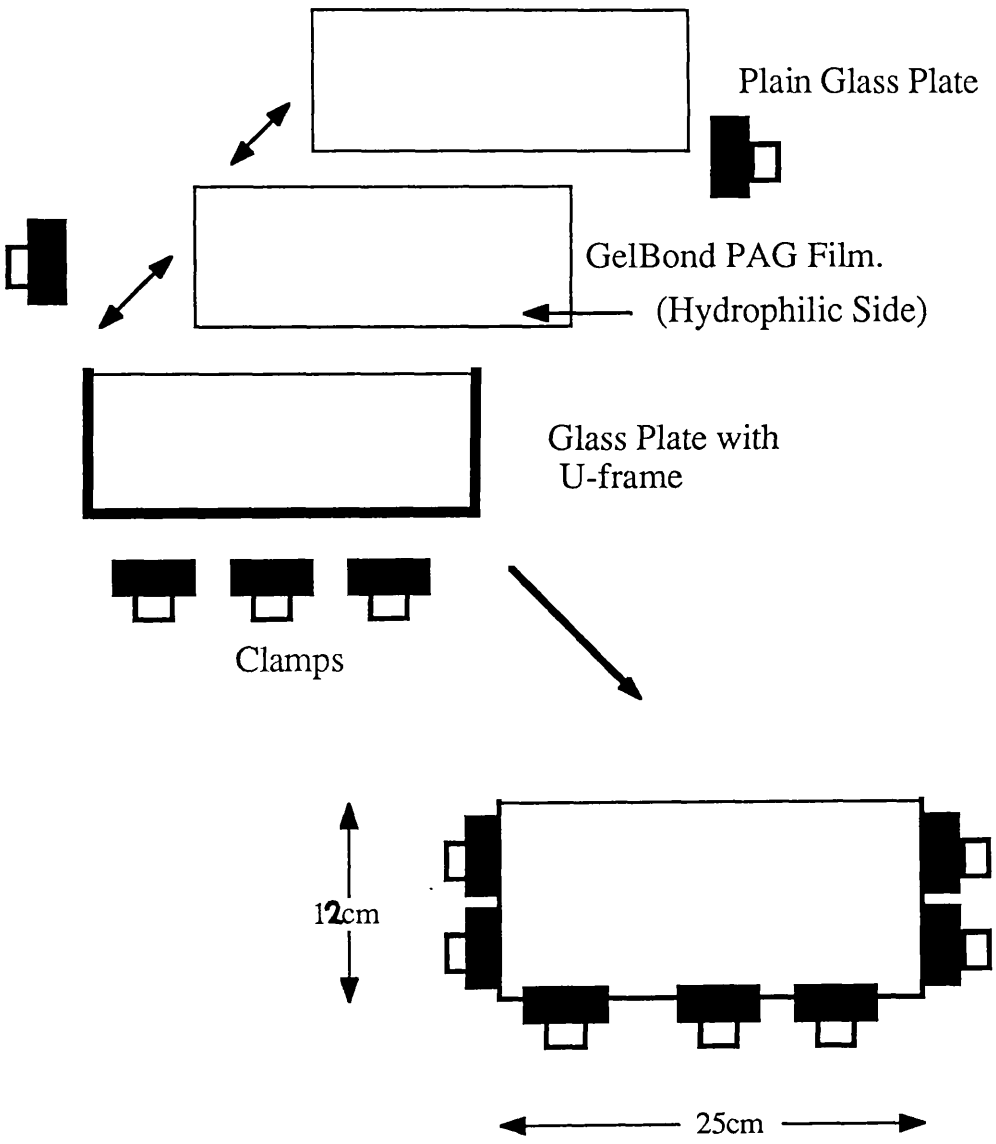
After applying a few drops of ethanol to the surface of a plain glass plate of dimensions 3.0 X 120 X 250mm (thus ensuring good contact) a sheet of GelBond PAG film was placed on the surface of the plate with its hydrophilic side facing up. This glass plate and the glass plate bearing a U-frame were then clamped together with the GelBond PAG film sandwiched in between. This set-up is illustrated in Fig. 2.3. Finally, the assembled mould was cooled to $4^{o}C$ prior to use in order to decrease the rate of polymerisation when the gel was being poured.

2.5.2. Preparation of IPG's

The following stock solutions were prepared:

(A) Immobiline solutions were prepared by the addition of 25 ml of distilled water to the contents of each bottle of Immobiline. This produced 0.2M solutions which were then aliquoted as 1ml portions and stored at $-20^{o}C$ for up to 6 months. After thawing, they were stored at $4^{o}C$ for periods

Figure 2.3. Preparation of a Gel Mould for Gels Cast on GelBond PAG Film.



of up to 6 weeks. The acidic acyrylamido derivative (pK 0.8, M_r 207) was also dissolved in distilled water to make a 0.2M solution. It was then stored in the same way as the Immobilines. Storage of these solutions for longer periods was avoided because Immobilines, particularly the alkaline ones (pK's 6.2, 7.0, 8.2 and 9.3) are susceptible to hydrolysis and autopolymerisation (Pietta et al., 1985; Astrua-Testori et al., 1986).

Following the report by Gåveby et al. (1988) that reconstituting Immobilines in n-propanol rather than water prevents the hydrolysis of the amide bond and the occurence of autopolymerisation; in the latter part of 1988, "Immobiline II" was introduced. This new generation of Immobilines was supplied as 10ml aliquots of 0.2M solutions. The alkaline Immobilines were supplied in solution in n-propanol and the acidic Immobilines in an inhibitor-containing water.

(B) Acrylamide/bisacrylamide solution ("30:08") consisted of 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide.

(C) 10% (w/v) ammonium persulphate.

(D) 10% (v/v) TEMED.

(E) 95% (w/w) glycerol solution.

Linear pH gradients were generated by mixing two solutions, an acidic and dense solution, and a basic and light one, in a Bio-Rad Model 385 gradient former (Fig. 2.2).

2.5.2.(i) 0.5mm Thick gels

IPG's which were subsequently to be stained were cast on GelBond film. These gels were 0.5mm thick.

IPG's of three pH ranges, i.e. pH 4-9, pH 2.8-4.5 and pH 3.5-5.0, were prepared using a modification of the procedure recommended by LKB Application Note No. 324 and the LKB Laboratory Manual for the Multiphor II Electrophoresis System.

Sixteen ml each of acidic and basic solutions, sufficient for two gels, were prepared. Although the LKB Application Note recommended preparing 15ml of each of these solutions, enough for two gels, and subsequently pipetting 7.5ml of each into the chambers of the gradient former for making one gel, in practice it was found that small volumes of the solutions were retained in the beakers in which they were made up and also in the chambers of the gradient former. Therefore 16ml, rather than 15ml, of each solution was prepared so that 7.8ml of each could be pipetted into the chambers of the gradient former.

The solutions for pH 4-9 IPG's were prepared according to Table 2.1. The solutions for pH 3.5-5.0 IPG's were prepared according to Table 2.2.

The solutions for pH 2.8-4.5 IPG's were prepared as recommended by Gianazza et al. (1987), and are described in Table 2.3.

Basic, light solution (7.8ml) was pipetted into the reservoir chamber of the gradient former and an equal volume of the acid, dense solution into the mixing chamber. Twenty

Table 2.1. Solutions for pH 4-9 IPG's of 0.5mm thickness

Acidic/dense solution (pH 4)	Volume	Basic/light solution (pH 9)	Volume
Immobiline pK 3.6	884µl		157µl
Immobiline pK 4.6	251µl		452µl
Immobiline pK 6.2	247µl		384µl
Immobiline pK 7.0	23µl		316µl
Immobiline pK 8.2	267µl		76µl
Immobiline pK 9.3	236µl		707µl
Stock solution A	2.67ml		2.67ml
Stock solution E	4.48ml		---
Distilled water	6.94ml		11.24ml

Table 2.2. Solutions for pH 3.5-5.0 IPG's of 0.5mm thickness

Acidic/dense solution (pH 3.5)	Volume	Basic/light solution (pH 5.0)	Volume
Immobiline pK 3.6	319µl		226µl
Immobiline pK 4.6	238µl		331µl
Immobiline pK 6.2	167µl		496µl
Stock solution A	2.67ml		2.67ml
Stock solution E	4.48ml		---
Distilled water	8.13ml		12.28ml

Table 2.3. Solutions for pH 2.8-4.5 IPG's of 0.5mm thickness

Acidic/dense solution (pH 2.8)	Volume	Basic/light solution (pH 4.5) Volume
Acrylamido-derivative pK 0.8	550µl	---
Immobiline pK 3.6	813µl	613µl
Immobiline pK 4.6	292µl	977µl
Immobiline pK 6.2	365µl	1025µl
0.2M NaOH	1058µl	---
Stock solution A	2.67ml	2.67ml
Stock solution E	4.48ml	---
Distilled water	5.78ml	10.72ml

μl each of the TEMED and ammonium persulphate stock solutions were added to the solutions in both chambers before they were poured into the pre-cooled mould.

After the gel had been poured, it was allowed to stand for 10 minutes to enable the gradient to stabilise before being polymerised at 50^{o}C for 1 hour.

pH 4-9 and pH 3.5-5.0 IPG's were removed from the mould and washed for 2 X 20 minutes with distilled water to remove the remaining catalysts and unreacted Immobilines, followed by a 20 minutes wash with 1% (w/v) glycerol. The gels were then dried overnight at room temperature in a dust-free atmosphere, wrapped in plastic bags and stored at -20^{o}C.

pH 2.8-4.5 IPG's were treated differently after polymerisation. They were not washed so that the glycerol density gradient, which is beneficial to IPG's of extremely low pH ranges, was not lost. Instead, they were wrapped in plastic bags and stored at 4^{o}C for up to a week.

Immobilines for focusing below pH 3.5 are not commercially available. In order to focus below this pH, an "Immobiline-type" acrylamido derivative (pK 0.8) was used.

Strongly acidic IPG's have a tendency to burn because of problems arising from the substantial difference between the conductivities of the Immobiline matrix and the bulk liquid, and also from electroosmosis due to the net negative charge around pH 3 which leads to the drying of anodic end of the gel (Righetti et al., 1988). Accordingly, the treat-

ment and focusing of pH 2.8-4.5 IPG's differed from that of pH 4-9 and pH 3.5-5.0 IPG's in the following ways:

(a) The gels were focused without prior washing so that they contained a glycerol gradient with the dense gradient located at the anodic end. The gradient acts as a "conductivity-quencher" which smoothes the voltage across the gel and prevents the anodic end of the gel from drying as a result of electroendosmosis.

(b) The gels were focused at a lower voltage than the other IPG's.

Before focusing, the dried pH 4-9 and pH 3.5-5.0 IPG's were placed in an LKB reswelling cassette and rehydrated for at least 4 hours as follows:

(1) pH 4-9 IPG's were rehydrated with 0.5% (w/v) pH 3.5-10.0 Ampholine in 20% (w/v) glycerol.

(2) pH 3.5-5.0 IPG's were rehydrated with a solution of 8M urea containing 0.5% (w/v) pH 3.5-5.0 Ampholine and 20% (w/v) glycerol.

Glycerol inhibits liquid exudation from the surface of the gels. This exudation of liquid, or "sweating", may adversely affect the final band pattern (Altland et al., 1986).

A limitation of IPG's is their inherently low conductivity which causes difficulties such as slow sample entry, lateral zone spreading and the formation of salt fronts which halt protein migration. Incorporation of carrier ampholyte into IPG's, known as "hybrid" or mixed carrier

ampholyte-immobilised pH gradient gels, helps to overcome the problems which result from the low conductivity of IPG's (Fawcett and Chrambach, 1986a; Altland et al., 1987).

Spaces (0.5cm) were cut between 1.0cm wide tracks in order to prevent lateral dispersion of samples.

2.5.2.(ii) 1mm Thick gels

Prior to electroblotting, IPG's must be removed from their backings so that they may be transferred to the blotting membranes. The presence of charged groups in the Immobiline gels make them adhere tenaciously to any surface, especially to the hydrophilic surface of the GelBond film. Even by using an LKB FilmRemover apparatus, it is often impossible to remove the IPG's from their GelBond backing without damaging them.

In view of this difficulty, the IPG's which were to be electroblotted were cast on a glass, rather than GelBond, surface. They were cast on plain glass plates of dimensions 1.0 X 120 X 250mm. In addition, in order to minimise the possibility of damaging the gel during transfer from the electrophoresis apparatus to the electroblotting apparatus, gels of 1mm thickness were prepared.

Electroblotting was performed only on pH 3.5-5.0 IPG's, which were incorporated with 0.5% (w/v) pH 3.5-5 Ampholine. In addition, the IPG's also contained 4.46M urea to optimise the resolution of the protein bands.

Acidic and basic solutions (15ml of each) sufficient

for preparing one gel, were prepared. The solutions for .1mm thick pH 3.5-5.0 IPG's were prepared according to Table 2.4.

Basic, light solution (14.1ml) was pipetted into the reservoir chamber of the gradient former; and an equal volume of the acidic, dense solution into the mixing chamber. Forty μl each of TEMED and ammonium persulphate stock solutions were added to the solutions in both chambers before these were poured into the pre-cooled mould.

After the gel had been poured, it was allowed to stand for 10 minutes to enable the gradient to stabilise before being polymerised at 50^{o}C for 1 hour, after which it was removed from the mould, wrapped in a plastic bag, and stored for up to 1 week at 4^{0}C before use. One mm thick gels were run without the washing and rehydration steps to which the 0.5mm thick gels were subjected.

2.5.3. Preparation of Samples and Their Application to the Gels

Parotid saliva samples, collected and stored as described in Section 2.2 could be applied to IPG's without prior desalting. For protein loads of 50μg and above, it was necessary to concentrate the samples by lyophilisation and reconstitution with distilled water to a fifth of their original volumes, followed by centrifugation at 10,000g for 20 minutes.

Sample volumes of up to 50 μl were applied to the surface of the IPG's. The optimum protein load was determined

Table 2.4. Solutions for pH 3.5-5.0 IPG's of 1mm thickness.

Acidic/dense solution (pH 3.5)	Volume	Basic/light solution (pH 5.0)	Volume
Immobiline pK 3.6	299µl		212µl
Immobiline pK 4.6	223µl		310µl
Immobiline pK 6.2	157µl		465µl
pH 3.5-5 Ampholine	188µl		188µl
9M urea solution	7.43ml		7.43ml
Stock solution A	2.50ml		2.50ml
Stock solution E	4.20ml		---
Distilled water	---		3.89ml

by the application of various protein loads (from 10-250μg) on a pH 4-9 IPG which was subsequently stained with Coomassie Brilliant Blue G250 followed by a silver stain technique.

The effect of the application position of the sample on the focused pattern was investigated by applying 20μg of proteins (for pH 4-9 IPG's) and 100μg (for pH 3.5-5.0 IPG's) at various positions on the gel. After focusing and staining, the application point which caused the least distortion was determined.

2.5.3. Running Conditions

IPG's were placed on the cooling plate of an LKB 2217 electrofocusing unit. The anolyte was 10mM glutamic acid and the catholyte was 10mM sodium hydroxide.

If salts are present in the samples, application of a voltage across the gel results in the formation of strongly alkaline and acidic boundaries. This may induce protein modification or denaturation. This effect was reduced by initially focusing the gel at low voltage to allow the ionic boundaries to migrate slowly out of the sample application points. pH 4-9 and pH 3.5-5.0 IPG's were initially focused at 500V (constant voltage) for 1 hour thereafter the focusing was performed at maximum voltage 5000V, maximum current 5mA and 5W (constant wattage) at a cooling temperature of 10^{o}C overnight (Righetti et al., 1988). An LKB 2297 Macrodrive 5 constant power unit system was used.

For pH 2.8-4.5 IPG's, a different set of conditions was applied, i.e. 200V (constant voltage) for 5 hours, followed by 1000V (constant voltage) overnight (Gianazza et al., 1987).

2.5.4. Measurement of the pH Gradient

Coloured protein markers were used to determine the pH gradient in IPG's. Wide range standards, pH 4.7-10.6, were loaded onto pH 4-9 IPG's, and narrow range standards, pH 2.4-5.65, onto pH 3.5-5.0 and pH 2.8-4.5 IPG's. Each vial of markers was reconstituted with 100μl of distilled water and 10μl was loaded onto each gel.

The wide-range pI markers (pH 4.7-10.6) consisted of the following proteins:

Protein	pI	M_r
C-Phycocyanin (Asperigillus nidulans)	4.75, 4.85	232,000
Azurin (Pseudomonas aeruginosa)	5.65	14,000
Trifluoroacetylated Myoglobin Met (Porcine)	5.90	17,000
Myoglobin Met (Porcine)	6.45	17,569
Myoglobin Met (Equine)	7.30	17,567
Myoglobin Met (Sperm Whale)	8.30	17,815
Cytochrome C (Horse Heart)	10.60	12,360

The narrow-range pI markers (pH 2.4-5.65) consisted of the following components, which, with the exception of the dye Patent Blue, are proteins:

Protein/dye	pI	M_r
Patent Blue (VF), sodium salt	2.40	567
Amyloglucosidase (Aspergillus)	3.65	99,000
Acetylated Cytochrome C (Horse Heart)	3.95	13,000
Glucose Oxidase (Fungal)	4.25	153,000
C-Phycocyanin (A. nidulans)	4.75, 4.85	232,000
β-Lactoglobulin (A) (Cow's Milk)	5.25	18,363
β-Lactoglobulin (B) (Cow's Milk)	5.35	18,277
Azurin (P. aeruginosa)	5.65	14,000

2.5.5. Effects of Salt Content on the Focused Pattern

Salts contained in samples fractionated in IPG's have been reported to cause protein modification even at low levels (Righetti et al., 1988; Strahler et al., 1988). Oxidation of the iron moiety of haemoglobin occurred at sodium chloride concentrations of above 5mM, and protein precipitation occurred above 50mM sodium chloride (Righetti et al., 1988).

The following experiment was therefore designed to determine the salt concentration at which denaturation of parotid salivary proteins would occur:

Twelve ml of parotid saliva from one individual was

desalted by overnight dialysis against 1 litre of distilled water in a microdialysis unit and concentrated by further overnight dialysis against 30% (w/v) polyethylene glycol. The protein concentration was then determined, and volumes containing 25 μg of protein were mixed with increasing concentrations of sodium chloride (0-600mM). This was accomplished by adding measured volumes of 50mM, 0.1M, 0.5M and 1.0M sodium chloride solutions to fixed volumes of the concentrated saliva. These were then loaded onto a pH 4-9 IPG.

An experiment was also performed to compare the patterns of undialysed and dialysed lyophilised concentrates: Seven 1ml aliquots of parotid saliva from one individual were lyophilised and reconstituted with distilled water in order to prepare solutions of the following concentrations: X1 (i.e. unconcentrated), X2, X3, X4, X5, X6 and X7. Half of each sample was desalted by overnight dialysis, in a microdialysis unit, against distilled water. The IEF patterns of undialysed and dialysed samples were compared on adjacent tracks on a pH 4-9 IPG in order of increasing concentration. Twenty five μg of protein was applied to each track.

The sodium, potassium and calcium concentrations of the unconcentrated and undialysed parotid saliva were determined using a Corning flame photometer and were found to be 18mM, 16mM and 0.2mM respectively.

2.5.6. Preparation of IPG's Containing Urea Concentration Gradients Perpendicular to the pH Axis

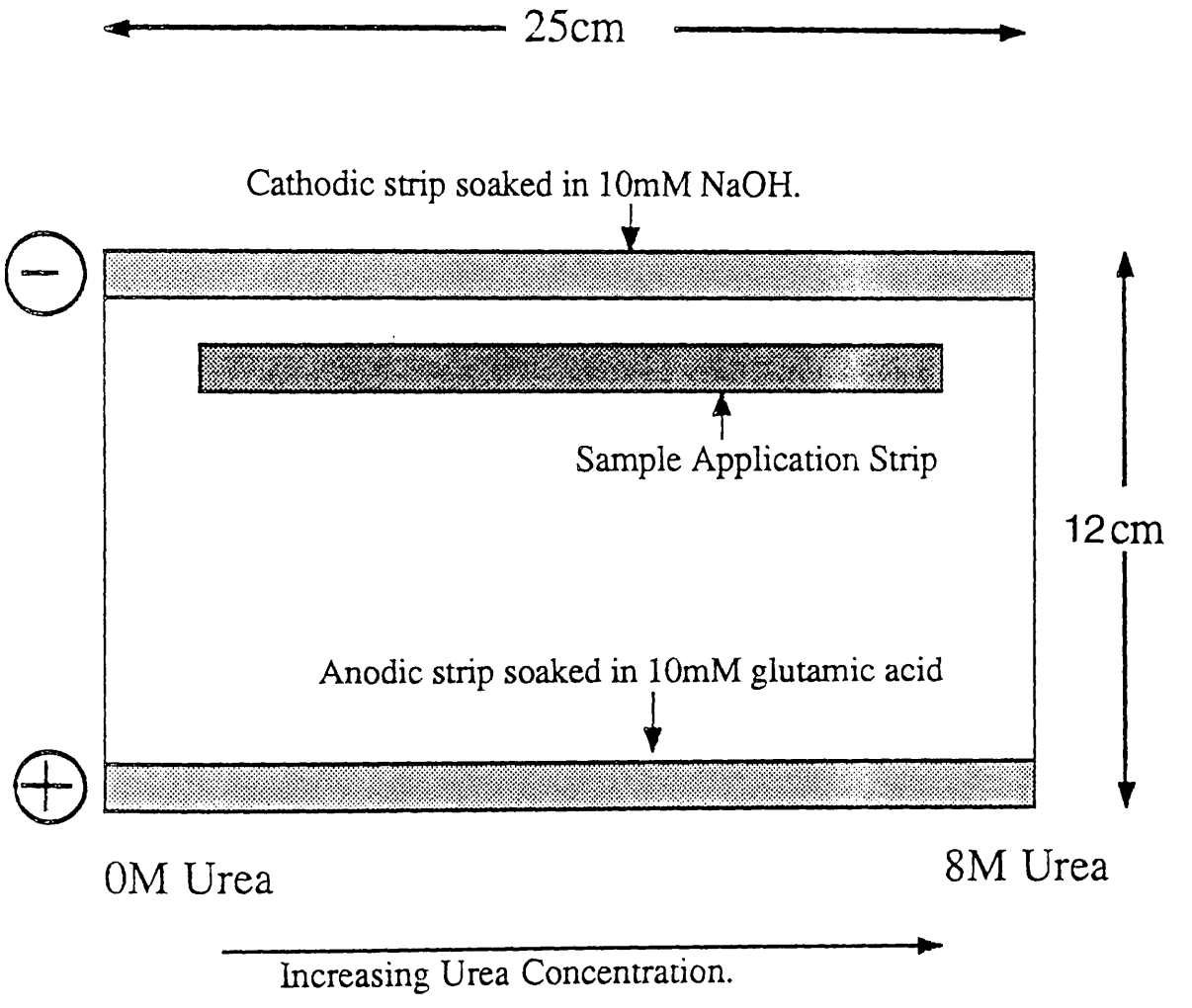
In order to investigate the effects of increasing urea concentrations on the isoelectric points of salivary proteins, dried pH 4-9 and pH 3.5-5.0 IPG's were placed in an LKB re-swelling cassette and rehydrated, using a gradient maker, with a urea concentration gradient of 0-8 M perpendicular to the pH gradient. The reservoir chamber of the gradient former was filled with 8.3ml of distilled water and the mixing chamber was filled with an equal volume of 8M urea solution. After the solutions had been poured, the gel was allowed to rehydrate for 4 hours.

A continuous zone of human parotid saliva was applied 2 cm from the cathode (Fig. 2.4) throughout the length of the gel using a piece of Whatman No.1 filter paper of dimensions 22.0 X 0.5cm as the sample application strip. A volume of unconcentrated parotid saliva (approximately 1ml) containing 1mg protein was applied to the pH 4-9 IPG and a volume of X5 concentrated parotid saliva (approximately 0.2ml, previously concentrated by lyophilisation and reconstitution with distilled water) containing 10.5mg protein was applied to the pH 3.5-5.0 IPG.

2.6. 2-D PAGE using Isoelectric Focusing in Carrier ampholyte-Containing Gels in the First Dimension

The method described is based on an adaptation of O'Farrell's (1975) 2-D procedure (Bio-Rad Bulletin 1244).

Figure 2.4. Diagram of an IPG with a 0 - 8M Urea Gradient Superimposed Perpendicular to the pH Gradient.



2.6.1. Stock Solutions

Stock solutions were prepared as follows:

(A) Acrylamide ("30:08") solution containing 30% (w/v) acrylamide and 0.8%(w/v) N,N'-methylenebisacrylamide.

(B) 10%(v/v) Triton X-100.

(C) 10%(w/v) SDS.

(D) 10%(w/v) APS.

(E) The IEF gel solution consisted of 48.6g urea. 28.8ml distilled water, 11.8ml of solution (A), 20.3ml solution of (B) 4.5ml pH 5-7 Ampholine, and 0.5ml pH 3.5-10.0 Ampholine. This was stored as 5.5ml aliquots at -20°C.

(F) The overlay buffer, consisted of 3g urea, 2ml of solution (B) 0.45ml Ampholine, pH 5-7, and 0.05ml Ampholine, pH 3.5-10.0 made up to 10 ml with distilled water.

(G) The IEF sample concentrate consisted of 0.1ml of solution (C), 0.02ml pH 3.5-10.0 Ampholine, 0.18ml pH 5-7 Ampholine, 0.1ml of 2-mercaptoethanol and 0.2ml of undiluted Triton X-100.

(H) 0.1M sodium hydroxide solution was used as the catholyte.

(I) 0.06% (w/v) phosphoric acid solution was used as the anolyte.

(J) The IEF gel equilibration buffer consisted of 12.5ml of 0.5M Tris-HCl, pH 8.8, 5.5 ml of 2-mercaptoethanol solution, 10ml of solution (C) and 0.01g of bromophenol blue made up to 100ml with distilled water.

Solutions (H) and (I) were degassed prior to use.

2.6.2. Preparation of First Dimension Gels

Solution (E) 5.5ml was thawed and degassed for 1 minute, followed by the addition of 5 µl of TEMED and 10 µl of APS solution (D). This solution was then used to fill a 10ml syringe fitted with 25cm of fine tubing. The tubing was inserted to the bottom of 18cm long glass tubes (1.5mm internal diameter), the bases of the tubes had been previously sealed with 2 layers of parafilm. The tubes were slowly filled to a height of 10cm, with care being taken to dislodge any bubbles trapped at the base. Another syringe was used to overlay the tubes with 20 µl of solution (F). Polymerisation was usually complete within 1 hour.

2.6.3. Preparation and Loading of the Samples

Ten ml aliquots of parotid saliva were desalted by overnight dialysis against 1 litre of distilled water, concentrated by using Aquacide and centrifuged at 10,000g for 20 minutes at 4^{0}C. The protein concentrations of the samples were then determined, and a volume of saliva containing 100 µg of protein was added to an equal volume of glycerol. Urea (0.5mg) was added and mixed, followed by 1 µl of solution (G) per 5 µl of sample.

The parafilm and overlay solution were removed from the rod gels and the saliva samples were applied to the top of the gels using a Hamilton microlitre syringe. After sample

application, 10 μl of solution (F) was overlayed onto each tube. These were then filled to the top with 0.1M sodium hydroxide solution. This system is illustrated in Fig. 2.5.

2.6.4. Running Conditions

Phosphoric acid solution (2.5 litres, 0.06%(w/v)) was added to the lower reservoir and 0.5 litres of 0.1M sodium hydroxide solution to the upper reservoir. A Bio-Rad Protean II electrophoresis apparatus was used in conjunction with an LKB 2103 constant power unit. IEF was carried out at 400V for 12-15 hours, followed by 800V for 2 hours.

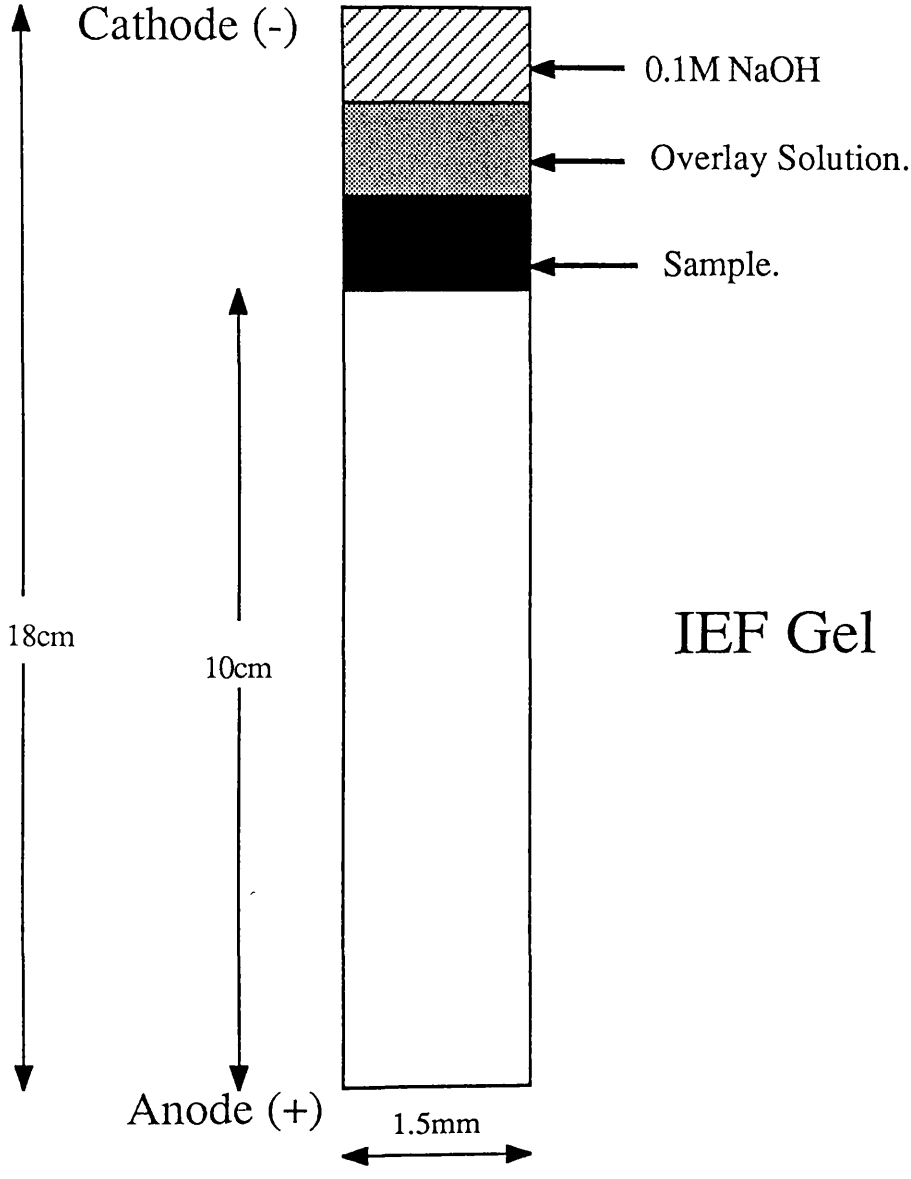
2.6.5. Removal and Equilibration of the IEF Rod Gels

The rod gels were removed by first loosening them with a solution of 10% (w/v) glycerol. This was applied using a syringe with a short length of fine tubing. Each tube was then connected to a low pressure air line and the gels carefully extruded. Each gel was shaken for 10 minutes with 3 ml of IEF equilibration buffer (J), after which the solution was removed and the gel was either applied directly to the SDS gel or stored at -20^{o}C.

2.6.6. SDS-PAGE

SDS-PAGE was performed on either 12.5 %T polyacrylamide gels or 5-20 %T linear gradient polyacrylamide gels. Electrophoresis was carried out at a constant voltage of 60V and a cooling temperature of 4^{o}C overnight, and con-

Figure 2.5. IEF Rod Gel for The First Dimension Separation of Proteins In O'Farrell-Type Two-Dimensional PAGE.



tinued until the dye front reached the bottom of the gel. A Shandon Vokam SAE 2761 power supply unit was used.

2.7. 2-D PAGE with IPG's in the First Dimension

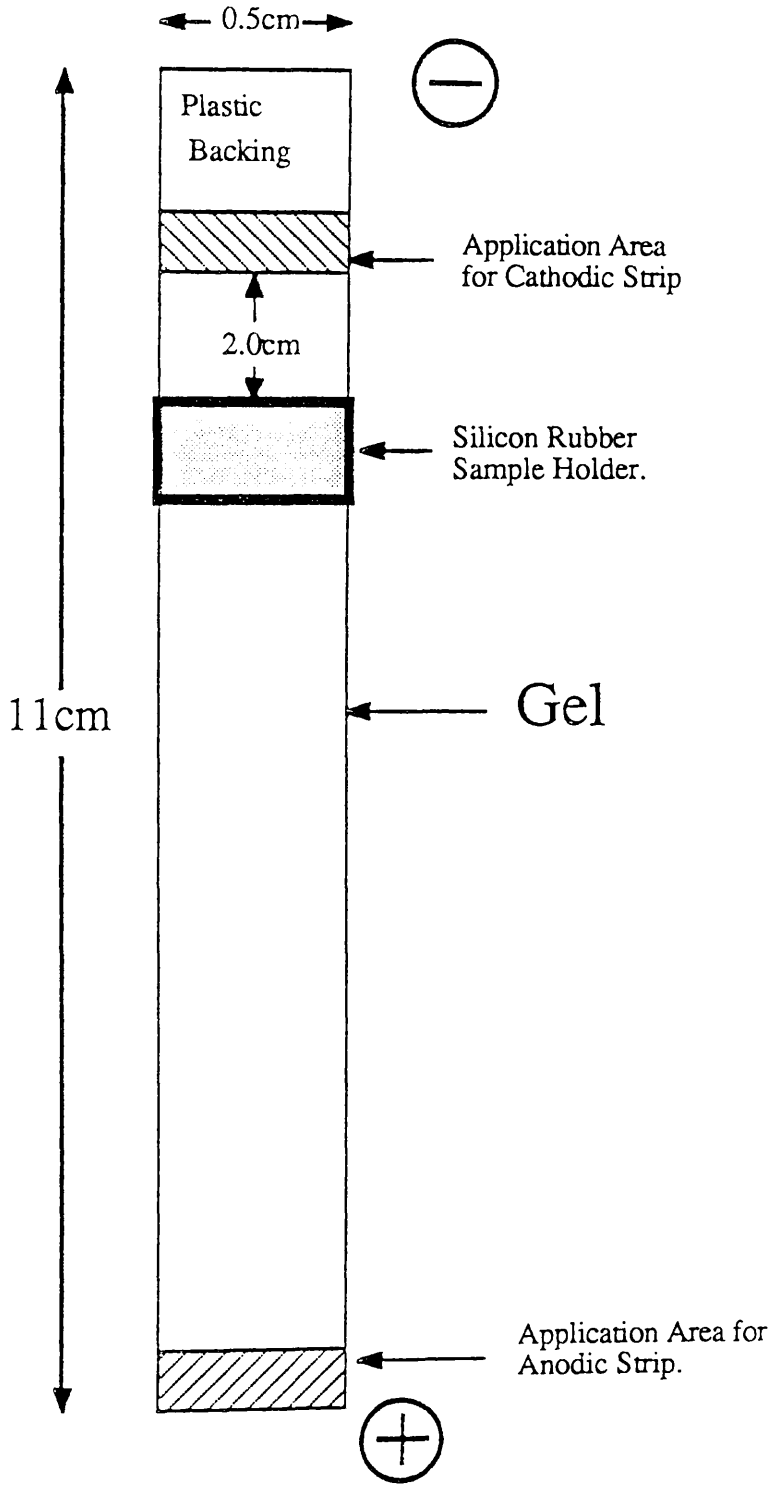
2.7.1. First Dimension

2.7.1(i) Preparation of the Gels

0.5mm thick pH 4-9 and pH 3.5-5.0 IPG's were prepared, washed and dried as described in Section 2.5.2. The dried IPG's were cut into 5mm wide strips, using a Rotatrim precision paper cutter (Görg et al.,1985). 20 strips were prepared for each run. The IPG strips were rehydrated in an LKB rehydrating cassette for at least 4 hours before use. The rehydration solutions for the IPG strips consisted of 8M urea and 0.5 % (w/v) Ampholines dissolved in 20% (w/v) glycerol solution. pH 3.5-10.0 Ampholines were used for the pH 4-9 IPG's and pH 3.5-5 Ampholines for the pH 3.5-5.0 IPG's.

After the IPG strips had been rehydrated, they were lightly pressed between 2 sheets of moistened Whatman No.1 filter paper in order to remove excess urea solution. They were then placed 2mm apart on the cooling plate of an LKB 2217 Ultrophor electrofocusing unit. Pharmacia-LKB silicon rubber sample applicators, which can contain sample volumes of up to 40μl, were placed on the IPG strips at a distance of 2cm from the cathode (Fig. 2.6). The saliva samples were applied in the wells of the sample applicators using micropipettes.

Figure 2.6. IPG Strip for the First Dimension Separation of Proteins in 2D-PAGE.



2.7.1.(ii) Running Conditions

An LKB 2297 Macrodrive 5 constant power supply unit was connected to the electrofocusing unit. For improved sample entry, a low constant voltage (300V) was applied for the first hour of IEF, after which the conditions were changed to 3000V (maximum voltage), 2mA (maximum current) and 5W (constant power) overnight at a cooling temperature of 10^{0}C (Görg et al., 1988). Glutamic acid solution (10mM) was used as the anolyte and 10mM sodium hydroxide as the catholyte.

2.7.1(iii) Equilibration of the IPG Strips

After focusing, each IPG strip was placed in a test tube and equilibrated, using a mechanical shaker, for 15 min in 10ml of an equilibration buffer of 0.05M Tris-HCl, pH 6.8 containing 2% (w/v) SDS, 65mM DTT, 6M urea and 30% (w/v) glycerol containing a few grains of bromophenol blue (Görg et al., 1987). This was followed by shaking for a further 15 minutes in 10ml of fresh equilibration buffer containing 260mM iodoacetamide.

Thiol-reducing agents such as DTT are capable of partially solubilising particulates which eventually cause the point-streaking observed in silver stained 2-D gels. In order to overcome this problem, iodoacetamide was added to the equilibration solution. Iodoacetamide carboxymethylates excess DTT in the equilibration solution, and thus reduces the

occurrence of point-streaking.

After the IPG strips had been equilibrated, they were placed on edge on a dry filter paper and allowed to drain thoroughly. If not used immediately, they were frozen and stored at -20^{o}C.

2.7.2. Second Dimension

2.7.2(i) Linear Polyacrylamide Gradient SDS-PAGE

For the second dimension of protein fractionation, the equilibrated IPG strip was placed on the surface of a horizontal linear polyacrylamide gradient SDS-PAGE gel.

2.7.2(ii) Stock Solutions

The following stock solutions were prepared:

(A) Acrylamide ("30:08") solution containing 30% (w/v) acrylamide and 0.8 (w/v) N,N'-methylenebisacrylamide.

(B) 3M Tris-HCl buffer, pH 8.8

(C) 10% (w/v) SDS solution.

(D) 95% (w/w) glycerol solution.

(E) 20% (w/v) ammonium persulphate solution.

(F) 10% (v/v) TEMED.

2.7.2.(iii) Preparation of the Gradient Gels

The gel mould was prepared as described in Section 2.5.1.. All gels were cast on GelBond PAG film.

A gradient former was used to pour the 3-15 %T linear gradient polyacrylamide gels. Sixteen ml of each solution, sufficient for two gels, was prepared according to Table

2.5.

Light solution (7.8ml) was pipetted into the reservoir chamber of the gradient former and an equal volume of the dense solution into the mixing chamber, followed by 20 μl each of TEMED and ammonium persulphate solutions into each.

After the gel had been poured, it was allowed to stand for 10 minutes before it was polymerised at 50^{o}C for 1 hour. Upon the removal of the gel from the mould, unless immediately used, it was sealed in a plastic bag and stored at 4^{o}C for up to a week.

2.7.2.(iv) Transfer of IPG Strips to SDS gels

Two IPG strips were placed on an SDS gel. Each strip was placed on the SDS gel with its gel-side facing downwards and its anodic end on the left hand side (Fig. 2.7).

2.7.2.(v) Running Conditions

An LKB 2217 Multiphor electrophoresis unit was used in conjunction with an LKB 2103 power supply system. The reservoir buffer was 0.1% (w/v) SDS, 0.025M Tris, 0.192M glycine and 0.01% (w/v) sodium azide, pH 8.3. The electrode wicks were placed so that they covered 2cm of each end of the gel (Fig 2.8). The cooling temperature was 10^{o}C.

For the first 90 minutes, a maximum voltage of 200V was applied to facilitate the slow entry of proteins from the IPG strips into the matrix of the gel. After this, the IPG strips were removed and the cathode wick was repositioned so

Table 2.5. Solutions for linear gradient SDS-PAGE gels for the second dimension of 2-D PAGE

Light solution (3 %T)

Solution	Volume (ml)
Stock solution A	1.60
Stock solution B	2.00
Stock solution C	0.16
Distilled water	12.24

Dense solution (15 %T)

Solution	Volume (ml)
Stock solution A	8.00
Stock solution B	2.00
Stock solution C	1.60
Distilled water	1.36
Stock solution D	4.48

Figure 2.7. Positioning of the IPG Strips on an SDS Polyacrylamide Gel for Two-Dimensional PAGE.

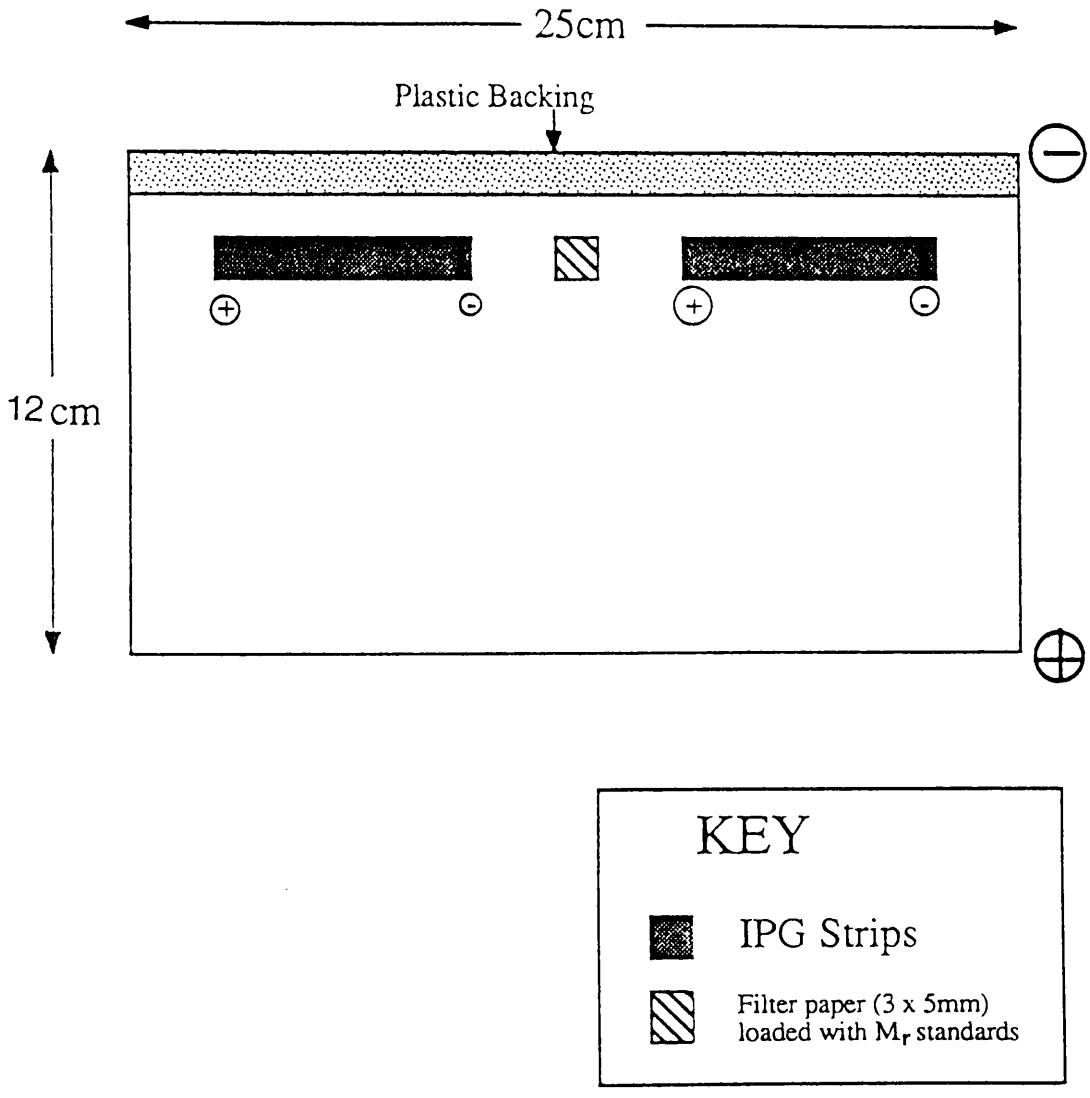
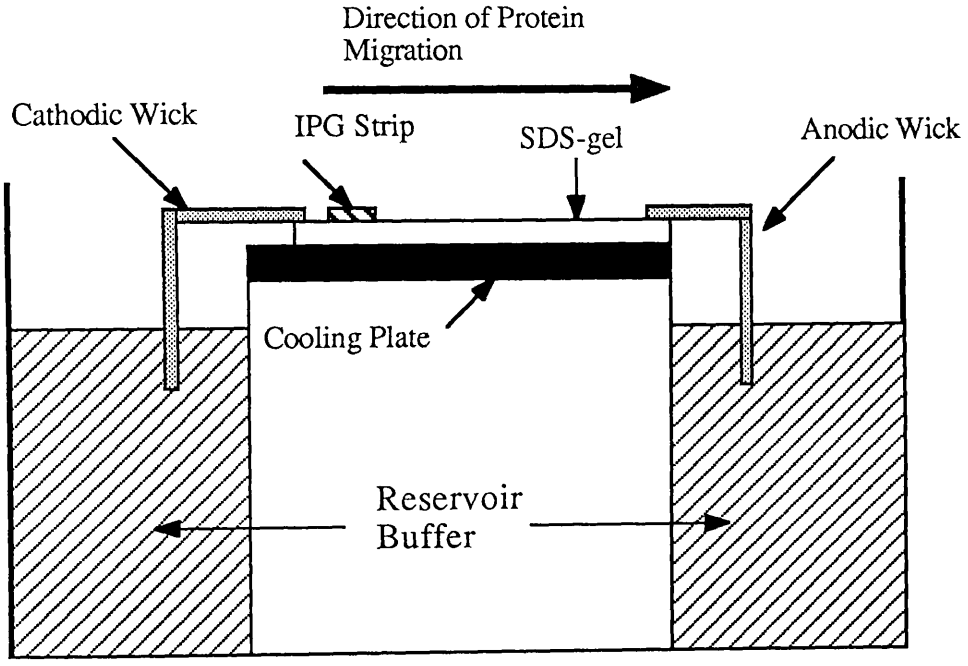


Figure 2.8. Cross-Section , Parallel to the Direction of Protein Migration, of an Electrophoresis Tank for the Second Dimension of Two-Dimensional-PAGE.



that it covered the application sites of the IPG strips on the gel. The running conditions were then changed to 600V (maximum voltage), 30mA (constant current) and 30W (maximum wattage), until the dye front reached the anodic of the gel. This took approximately 90 minutes.

2.8. Staining Procedures

2.8.1. Coomassie Brilliant Blue G250 Stain

A colloidal suspension of CBB G250 in trichloroacetic acid (TCA) and perchloric acid (PCA) was prepared as described by Mairs (1981; p.65):

Two g of CBB G250 was dissolved in 100ml of distilled water. One hundred ml of 1M sulphuric acid solution was added and the mixture was stirred for 1 hour. The precipitate formed was allowed to settle overnight, and the solution was passed through a Whatman No. 1 filter paper. The pH of the filtrate was raised to 5.5 by the addition of 10M potassium hydroxide solution. Finally, PCA, TCA and distilled water were added to give a total volume of 400ml and final concentrations of 0.5% (w/v) CBB G250, 3.5% (w/v) PCA and 10% (w/v) TCA.

Gels were fixed and stained in this solution for 1 hour before being destained in 10% (v/v) acetic acid.

2.8.2. Comassie Brilliant Blue R250 Stain

Half mm thick gels were fixed for 30 minutes in 10% (w/v) TCA containing 3.5% (w/v) PCA prior to staining, while

1.5mm thick gels were Coomassie-stained without prior fixing.

The staining and destaining procedures were performed as described by Lindsay and Beeley (1986):

The gel was fixed and stained in a solution of 0.1% (w/v) CBB R250 in 40% (v/v) ethanol containing 10% (v/v) acetic acid for 3 hours, after which it was destained with several changes of 10% (v/v) acetic acid over a period of 2-3 days.

2.8.3. Silver Stain

The procedure for silver stain is a modification of the procedure of Damerval et al. (1987) and is outlined in Table 2.6.

2.8.4. PAS Stain for Glycoproteins

2.8.4 (i) Preparation of Schiff's Reagent

The Schiff's reagent was prepared according to the method of Kapitany and Zebrowski (1973).

One g of pararosaniline hydrochloride was dissolved in 200ml of boiled, distilled water, removing the flask from the heat just before addition of the dye. The solution was cooled to 50^{o}C in cold water and 2g of potassium metabisulphite was added. After cooling to room temperature, 2ml of concentrated hydrocholoric acid was added and the solution mixed and allowed to stand overnight in the dark. Powdered, activated charcoal (0.2g) was added and the flask shaken for

Table 2.6. Procedure for silver staining.

Step	Solution	Duration
1. Fix.	10% (w/v) trichloroacetic acid containing 10%(w/v) perchloric acid.	30min.
2. Fix.	2.5% (w/v) glutaraldehyde in 1M sodium acetate solution.	30min.
3. Wash.	distilled water.	4 X 30min.
4. Sensitise.	Farmer's Reducer consisting of 0.15% (w/v) potassium hexacyanoferrate III, 0.3% (w/v) sodium thiosulphate and 0.05% (w/v) sodium carbonate.	1min.
5. Wash.	distilled water.	2 X 15min.
6. Silver.	0.1% (w/v) silver nitrate.	30min.
7. Rinse.	distilled water.	2 X 10sec.
8. Rinse.	developer (0.5% (w/v) sodium carbonate in 0.2% (w/v) formaldehyde).	2 X 10sec.
9.Develop.	developer (as above).	as required, normally 3-8mins.
10. Stop.	5% (v/v) acetic acid.	10min.
11. Rinse.	distilled water.	2 X 10min.
12. Store.	5% (w/v) glycerol.	

10 minutes. The mixture was filtered through a Whatman No. 1 filter paper. The charcoal treatment was repeated if necessary until the solution was pale yellow to clear in colour. The reagent was stored in a dark bottle at 4°C.

2.8.4(ii) Schiff Staining Procedure

The method of Glossmann and Neville (1971), which involves elution of SDS from the gel, was used. Other procedures such as that of Kapitany and Zebrowski (1973) do not work well with SDS-containing gels because SDS causes high background staining. The procedure, to which the gels are subjected immediately after electrophoresis, is outlined in Table 2.7.

2.9. Protein Blotting

2.9.1. Electroblotting Procedure

An LKB 2117-005 Multiphor II Novablot System, which is a unit for semi-dry horizontal electrophoretic transfer, was used. A semi-dry transfer technique is one which uses filter paper soaked in electrode solution as the only reservoir buffer.

2.9.2. (i) Electroblotting of Proteins onto Nitrocellullose Membranes

The stock solutions consisted of the following, as recommended by the LKB Multiphor II Novablot Instructions Manual:

(1) For the transfer of proteins from SDS gels, a continuous

Table 2.7. Procedure for Schiff stain.

Step	Solution	Duration and conditions
1. Wash.	40% (v/v) methanol in 7% (v/v) acetic acid.	overnight.
2. Wash.	40% (v/v) methanol in 7% (v/v) acetic acid.	8hrs.
3. Oxidise.	1% (v/v) periodic acid in 7% (v/v) acetic acid.	1hr. at 4°C in the dark.
4. Incubate.	Schiff's Reagent.	1hr. at 4°C in the dark.
5. Wash.	1% (w/v) sodium thiosulphate in 0.1M hydrochloric acid.	4 X 10min.
6. Store.	Schiff's Reagent.	

buffer consisting of 39mM glycine, 48mM Tris and 0.035% (w/v) SDS in 20% methanol was used.

(2) For the transfer of proteins from IEF gels, a discontinuous buffer system, consisting of these solutions, was used:

Anode solution No.1 (pH 10.4) was 0.3M Tris,

Anode solution No.2 (pH 10.4) was 0.1M Tris, and

Cathode solution (pH 10.5) was 0.1M arginine in 0.01%(w/v) SDS.

As gels cast on GelBond PAG film tend to adhere tenaciously to their backings they had to be removed using an LKB FilmRemover Unit which separates the gel from its backing by drawing a thin wire between the gel and the backing.

The transfer unit assembly is illustrated in Fig. 2.9. A current of 0.8mA per cm^2 of the membrane was applied for 2 hours.

2.9.2. (ii) Electroblotting of Proteins onto Polyvinylidene difluoride Membranes

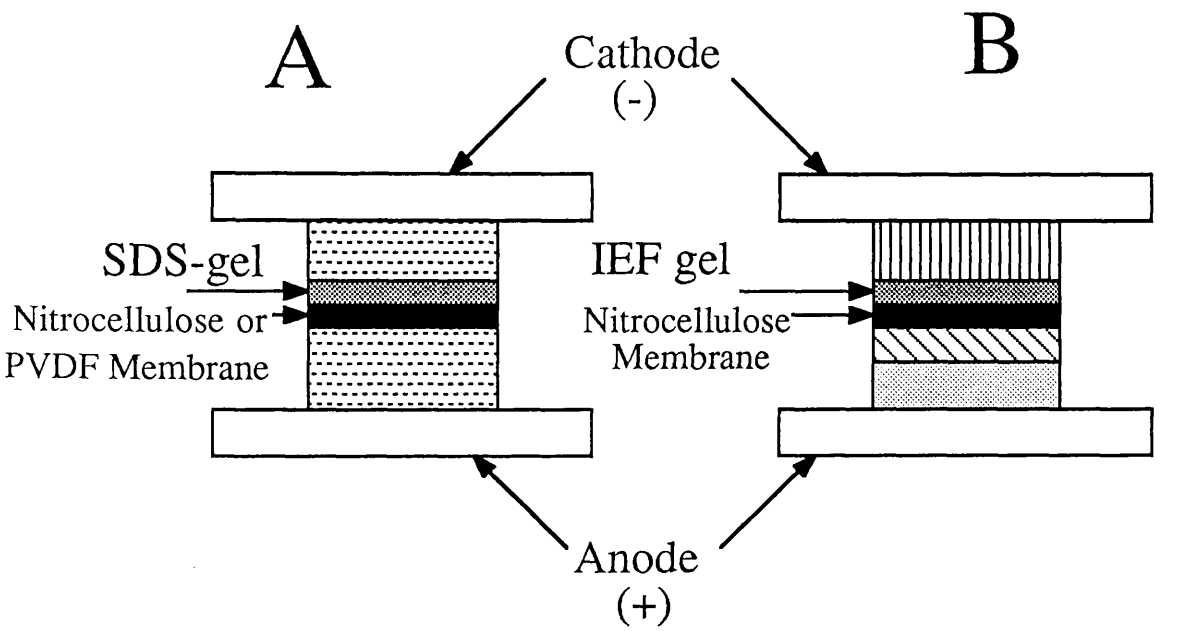
The procedure of Matsudaira (1987) was used. The transfer buffer consisted of 10mM 3-(cyclohexylamino)-1-propane sulphonic acid (CAPS), pH 11.0 (titrated to this pH value with 2M sodium hydroxide solution) in 10% (v/v) methanol. A piece of PVDF membrane was rinsed briefly in pure methanol and washed for 5 minutes in the transfer buffer.

Following SDS-PAGE, the gel was soaked for 5 minutes in the transfer buffer in order to reduce its content of Tris

Figure 2.9. Assembly of Electroblotting Transfer Unit.

A. For blotting of SDS Gels

B. For blotting of IEF Gels.



KEY.

9 layers of filter paper soaked in blotting buffer

9 layers of filter paper soaked in Cathode Solution

3 layers of filter paper soaked in Anode Solution No. 2

6 layers of filter paper soaked in Anode Solution No. 1.

and glycine, after which it was sandwiched between a sheet of PVDF membrane and 9 sheets of Whatman No. 1 filter paper on each side. A current of 0.8mA per cm^2 of the membrane was applied for 2 hours.

2.9.3. Methods for the Detection of Proteins on Blots

2.9.3.(i) General Staining of Proteins by India Ink

In order to check the efficiency of the electroblot and for the precise location of the protein bands or spots, it is necessary to stain all the proteins which have been electroblotted onto the nitrocellulose membrane. For this, the India ink staining method of Sutherland and Skerritt (1986) was used. The procedure for the treatment of nitrocellulose membranes to be stained after electroblotting is outlined in Table 2.8.

2.9.3.(ii) Coomassie Brilliant Blue Staining of Proteins Electroblotted onto PVDF

The procedure recommended by Matsudaira (1987) was used: after electroblotting had been carried out, the PVDF membrane was washed for 5 minutes in distilled water, stained with 0.1% (w/v) CBB R250 in 50% (v/v) methanol for 5 minutes and subsequently destained in several changes of 50% (v/v) methanol and 10% (v/v) acetic acid for 10 min. The membrane was washed for 3 X 5 minutes with HPLC-grade water and air-dried in a dust-free atmosphere. The stained protein bands of interest were cut out using a clean scalpel and

Table 2.8. Procedure for India Ink staining of proteins following electroblotting onto nitrocellulose membranes

Step	Solution	Duration
1. Sensitise.	1% (w/v) potassium hydroxide.	5min.
2. Wash.	10% (v/v) methanol in PBS for SDS-PAGE gels, PBS for non-SDS PAGE gels.	2 X 10min.
3. Stain.	0.5% (v/v) India ink and 1% (w/v) Tween 20 in PBS.	1hr.
4. Wash.	PBS.	2 X 10min.

stored in Eppendorf tubes at -20°C.

2.9.3.(iii) Detection of Antigens by Alkaline Phophatase-Conjugated Anti-Antibody

A modification of the method of Eap and Baumann (1988), based on the procedure of Blake et al. (1984) was used.

Stock 5 mg/ml nitroblue tetrazolium (NBT) and 3 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solutions were prepared, stored frozen at -20°C, and thawed just prior to use. The alkaline phosphatase substrate solution, which was prepared just before use, consisted of the following reagents: 2ml of NBT stock solution, 1.6ml of BCIP stock solution, 1.2ml of 0.1 M magnesium chloride, 1.2 ml of 0.1M zinc chloride and 94ml of 0.05 M sodium carbonate-sodium hydrogen carbonate buffer, pH 9.6.

The alkaline phosphatase-conjugated anti-antibody method for the detection of proteins electroblotted onto the nitrocellulose membranes is outlined in Table 2.9.

2.9.3.(iv) Detection of Antigens Using Immune Complexes of Alkaline Phosphatase and Monoclonal Anti-Alkaline Phosphatase (APAAP Complexes)

Western blotting followed by the detection of proteins by the alkaline phosphatase-conjugated anti-antibodies occasionally results in weak staining reactions, particularly if the antigen is present in small amounts (< 1μg). In order to intensify staining, an immune complex of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP

Table 2.9. Procedure for the detection of antigens by alkaline phophatase-conjugated anti-antibody

Step	Solution	Duration
1. "Quenching" of the remaining sites of attachment.	1% (w/v) Tween 20 in PBS (Tween-PBS solution)	2 X 30min.
2. Incubation in a specific antiserum to antigen	1:1000 dilution of the antiserum in Tween-PBS.	3hrs.
3. Wash.	Tween-PBS.	3 X 10min.
4. Incubation in alkaline phosphatase-conjugated anti-antibody.	1:1000 dilution of the conjugate in Tween-PBS.	2hrs.
5. Wash.	Tween-PBS.	3 X 10min.
6. Wash.	0.05M sodium carbonate-sodium hydrogen carbonate buffer, pH 9.6.	2 X 5min.
7. Develop.	Alkaline phosphatase substrate solution.	Up to 2hrs.at 37^{0}C.
8. Wash.	distilled water.	2 X 10min.

complex) may be used (Cordell et al., 1984). The APAAP complex consists of 4 molecules of alkaline phosphatase enzyme complexed to an equal number of mouse monoclonal antibodies. The rabbit antibody against mouse IgG is used as a "bridge" between two APAAP complexes. One stage of incubation in the APAAP complex would theoretically attach 4 molecules of alkaline phosphatase to each molecule of the first antibody, while an additional one and two stages would add 16 and 54 molecules, respectively, of alkaline phosphatase molecules.

The APAAP method for the detection of proteins which have been electroblotted onto nitrocellulose membranes is outlined in Table 2.10.

2.9.3.(v) Detection of Concanavalin A-Binding Glycoproteins

Concanavalin A (ConA) is a lectin which preferentially binds to α-D-mannopyranosyl and α-D-glucopyranosyl residues. Hawkes (1982) described a method in which ConA was used as a probe for glycoproteins which had been electroblotted onto nitrocellulose. In the second stage, horse-radish peroxidase (HRP), a ConA-binding glycoprotein was added. Finally, the membrane was incubated in a substrate solution for HRP. A modification of this method was used.

As ConA is capable of binding small hydrophobic molecules at a different site from that which binds carbohydrates (Edelman and Wang, 1978), a control experiment was performed in which α-D-methyl mannoside, a sugar which

Table 2.10. Detection of antigens using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes)

Step	Solution	Duration
1. "Quenching" of the remaining sites of attachment.	1% (w/v) Tween 20 in PBS (Tween-PBS solution).	2 X 30min.
2. Incubation in goat antiserum to antigen	1:1000 dilution of the antiserum in Tween-PBS.	3hrs.
3. Wash.	Tween-PBS.	3 X 10min.
4. Incubation in mouse monoclonal antibody against goat IgG.	1:1000 dilution of the antibody in Tween-PBS.	2 hrs.
5. Wash.	Tween-PBS.	3 X 10min.
6. Incubation in rabbit antiserum against mouse IgG.	1:1000 dilution of the antibody in Tween-PBS.	2hrs.
7. Wash.	Tween-PBS.	3 X 10min.
8. Incubation in APAAP complex.	1:1000 dilution of the complex in Tween-PBS.	2hrs.
9. Wash.	Tween-PBS.	3 X 10min.
10. Repeat Step 6.		
11. Wash.	Tween-PBS.	3 X 10min.
12. Repeat Step 8.		
13. Wash.	Tween-PBS.	3 X 10min.
14. Repeat Step 6.		
15. Wash.	Tween-PBS.	3 X 10min.
16. Repeat Step 8.		
17. Wash.	Tween-PBS.	3 X 10min.
18. Wash.	0.05M sodium carbonate-sodium hydrogencarbonate buffer, pH 9.6.	2 X 5min.
21. Develop.	Alkaline phosphatase substrate solution.	Up to 2hrs at 37^{0}C.
22. Wash.	distilled water.	2 X 10min.

N.B. As a result of the number of steps involved, it was necessary to carry out the whole procedure over a period of 2-3 days. Thus when it was necessary, the incubation in an antibody- or APAAP complex-containing solution was performed overnight at 4^{0}C, and the procedure was continued the following day.

has a strong inhibitory effect on ConA-binding, was added to the ConA incubating solution.

The following stock solutions were prepared:

(a) 0.2M sodium chloride in 50mM Tris-buffered saline (TBS), pH 7.4.

(b) 0.1M sodium acetate-acetic acid buffer solution, pH 5.6.

(c) 0.3% (w/v) 4-chloro-1-napthol solution in methanol. This was stored in the dark at 4^{0}C.

(d) Horse-radish peroxidase substrate (HRP) solution consisting of 0.06% (w/v) 4-chloro-1-naphthol and 0.01% (v/v) hydrogen peroxide in 0.1M sodium acetate-acetic acid buffer solution, pH 5.6.

The procedure for the detection of glycoproteins which had been electroblotted onto nitrocelluose membranes is outlined in Table 2.11.

2.10. Immunochemical Techniques

2.10.1. Double Immunodiffusion

A modification of the procedure recommended by Johnstone and Thorpe (1987; pp. 133-135), which is a variation of the original method of Ouchterlony (1948), was used.

GelBond PAG film was cut into 26 X 76mm-sized pieces. A 1% (w/v) agar solution was prepared by heating 1g of agar in PBS-azide until completely dissolved. When the agar solution had cooled to 45^{o}C, 3.5ml was pipetted onto the hydrophilic side of each piece of GelBond film which had been placed on a levelling table. A template was placed over

Table 2.11. Procedure for the detection of ConA-binding glycoproteins following electroblotting onto nitrocellulose membranes

Step	Solution	Duration
1. "Quenching" of the remaining binding sites.	1% (w/v) gelatin in Tris-buffered saline (Gelatin-TBS).	2 X 30min.
2. Incubation in ConA-containing solution.	50μg/ml ConA in Gelatin-TBS. In the control, this solution also contained 0.1M α-D-methyl mannoside.	1hr.
3. Wash.	Gelatin-TBS.	3 X 10min.
4. Incubation in horse-radish peroxidase (HRP)-containing solution.	50μg/ml HRP in Gelatin-TBS.	1hr.
5. Wash.	Gelatin-TBS.	3 X 10min.
6. Wash.	0.1M sodium acetate-acetic acid buffer, pH 5.6.	10min.
7. Develop.	HRP substrate solution.	up to 30min.
8. Wash.	distilled water.	2 X 10min.

the solidified agar, and holes were punched into the agar using a gel punch. Each well had a capacity of 20 μl. The centre well was filled with the undiluted antigen, and the six surrounding wells were filled with varying dilutions of the antiserum; i.e. undiluted, 1/2, 1/4, 1/8, 1/16 and 1/32; and incubated overnight in a humid box at room temperature, after which the gel was checked for immunoprecipitin lines. If staining was necessary to visualise the immunoprecipitin lines, the gel was washed five times over 2 days with 100ml of PBS-azide, stained for 2 hours with 0.1% (w/v) CBB R250 in 40% (v/v) ethanol containing 10% (v/v) acetic acid, and destained with several changes of 10% (v/v) acetic acid over 2-3 days.

2.10.2. Latex Slide Test for Rheumatoid Factors

The Latex RF (rheumatoid factor reagent) contains latex particles coated with human γ-globulins which agglutinate in the presence of rheumatoid factors.

Parotid saliva samples were lyophilised and reconstituted with distilled water to a fifth of their original volumes and centrifuged as described in Section 2.2. 20 μl of each concentrated sample was mixed with an equal volume of the Latex RF reagent. A positive reaction was indicated by agglutination while a negative reaction was indicated by a homogeneous suspension.

2.10.3. Production of a Rabbit Antiserum against Human Urinary Kallikrein

2.10.3. (i) Antiserum Production

The antiserum was produced using a modification of the the procedure of Catty and Raykundalia (1988).

Each vial of the commercially obtained kallikrein contained 0.2 Units (i.e. 40μg) of the enzyme dissolved in 0.5 ml of 50% glycerol buffered with 50mM Tris-HCl, pH 7.75. Half of this was mixed with 0.5ml of 0.15M sdoium chloride solution and 0.5ml of Freund's Complete Adjuvant (FCA), and an emulsion formed. This was injected subcutaneously into a rabbit at 4 different sites.

After 4 weeks, a booster injection containing an equal amount of kallikrein was administered in a similar manner, with the single difference that Freund's Incomplete Adjuvant (FIA) was used instead of FCA. One week after the booster dose, 10ml of serum was harvested by bleeding the rabbit from a marginal ear vein. A week later, a further 10ml was collected.

Following collection, the blood was allowed to clot for 1 hour at 37^{o}C, and left overnight at 4^{o}C to allow the clot to retract. The red cells were removed by centrifuging the serum at 1,500 r.p.m. at 4^{o}C for 20 minutes. The supernatant was removed and stored at -20^{o}C.

In accordance with the Animals (Scientific Procedures)Act, 1986, the procedures on the rabbits were carried out by personnel holding the necessary licenses.

The activity of the antiserum was tested by double im-

munodiffusion using human urinary kallikrein as the test antigen. Human serum albumin, human IgG and human colostral sIgA were used as control antigens.

2.10.3.(ii) Purification of Rabbit IgG

The isolation of IgG from the rabbit antiserum to urinary kallikrein was carried out as recommended by Johnstone and Thorpe (1987; pp.51-52).

The standard working temperature for all incubations and centrifugations was 25°C. Ten ml of serum was warmed to this temperature. Sodium sulphate was then added and dissolved by stirring to make an 18% (w/v) solution. This was then incubated for 30 minutes, and centrifuged at 3,000g for 30 minutes. The supernatant was discarded, and the protein precipitate was redissolved in distilled water to a final volume of 25ml. After warming the solution to the working temperature, sodium sulphate was added and dissolved to make a 14% (w/v) solution. This was then incubated for 30 minutes, and centrifuged at 3,000g for 30 minutes. Again, the supernatant was discarded and the precipitate was redissolved in water to a final volume of 3ml. This solution was then dialysed overnight against 10mM sodium phosphate buffer, pH 8.0. At this stage, a partially purified IgG solution was obtained.

The Whatman DE32 anion exchanger was equilibrated overnight against 0.07M sodium phosphate buffer, pH 8.0, and 15ml wet settled volume of the exchanger was added to the

partially purified IgG solution. This mixture was centrifuged at 3,000g for 30 minutes. The IgG molecules were contained in the supernatant which was collected and its antibody activity against human urinary kallikrein was tested by double immunodiffusion. It was then stored as 50 μl aliquots at -20^{o}C.

2.11. High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is an efficient method of fractionating mixtures of macromolecules. Proteins vary widely in their properties, and it is therefore possible to fractionate them according to their differences in charge, size, hydrophobicity and affinity for lectins. A charge fractionation method (ion-exchange chromatography) was used for fractionating salivary proteins.

In ion-exchange chromatography, a protein which has a region of charge on its surface, opposite in sign to the fixed charge of the stationary phase, will remain bound to the fixed charge of the ion-exchanger until it is displaced by buffer ions of sufficiently high ionic strength.

A Pharmacia Fast Protein Liquid Chromatography (FPLC) unit fitted with a Pharmacia Mono-Q anion exchange column (Model Mono-Q HR 5/5, dimensions 5 X 50mm) was used. Human parotid saliva samples were concentrated by dialysis in a microdialysis unit against 30% (w/v) polyethylene glycol 10,000 followed by an overnight dialysis against 20mM bis-

tris propane-hydrochloric acid buffer solution, pH 7.0. All solutions were prepared using HPLC-grade water.

As this study was mainly concerned with anionic proteins (proteins with pI's < 6), a column which had been packed with a strong anion-exchanger (Mono-Q) was used. The pH of the eluant, pH 7.0, was chosen so that the anionic proteins are negatively charged when they were being fractionated (Henry, 1989).

The flow-rate of the eluant through the column was 1 ml/min, and the chart speed of the recorder was 2 cm/min. A 214nm detector and a 500μl sample loop were fitted. The initial, low ionic strength buffer was 20mM bis-tris propane-hydrochloric acid buffer solution, pH 7.0 (buffer A); and the final, high ionic strength buffer was 20mM bis-tris propane-hydrochloric acid buffer solution, pH 7.0, containing 1M sodium chloride (buffer B). Just before use, the solutions were filtered through a 20μm Millipore filter.

The FPLC apparatus was operated in the programmed mode. The program was set as follows: the gradient, introduced at 3 minutes, was 0-30% B from 3 to 14 minutes, and 30-100% B from 14 to 16 minutes. The process was stopped at 20 minutes. 0.5ml-sized fractions were collected.

2.12. Enzymic Deglycosylation of Glycoproteins

Many proteins are conjugated proteins which contain non-amino acid substituents or prosthetic groups attached to the polypeptide chain. These proteins may be classified ac-

cording to the nature of their prosthetic groups; for example, glycoproteins, phosphoproteins and metalloproteins (Lehninger, 1975). Enzymes capable of catalysing the removal of the prosthetic groups may be useful in the elucidation of protein structure. For example, glycosidases facilitate the removal of monosaccharide or oligosaccharide groups from glycoproteins.

2.12.1. Incubation with Neuraminidase

2.12.1.(i) Introduction

The sialic acid α-N-acetylneuraminic acid often occurs as the peripheral residue of oligosaccharide side groups of glycoproteins which are N-linked to the polypeptide via asparagine. The presence of the carboxyl group located on C_1 of the α-N-acetylneuraminic acid molecule causes the sialic acid to have a significant effect on the pI of the glycoprotein. The pI's of the sialated forms of a glycoprotein are, therefore, lower than that of its asialo forms. In addition, differing amounts of sialic acid in the oligosaccharide side groups of a glycoprotein will result in microheterogeneity (Beeley, 1985).

Neuraminidase (E.C.3.2.1.18) is an exoglycosidase which hydrolyses the terminal ketosidic bonds which join sialic acid to oligosaccharide. Neuraminidase isolated from Vibrio cholerae will cleave the bond between α-N-acetylneuraminic acid and galactose at the following rates: NeuAc α-2-3 Gal > NeuAc α-2-4 Gal > NeuAc α-2-6 Gal

(Kobata, 1979).

2.12.1.(ii) Procedure

The incubation of parotid saliva with neuraminidase was carried out using a modification of the procedure of Ada et al. (1961):

Neuraminidase was supplied as a solution of 1U/1ml, and was stored at -20^{0}C as aliquots of 50mU/50 μl. A volume of parotid saliva, approximately 75-125μl, containing 100μg of protein was pipetted into an Eppendorf tube, followed by 50mU of neuraminidase, 10μl of 0.01M calcium chloride solution and 20μl of 0.1M pH 5.6 sodium acetate buffer, and overlaid with 40μl of toluene. The mixture was then incubated for 18 hours at 37^{0}C. Control samples were incubated under identical conditions, but in the absence of the enzyme.

2.12.2. Incubation with Endo-β-N-Acetylglucosaminidase F (Endo F)

2.12.2.(i) Introduction

Endo F (E.C. 3.2.1.96) cleaves high mannose and complex- type N-linked oligosaccharides from glycoproteins, leaving a single N-acetylglucosamine residue attached to the polypeptide (Elder and Alexander, 1982).

2.12.2.(ii) Procedure

The incubation with the enzyme was performed according

to the method of Elder and Alexander (1982):

Endo F was supplied as a lyophilisate (3U per vial). It was reconstituted to 0.3ml with 0.1M pH 6.1 sodium phosphate buffer (the buffer solution was prepared as described by Dawson et al. (1986)). It was stored at -20^{0}C as aliquots of 100mU/10 l. A volume of parotid saliva, approximately 75-125 μl, containing 100μg of protein was pipetted into an Eppendorf tube, followed by 100mU of Endo F, 10μl of 0.1M EDTA and 10μl of 5% (v/v) 2-mercaptoethanol; and overlaid with 40 μl of toluene. The mixture was then incubated for 18 hours at 37^{0}C. Control samples were incubated under identical conditions, but in the absence of the enzyme.

2.12.3. Incubation with Peptide: N-Glycosidase F (PNGase F)

2.12.3.(i) Introduction

PNGase F (E.C. 3.2.2.18) hydrolyses asparagine-linked glycans from glycoproteins. The cleavage occurs between the innermost residue of N,N'-diacetylchitobiose and the asparagine residue to which the oligosaccharide is linked. PNGase F has a broader substrate specificity than Endo F; whereas it hydrolyses all classes of asparagine-linked glycans (i.e. high mannose, hybrid, and bi-, tri- and tetraantennary oligosaccharides), Endo F is unable to remove tri- and tetraantennary complex oligosaccharides from glycoproteins. In addition, PNGase F cleaves biantennary complex oligosaccharides 30-fold faster than Endo F does.

While the glycosylation sites of native glycoproteins are inaccessible to most oligosacchride chain-cleaving enzymes, PNGase F could achieve this in many cases (Tarentino et al., 1985).

2.12.3.(ii) Procedure

A modification of the procedure of (Tarentino et al., 1985) was used to deglycosylate parotid salivary proteins in their native state:

PNGase F was supplied as a lyophilisate (20U per vial). It was reconstituted to 2ml with 0.25M sodium phosphate buffer, pH 8.0, and stored at -20^{0}C as aliquots of 100mU/ 10μl. A volume of parotid saliva, approximately 75-125μl, containing 100μg of protein, was pipetted into an Eppendorf tube, followed by 50 μl of 50mM EDTA and 20mM 2-mercaptoethanol dissolved in 0.25M sodium phosphate buffer, pH 8.0. 100mU of PNGase F was then added. The mixture was overlaid with 40μl of toluene. Incubation was carried out for 18 hours at 37^{0}C. Control samples were incubated under identical conditions, but in the absence of the enzyme.

2.13. Assay for Tissue Kallikrein Activity in Parotid Saliva

The kallikrein activity in human parotid saliva was determined using a modification of the procedure of Amundsen et al. (1979). Kallikrein hydrolyses the chromogenic tripeptide substrate H-D-Val-Leu-Arg-NH-pNA (S-2266), releasing p-nitroaniline which is water-soluble and produces a yellow

colour:

$$\text{H-D-Val-Leu-Arg-NH-pNA} + H_2O \xrightarrow{\text{kallikrein}} \text{H-D-Val-Leu-Arg-OH} + \text{p-nitroaniline}$$

The following stock solutions were prepared:

(a) 0.05M Tris-HCl buffer, pH 9.0.

(b) A 2mM solution of S-2266 was prepared by reconstituting 25mg of the substrate with 28.8ml of distilled water.

(c) 10 KIU/ml (Kallikrein Inhibitor Unit) trasylol (lyophilised aprotinin) in buffer (a). Aprotinin is a potent inhibitor of kallikrein. 1 KIU aprotinin = 0.17 μg of the protein (Biochemica Information, 1st edition, Boehringer Mannheim Biochemicals, Indiana, U.S.A., 1987).

All solutions to be used were equilibrated to 37^{o}C before use. 850 μl of 0.05M Tris-HCl buffer, pH 9.0, and 50 μl of parotid saliva were pipetted into a disposable cuvette and mixed. For the blank, 850 μl of 10 KIU/ml trasylol solution (stock solution (c)) was used instead of the buffer solution. S-2266 solution (200 μl) was added and mixed. The mixture was incubated for exactly 30 minutes, and stopped by the addition of 100 μl of 50% (v/v) acetic acid to the contents of each cuvette. The absorbance was then read at 405nm using a Pye Unicam SP8-100 uv/visible Spectrophotometer.

2.14. Densitometric Scans of pH 3.5-5.0 IPG's

Silver stained pH 3.5-5.0 IPG's were scanned using an LKB Ultroscan laser densitometer in order to compare the

band patterns of RA patients, SS patients and normal healthy individuals.

2.15. Photography

After development or destaining, the blots or gels were promptly photographed using a Nikon F3HP camera fitted with a Nikkor 55mm, f 2.8, macro lens. Kodak "Ektachrome 50" tungsten-balanced slide film was used. Two tungsten bulbs were used as the sources of illumination. When necessary, the slides were copied, using a slide-copier, onto Kodak "Tmax 100" black-and-white or Kodak "Kodacolor Gold 100" colour negative films.

RESULTS

3.1. Assay Methods for the Determination of the Protein Content of Human Parotid Saliva

The standard curves obtained by the Lowry-Folin and Bio-Rad assays (Section 2.3), using bovine serum albumin (BSA) and bovine α-chymotrypsinogen as the standards, are shown in Fig. 3.1 (a) and (b). The standard curves obtained by the BCA standard and microprotocols, using bovine serum albumin and bovine α-chymotrypsinogen as the standards are shown in Fig. 3.1 (c) and (d). In the BCA standard protocol, the samples are incubated at 37^{0}C, while in the microprotocol they are incubated at 60^{0}C. These graphs show that for all 3 assay methods, the estimation of protein concentration is dependent on the protein standard used within the range of protein concentrations used. However, the sensitivity (i.e. colour produced) is greater in the case of α-chymotrypsinogen with the Lowry-Folin and BCA assays, while in the case of the Bio-Rad assay and BSA, it is less.

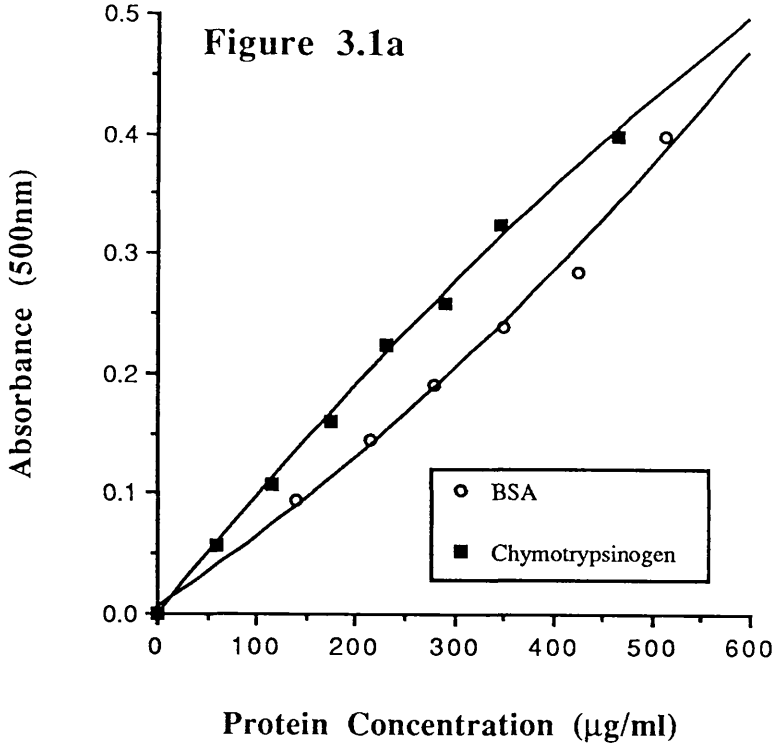
The principal on which the BCA assay is based is the reduction of copper (II) cations to copper (I) by reaction with the protein, followed by the chelation of the copper (I) cation by bicinchoninic acid (BCA), producing a purple colour with an absorbance maximum at 562nm.

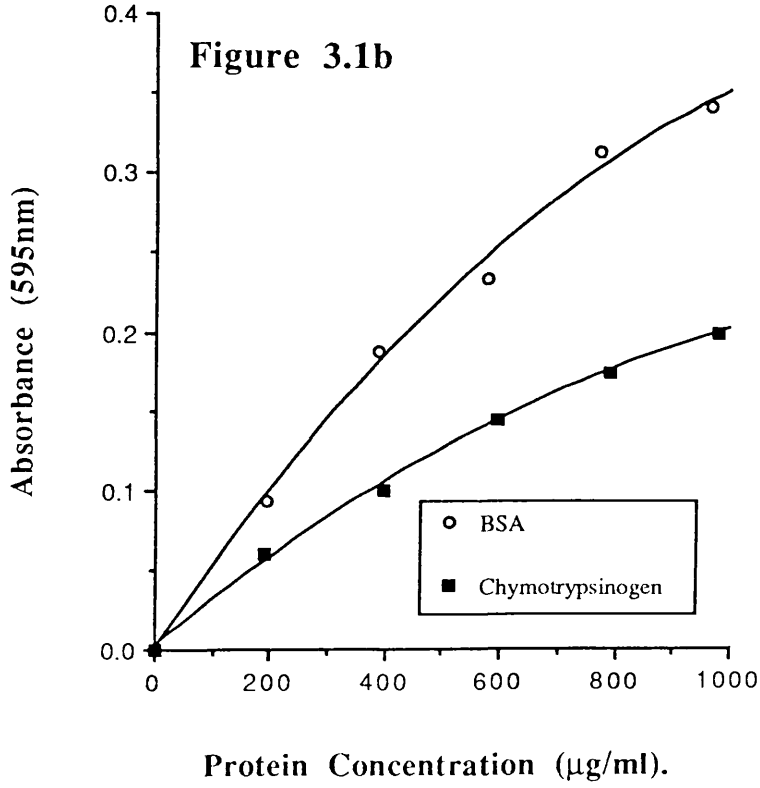
Fig. 3.2, which is a plot of absorbance values versus volume of parotid saliva used for the Lowry-Folin and Bio-

Fig. 3.1. Standard curves of the Lowry-Folin, Bio-Rad and BCA protein assays using α-chymotrypsinogen and bovine serum albumin as the standards

Panel (a) shows the standard curve of the Lowry-Folin protein assay method while panel (b) shows the standard curve of the Bio-Rad protein assay method.

Panel (c) shows the standard curve for BCA standard protocol, in which the samples were incubated at $37^{0}C$ for 30 minutes, while panel (d) shows the standard curve for BCA microprotocol, in which the samples were incubated at $60^{0}C$ for 30 minutes.





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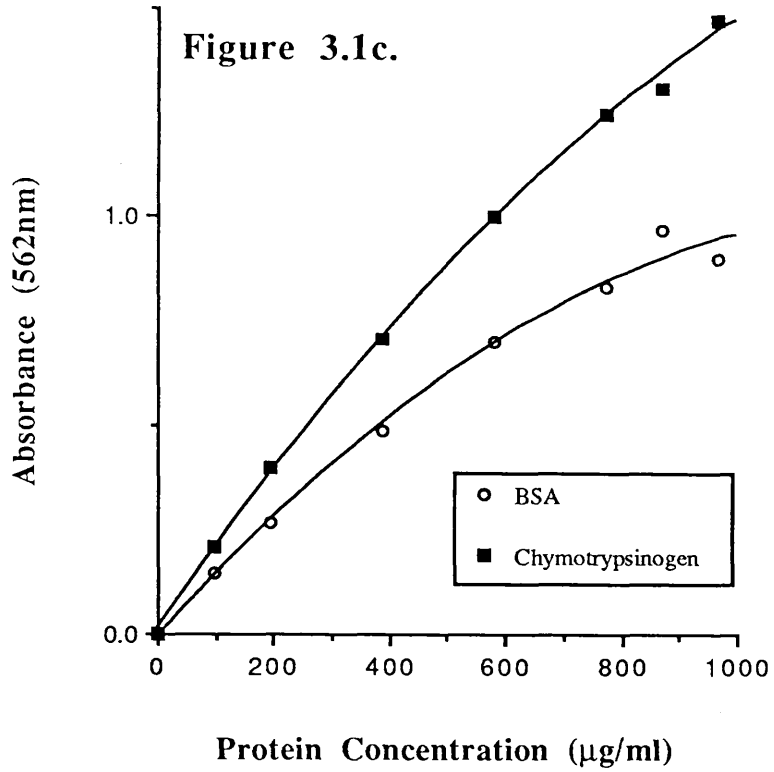


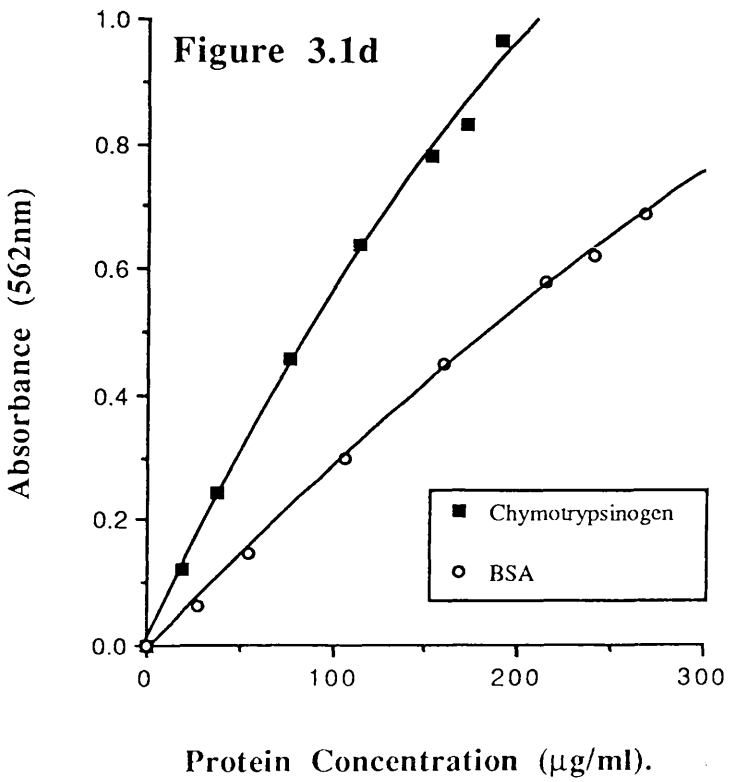
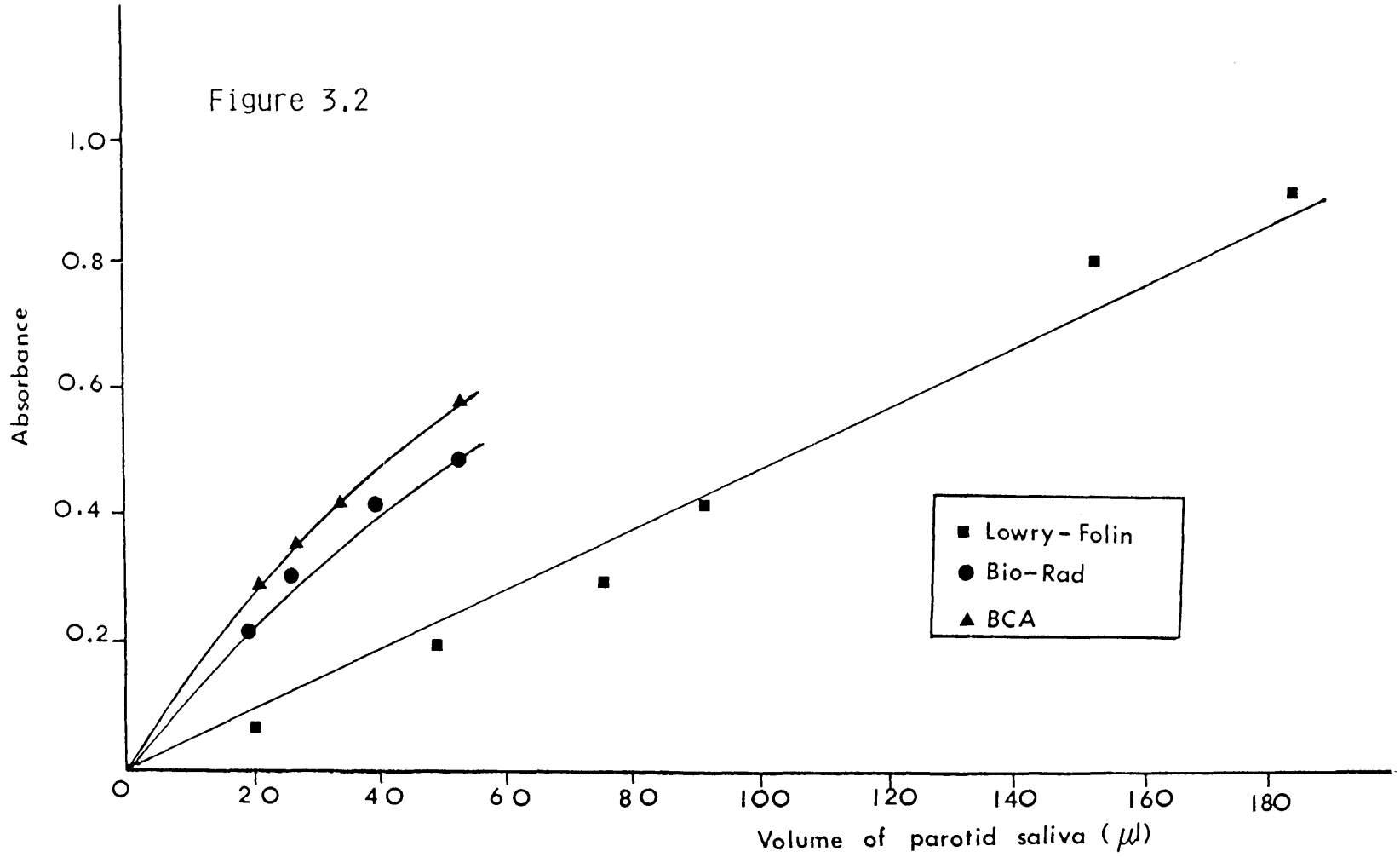


Fig. 3.2. Comparison of the standard curves of the Lowry-Folin, Bio-Rad and BCA protein assays

Plots of absorbance values versus volumes of parotid saliva used were obtained. The BCA standard protocol was used.

Figure 3.2


Absorbance
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
160
180
Volume of parotid saliva (μl)
Lowry - Folin
Bio-Rad
BCA

Rad assays and the BCA standard protocol, shows that the BCA standard protocol gives the highest absorbance per unit volume of saliva used; therefore it is the most sensitive assay method of the three for the determining the protein content of saliva.

Of the 3 assay methods, the BCA assay requires the smallest volumes of parotid saliva, 20 μl for the standard protocol and 5 μl for the microprotocol. In comparison, the Lowry assay requires 100 μl, and the Bio-Rad assay, 50 μl. While economy in the volume of saliva samples is usually not crucial when assaying samples from normal subjects with high salivary flow-rates, often in the case of RA and SS patients, many of whom have low flow-rates, only small volumes (frequently between 0.2 and 1ml) may be available. Thus the BCA microprotocol was chosen for the routine determination of the protein content of human parotid saliva for the reasons of its high sensitivity and low volumes of saliva it requires. The BCA assay may also be automated by using microtitre plates to determine the protein content of large numbers of samples.

The least time-consuming of the 3 assays is the Bio-Rad, the principle of which is the binding of the dye CBB G250 to the protein, thereby shifting the value of the absorbance maximum from 465nm to 595nm (Bradford, 1976). This process is rapid and takes approximately 2 minutes. The Lowry assay (Lowry et al., 1951) is the most time-consuming as it involves a number of steps in which the timing is

critical.

The major group of proteins in saliva are PRP's (Bennick, 1982; 1987), the properties of which may give rise to inaccuracies when certain protein assay methods are used. For example, they contain high levels of proline, glycine and glutamic acid but no tryptophan or tyrosine (Bennick, 1982). The Lowry-Folin assay (Lowry et al., 1951) depends on the reaction of the peptide bonds in the protein with copper in alkaline solution (i.e. the Biuret reaction) and also on the reduction of phosphomolybdic acid-phosphotungstic acid (Folin-Ciocalteau reagent) by tyrosine and tryptophan residues in the protein (Chou and Goldstein, 1960). Therefore the estimation of the protein concentration of saliva using this method is probably inaccurate. The lack of amino acids containing aromatic side-groups, such as tryptophan and tyrosine, in PRP's also makes the determination of the protein content of saliva using ultra-violet spectrophotometry (i.e. taking absorbance readings at 280nm) grossly inaccurate. PRP's have unusual staining properties towards CBB R250 (Shatzman and Henkin, 1983), and in the course of this study (Section 3.2.2) they have been shown to bind poorly to CBB G250; thus the Bio-Rad assay, which depends on the binding of CBB G250 to proteins, may also be inaccurate for determining the protein content of saliva.

The behaviour of PRP's towards the reagents used in the BCA assay has not been studied. However, in this study, it was the protein assay method of choice due to its sen-

sitivity and the need to use only small volumes of saliva samples. Athough the BCA assay is more time-consuming than the Bio-Rad assay, it is no more difficult to carry out.

3.2. Analysis of Human Parotid Salivary Proteins by SDS-PAGE

3.2.1. The Band Pattern Revealed by SDS-PAGE

SDS-PAGE of 40 μg of parotid salivary protein followed by staining of the gel with 0.1% (w/v) CBB R250 in 40% (v/v) ethanol and 10% (v/v) acetic acid, and destaining in several changes of 10% (v/v) acetic acid over 2-3 days (Lindsay and Beeley, 1986) resulted in approximately 6 blue-staining and 10-12 pink-staining bands in most normal healthy subjects. The metachromatic reaction, which resulted in some of the bands staining with a pink colour is characteristic of the proline-, glycine- and glutamic acid-rich phosphoproteins in human saliva (Shatzman and Henkin, 1983). Although the inclusion of ethanol or methanol in the destaining solution shortens the destaining time required, it resulted in poor staining, or even loss, of the pink-staining components, and as such no alcohol was included in the destaining solution. Metachromasia has also been noted in collagen, which is also proline-rich. It is thought to be due to the unique stacking of dye molecules and dye-dye interactions in regions of closely-spaced proline residues, causing a difference of approximately 30nm between the absorbance maxima for collagen-CBB R250 complexes and that of other protein-CBB R250 complexes (McCormick et al., 1979).

The pink-staining components have been designated the following nonmenclature: A (M_r 72,000), B (M_r 50,000), C_1 (M_r 45,000), C_2 (M_r 38,000), D_1 (M_r 31,000), D_2 (M_r 28,000), D_3 (M_r 26,000), E_1 (M_r 22,000), E_2 (M_r 19,000), E_3 (M_r ap-

proximately 17,000), E_4 (M_r approximately 14,000), E_5 (M_r approximately 12,000) and E_6 (M_r approximately 10,000). These are indicated in Fig. 3.3. C_1 appears to contain both blue- and pink-staining components. Blue-staining bands were also obtained; 3 of which have been identified in Section 3.2.4(ii) as the glycosylated and non-glycosylated isozymes of α-amylase (M_r's 63,000 and 59,000, respectively) and lactoferrin (M_r 90,000). In addition, there are blue-staining bands of low M_r, designated as F_1 and F_2 (M_r's < 10,000).

3.2.2. Comparison of Coomassie and Silver Staining

The metachromatic reaction did not, however, occur when the gels were stained with the dye CBB G250 and only blue-staining bands were visualised (Fig. 3.4 (a)). CBB G250 differs from CBB R250 in having 2 additional methyl groups which may prevent the dye-protein stacking which results in metachromasia.

In order to compare the sensitivities and staining properties of CBB R250, CBB G250 and silver stain towards salivary proteins, a range of loads of parotid salivary proteins was fractionated by SDS-PAGE, and the gels were stained accordingly (Figs. 3.4 (a), 3.4 (b) and 3.5). For CBB R250, a protein load of 40μg was optimal (Fig. 3.4 (b)). Even with a protein load of 90μg, most of the bands which were stained pink by CBB R250 were either weakly stained or not stained at all by CBB G250 (Fig. 3.4 (a)).

Although silver stain is one of the most sensitive

Fig. 3.3. SDS-PAGE of parotid salivary proteins from one individual, followed by CBB R250 stain

The track in the gel was loaded with 40μg of protein from a normal healthy individual.

The blue- and pink-staining bands were assigned the nonmenclature as indicated. The concentration of the gel was 12.5 %T.

Figure 3.3

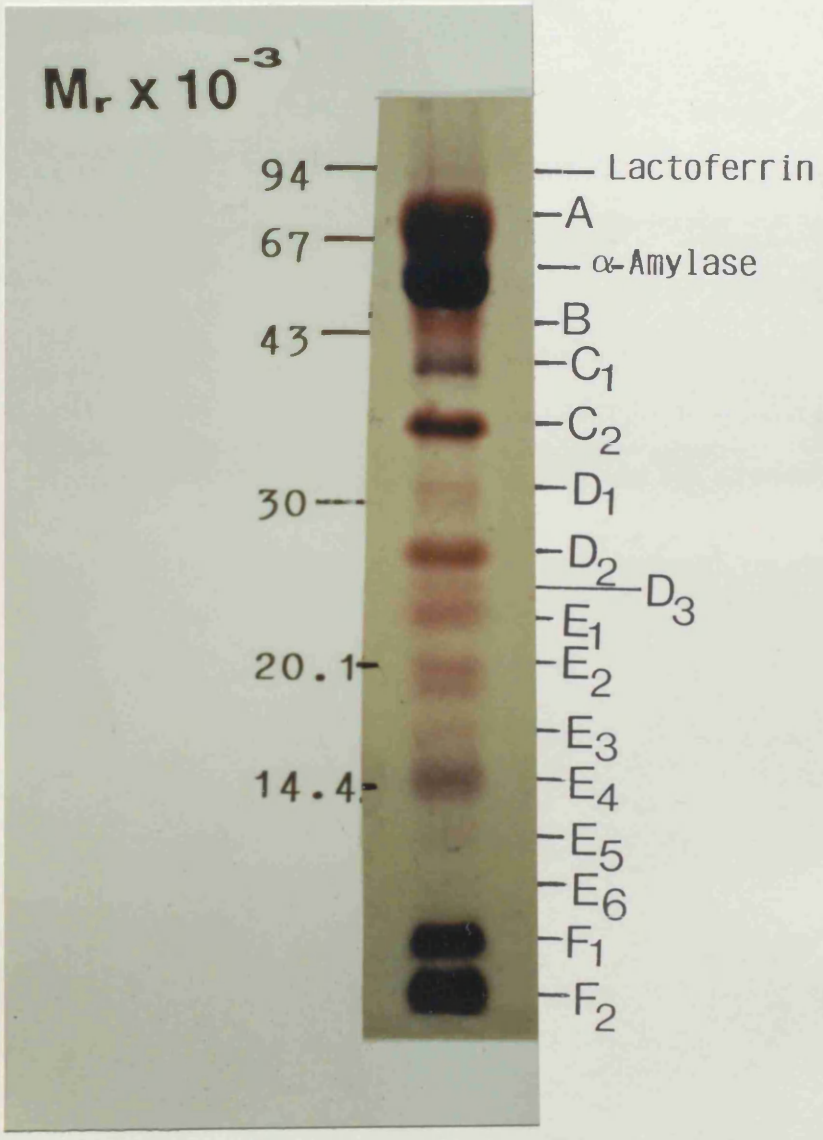

$M_r \times 10^{-3}$
94
67
43
30
20.1
14.4
Lactoferrin
A
α-Amylase
B
C_1
C_2
D_1
D_2
D_3
E_1
E_2
E_3
E_4
E_5
E_6
F_1
F_2

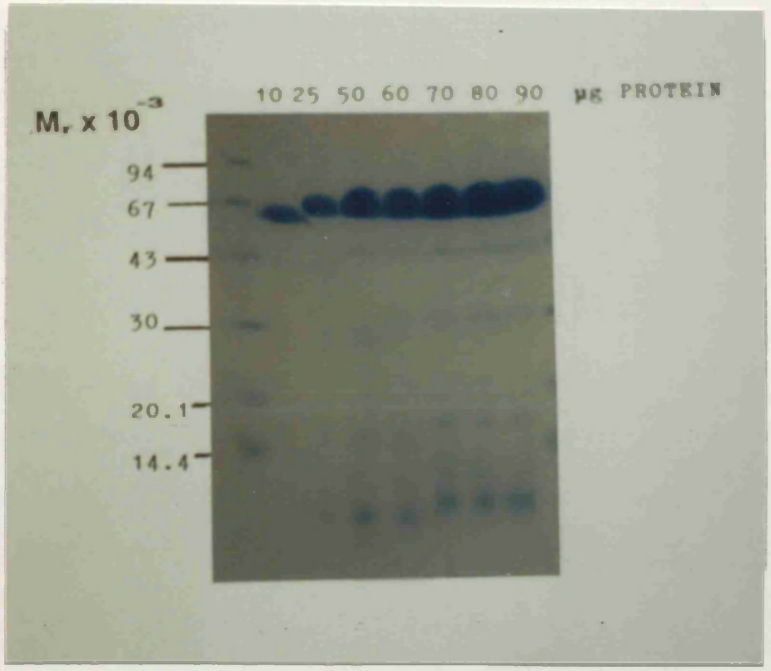
Fig. 3.4. SDS-PAGE of a range of parotid salivary protein loadings from one individual, followed by Coomassie stain

The gel in panel (a) was stained with CBB G250, while the gel in panel (b) was stained with CBB R250.

The protein loads are as indicated. The arrowheads in panel (b) indicate blue-staining bands. The first track in each gel was loaded with M_r marker proteins. The concentration of both gels was 12.5 %T.

Figure 3.4

(a)


10 25 50 60 70 80 90 µg PROTEIN
M, x 10⁻³
94
67
43
30
20.1
14.4

(b)

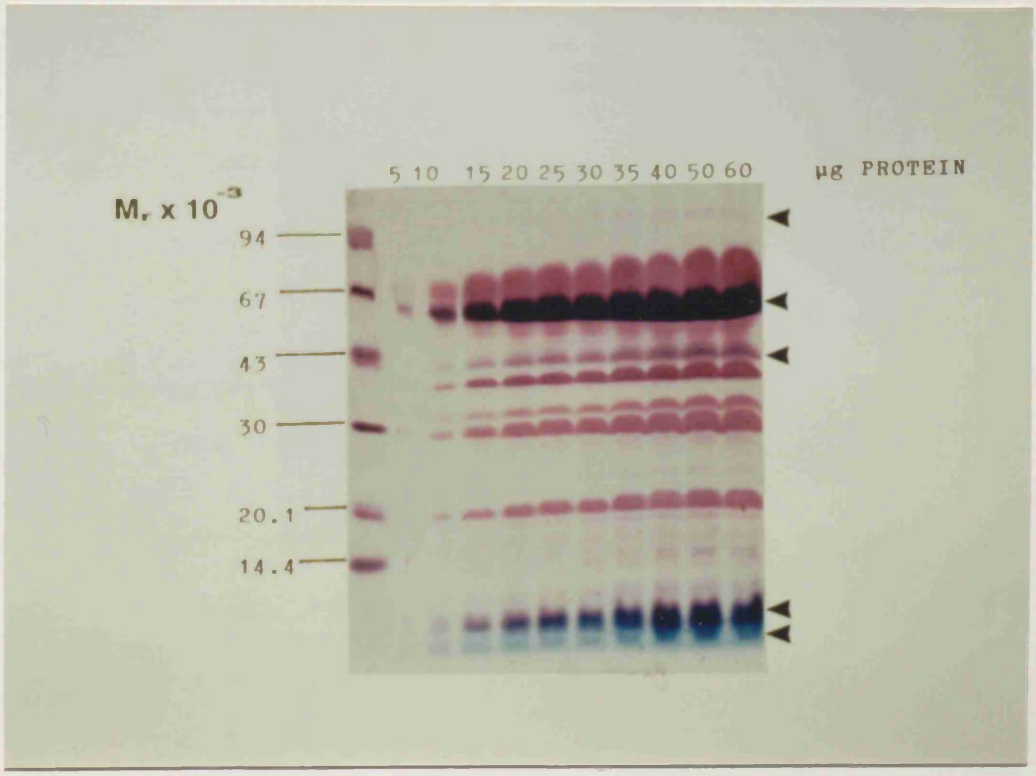
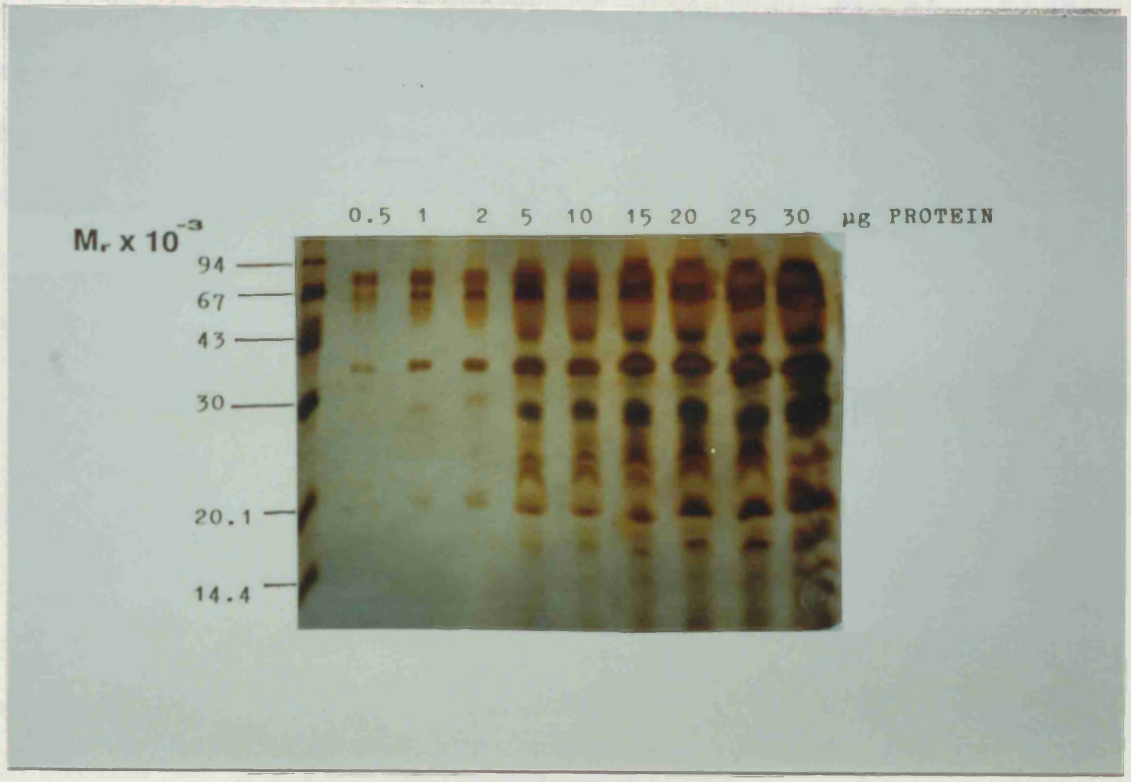

5 10 15 20 25 30 35 40 50 60 µg PROTEIN
M, x 10⁻³
94
67
43
30
20.1
14.4

Fig. 3.5. SDS-PAGE of a range of parotid salivary protein loadings from one individual, followed by silver stain

The protein loads are as indicated. The first track was loaded with M_r marker proteins. The concentration of the gel was 12.5 %T.

Figure 3.5



staining methods, salivary PRP's have been reported to stain poorly (Friedman, 1982). A number of different silver staining methods, including that of Sammons et al. (1981) and Morrisey (1981) were used to stain parotid salivary proteins after SDS-PAGE, but these methods produced a yellowish background which made it difficult to visualise the PRP's as they were stained with a yellowish-brown colour. However, a modification of the method of Damerval et al. (1987) produced a sufficiently clear background, thus allowing the PRP's to be visualised. It was found that the bands which were stained pink by CBB R250 were stained with a yellowish brown colour by silver stain, while the bands which were stained blue by CBB R250 were stained black or dark brown. A protein load of 5μg was optimal (Fig. 3.5).

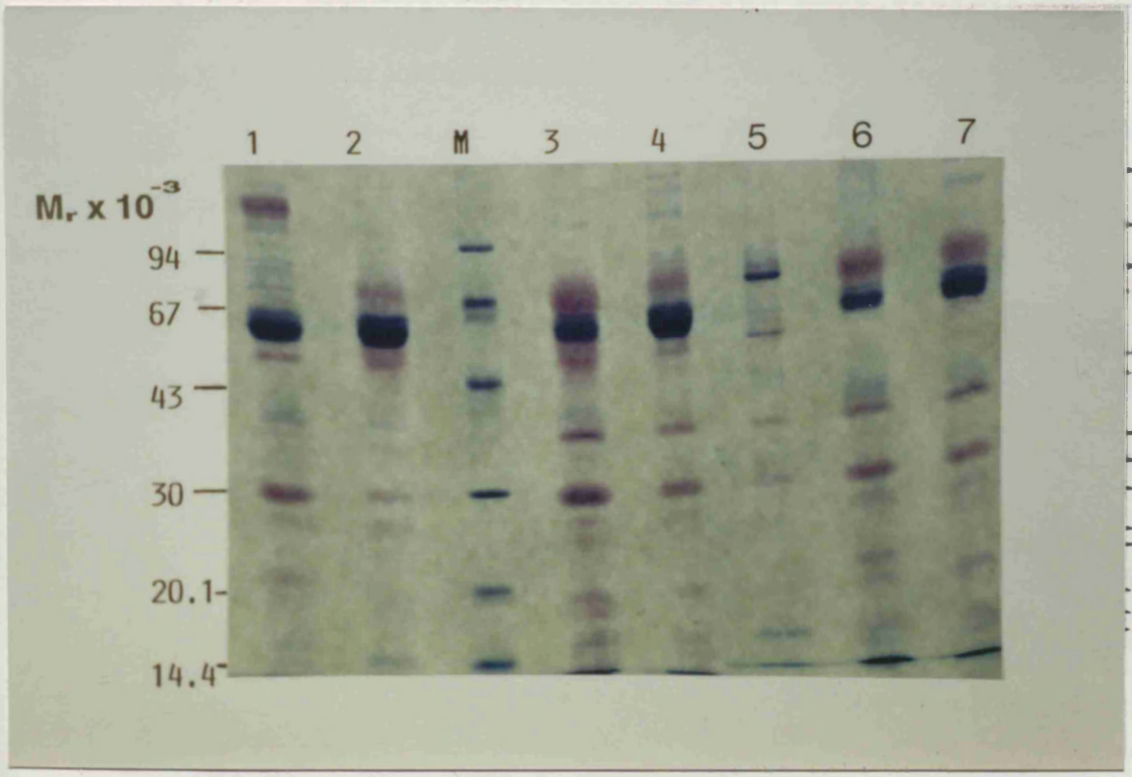
3.2.3. Band Patterns of Normal Healthy Individuals

The band pattern revealed by SDS-PAGE followed by staining with CBB R250 of 6 normal healthy subjects is shown in Fig. 3.6. There is considerable individual-to-individual variation in the pink-staining components, and certain bands which are strongly stained in some individuals are either less prominent, or not detectable in others. For example, the individual whose sample was loaded to track 1 displayed a pink-staining component of M_r approximately 100,000 which was not present in other samples, but did not exhibit band A (M_r 72,000). Bands A and B in Fig. 3.3, of M_r's 72,000 and 50,000, respectively, have been correlated, in Section

Fig. 3.6. SDS-PAGE of parotid salivary proteins from 7 different normal healthy individuals followed by CBB R250 stain

Tracks 1-7 were each loaded with 40 μg of protein from different normal healthy subjects, while track "M" was loaded with M_r marker proteins. The concentration of the gel was 12.5 %T.

Figure 3.6


1
2
M
3
4
5
6
7
$M_r \times 10^{-3}$
94
67
43
30
20.1
14.4
Lactoferrin
A
α- Amylase
B
C1
C2
D1
D2
D3
E1
E2
E3
F1
F2

3.2.4, to the glycosylated PRP of Levine et al. (1969). Thus it is possible that the component of M_r approximately 100,000 is a more glycosylated form of the component in band A. Another example is band C_1 (M_r 45,000) which is weakly stained in the samples in tracks 1 and 2, but more strongly stained in the others.

3.2.4. Identification of the Bands Revealed by SDS-PAGE

3.2.4. (i) Identification of the Pink-Staining Components

PRP's which have been isolated and partially characterised by other research groups were fractionated in SDS-PAGE gels which were subsequently stained by CBB R250. The acidic PRP's, designated as proteins "A" and "C" by Bennick and Connell (1971), most of the 9 non-glycosylated basic PRP's ("IB1"-"IB9") which have been described by Kauffman et al. (1986) and the glycosylated PRP of Levine et al. (1969) were loaded onto SDS-PAGE gels which were subsequently stained with CBB R250 (Fig. 3.7 (a) and (b)). Following SDS-PAGE, their apparent M_r's were determined and correlated with the bands which had been assigned alphabetical codes in Fig. 3.3. Their M_r values from the literature, where available, and the methods by which they were determined were also compared with the values obtained by SDS-PAGE. These results are summarised in Table 3.1.

Some of the purified PRP's appear to consist of two components. This could be due to fragmentation during the course of storage, or failure to purify the samples to

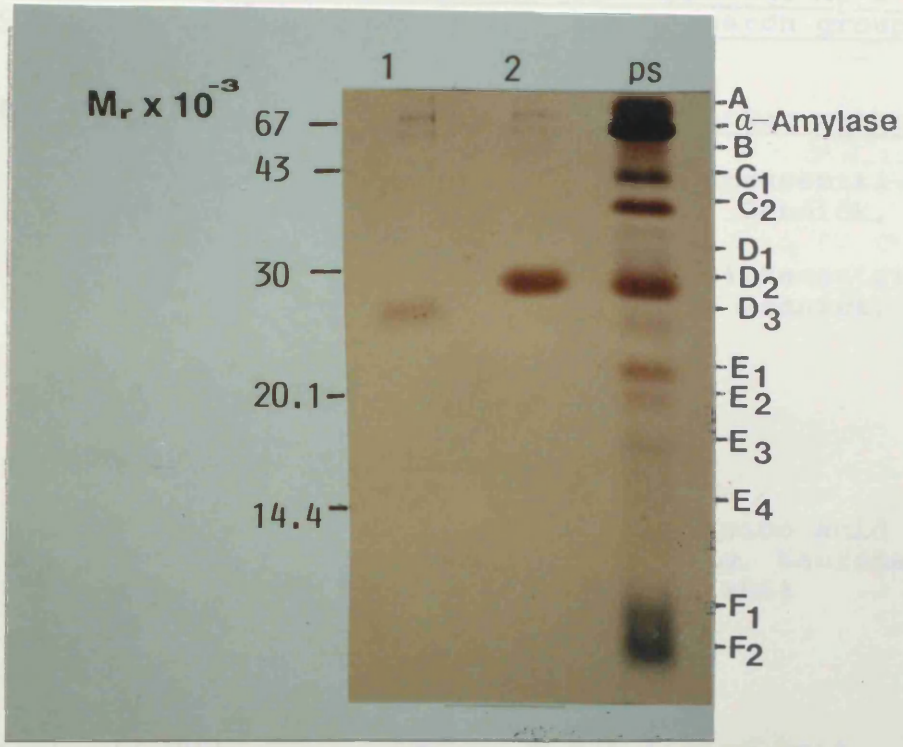
Fig. 3.7. SDS-PAGE of purified acidic PRP's and purified basic PRP's

Tracks 1 and 2 in panel (a) were each loaded with 2μg of the acidic PRP's "C" and "A" described by Bennick and Connell (1971), while tracks 3-10 in panel (b) were each loaded with 2μg of the non-glycosylated basic PRP's ("IB1"-"IB9" and "DEAE II") described by Kauffman et al. (1986) and track 11 panel (b) was loaded with 2μg of the glycosylated basic PRP described by Levine et al. (1969). The tracks labelled "ps" in panels (a) and (b) were each loaded with 40μg of parotid salivary proteins from normal healthy subjects, and track "M" was loaded with M_r marker proteins. Track 3 was loaded with "IB9", track 4 with "IB8", track 5 with "IB7", track 6 with "IB6", track 7 with "IB5", track 8 with "IB4", track 9 with "IB1", track 10 with "DEAE II" and track 11 with glycosylated basic PRP. The concentration of both gels was 12.5 %T, and they were stained with CBB R250.

"DEAE II" was a purified PRP sample, supplied together with "IB1"-"IB9" by Dr. P. Keller.

Figure 3.7

(a)


1
2
ps
Mr x 10-3
67
43
30
20.1
14.4
A
α–Amylase
B
C1
C2
D1
D2
D3
E1
E2
E3
E4
F1
F2

(b)

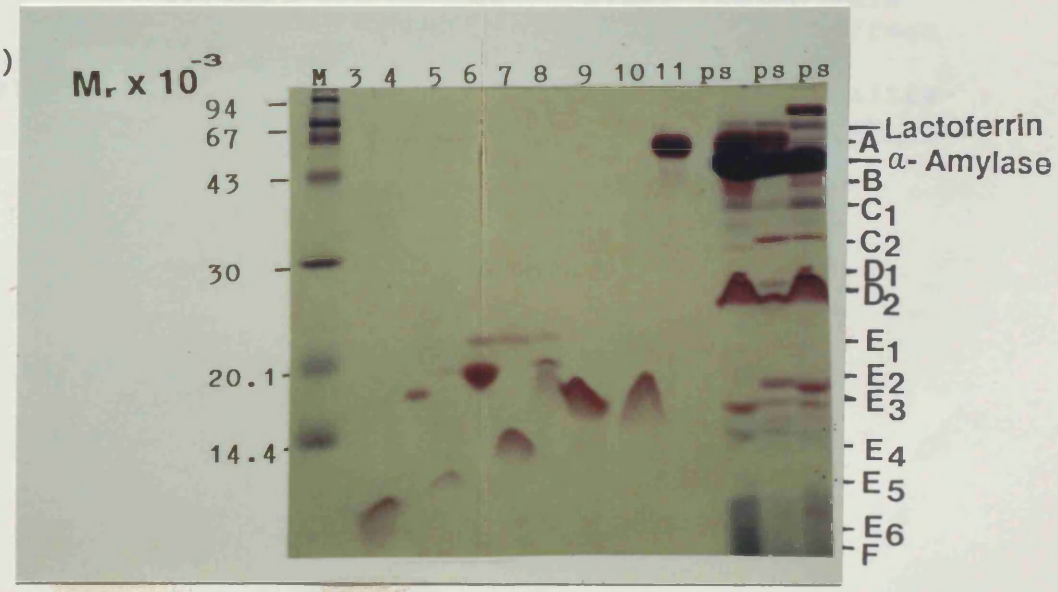

Mr x 10-3
M 3 4 5 6 7 8 9 10 11 ps ps ps
94
67
43
30
20.1
14.4
Lactoferrin
A
α- Amylase
B
C1
C2
D1
D2
E1
E2
E3
E4
E5
E6
F

Table 3.1. Determination by SDS-PAGE of the apparent M_r's of acidic and basic PRP's isolated by other research groups

Purified PRP's	SDS-PAGE M_r	Band	M_r by other methods
"A"	26,000	D_3	9,900 (ultracentrifugation, Bennick, 1982)
"C"	28,000	D_2	16,300 (ultracentrifugation, Bennick, 1982)
"IB9"	10,000	E_6	
"IB8"	10,000 and 17,000	E_6+E_3	
"IB7"	12,000 and 19,000	E_5+E_2	
"IB6"	19,000 and 22,000	E_1+E_2	11,530 (amino acid sequencing, Kauffman et al., 1986)
"IB5"	14,000 and 22,000	E_4+E_1	
"IB4"	19,000 and 22,000	E_1+E_2	
"IB1"	17,000	E_3	9,571 (amino acid seqencing, Kauffman et al.,1986
"Glycosylated PRP"	50,000 and 72,000	A+B*	36,400 (gel filtration, Levine et al., 1969).

N.B. * Band B is probably a contaminant of band A.

homogeneity.

The M_r's determined by SDS-PAGE differ significantly from the values determined by other methods such as gel filtration, ultracentrifugation and amino acid sequencing.

Bands A and B in Fig. 3.3, of M_r's 72,000 and 50,000, respectively, have been correlated to the glycosylated PRP of Levine et al. (1969). They were stained by the PAS glycoprotein stain (Fig. 3.8). This staining method locates glycoproteins in polyacrylamide gels and distinguishes them from non-glycosylated proteins. The individual whose saliva sample was loaded to track 6 in Fig. 3.8 exhibited a PAS-positive band of M_r approximately 100,000. This was the same individual whose sample was loaded to track 1 in Figure 3.6, who exhibited a pink-staining band of M_r approximately 100,000.

In addition, bands A and B in Fig. 3.3, of M_r's 72,000 and 50,000, have been shown to bind to the lectin ConA (Fig. 3.9). ConA binds preferentially to α-D-mannopyrannosyl and α-D-glucopyrannosyl residues (Hawkes, 1982). The individual whose saliva sample was loaded onto track 1 in Fig. 3.9 exhibited a ConA-binding band of M_r approximately 100,000. Again, this was the same individual whose sample was loaded onto track 1 in Figure 3.6, and who exhibited a pink-staining band of the same M_r. In the control experiment, in which 0.1M α-D-methyl mannoside, a ConA-inhibiting sugar, was included in the ConA-incubating solution, no bands were visualised when the blot was developed in the HRP substrate

Fig. 3.8. SDS-PAGE of parotid salivary proteins followed by PAS stain

Tracks 1-3 were each loaded with 40 μg of protein from RA patients, while tracks 4-6 were each loaded with 40 μg of protein from normal subjects. The concentration of the gel was 12.5 %T.

Figure 3.8

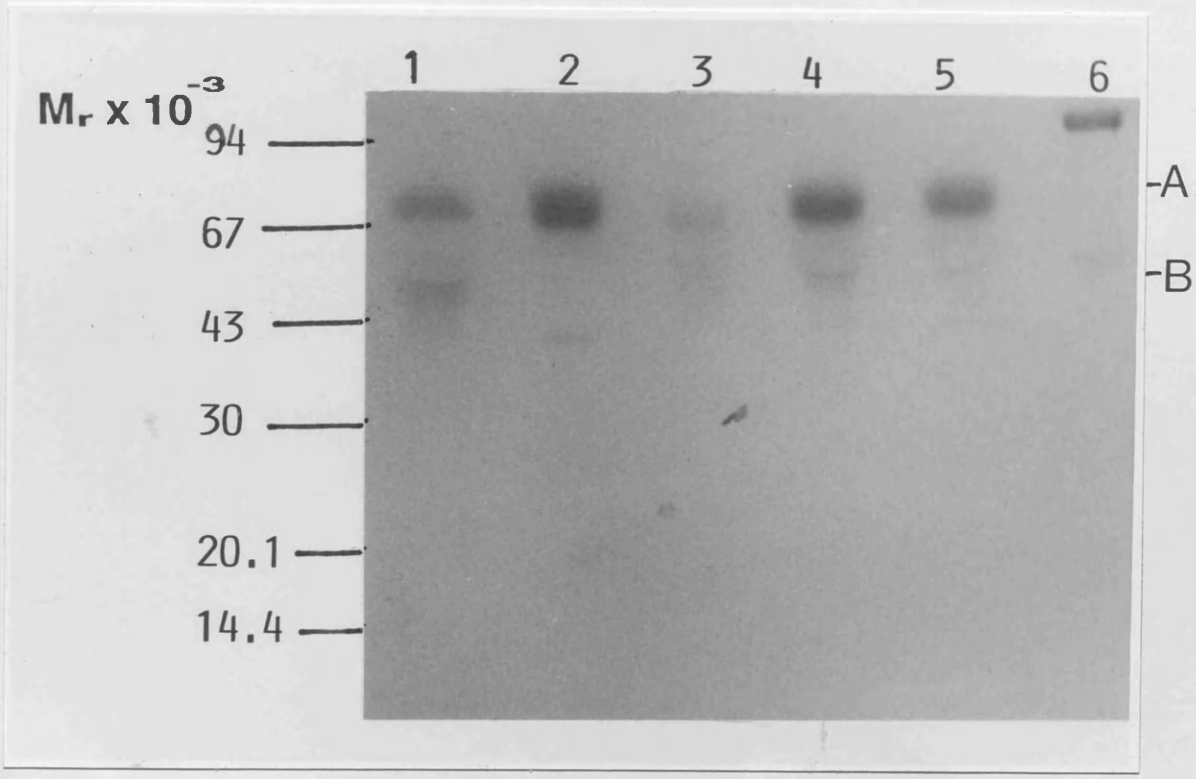
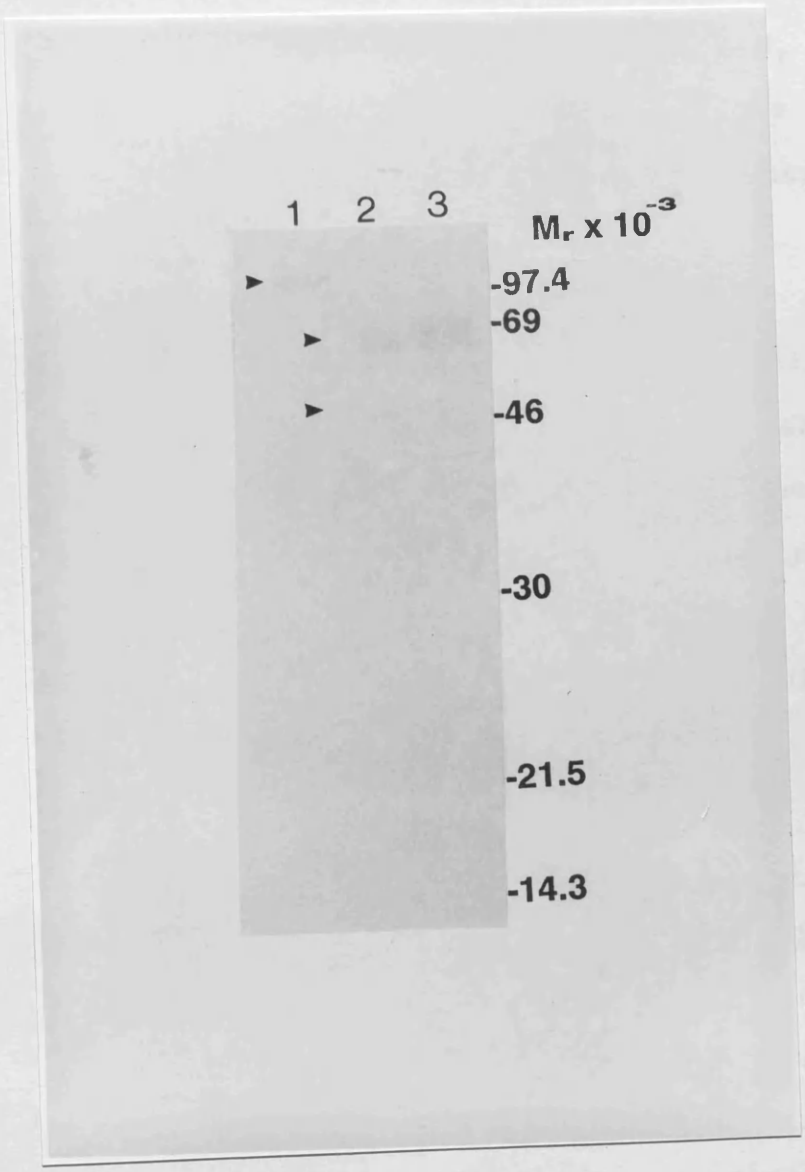


Fig. 3.9. SDS-PAGE of parotid salivary proteins followed by ConA blotting

Tracks 1-3 were each loaded with 40μg of protein from different normal healthy subjects. After electroblotting, the nitrocellulose membrane was incubated in a ConA-containing solution, followed by an HRP-containing solution. It was then developed in the HRP substrate solution. The bands visualised are indicated by arrowheads.

The concentration of the gel was 12.5 %T

Figure 3.9



solution.

3.2.4. (ii) Identification of the Blue-Staining Components

The bands of M_r's 63,000 and 59,000 have been shown, by immunoblotting, to cross-react with sheep antiserum to human salivary α-amylase (Fig. 3.10 (a)) which would appear to correspond to the glycosylated (M_r 63,000) and non-glycosylated (M_r 59,000)isozymes of the enzyme. The band of M_r 90,000 cross-reacted with rabbit antiserum to human lactoferrin (Fig. 3.10 (b)).

Control experiments were carried out in which after SDS-PAGE and electroblotting of the salivary proteins, the blots were incubated with the anti-rabbit and anti-sheep immunoglobulin-alkaline phosphatase conjugates, and developed in alkaline phosphatase substrate solution, omitting the stage where the blots were incubated with the primary antisera. In each case, no staining reaction was obtained.

3.2.5. Analysis of the Parotid Salivary Proteins of Rheumatoid Arthritis and Sjögren's Syndrome Patients

Parotid salivary proteins from normal subjects, RA patients and SS patients were fractionated by SDS-PAGE in gels of concentrations 12.5 %T and 20 %T. While 12.5 %T gels produced optimal resolution of most of the bands, 20 %T gels resulted in better resolution of the bands of lower M_r, particularly those with M_r < 40,000. These gels were stained

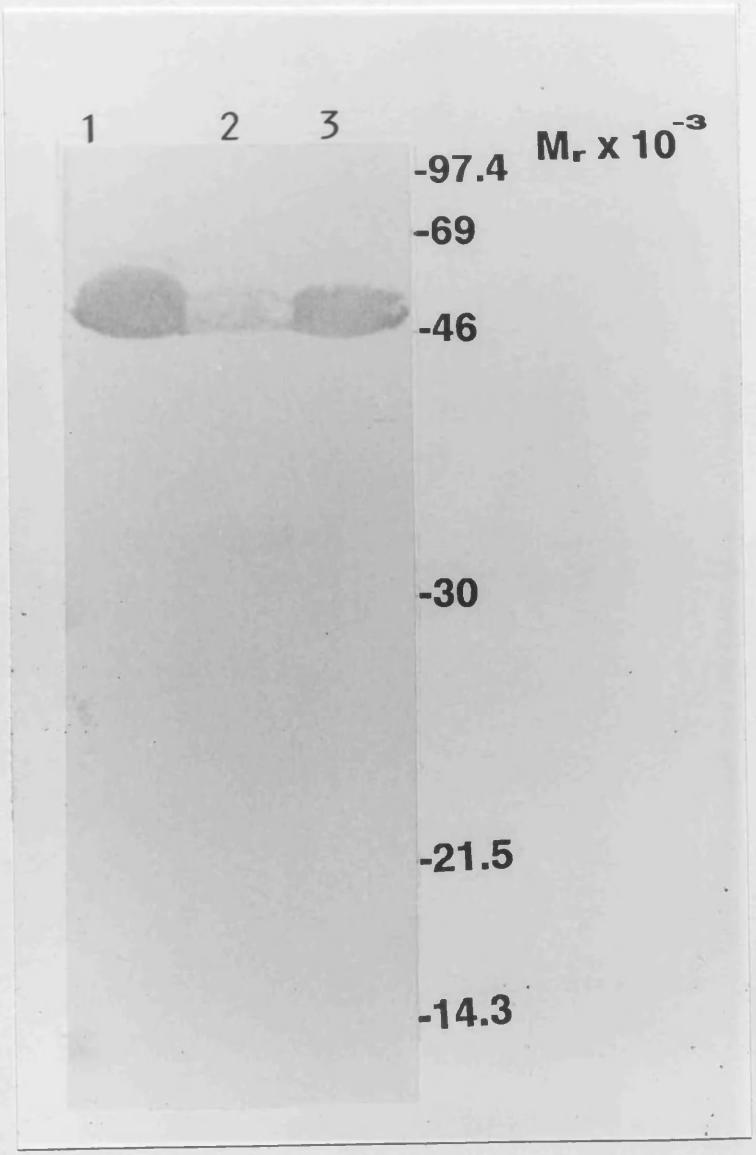
Fig. 3.10. SDS-PAGE of parotid salivary proteins followed by immunoblotting for α-amylase and lactoferrin

Panel (a) shows an immunoblot for α-amylase. 40μg of parotid salivary protein was applied to each track. The primary antibody was sheep antiserum to human amylase, and the detecting antibody was anti-sheep immunoglobulin-alkaline phosphatase conjugate.

Panel (b) shows an immunoblot for lactoferrin. 40 μg of parotid salivary protein was applied to the track labelled "PS" while 2μg of human lactoferrin was applied to each of the tracks labelled "Lf". The primary antibody was rabbit antiserum to human lactoferrin, and the detecting antibody was anti-rabbit immunoglobulin-alkaline phosphatase conjugate. The bands visualised are indicated by arrowheads. The concentration of both gels was 12.5 %T.

Figure 3.10

(a)

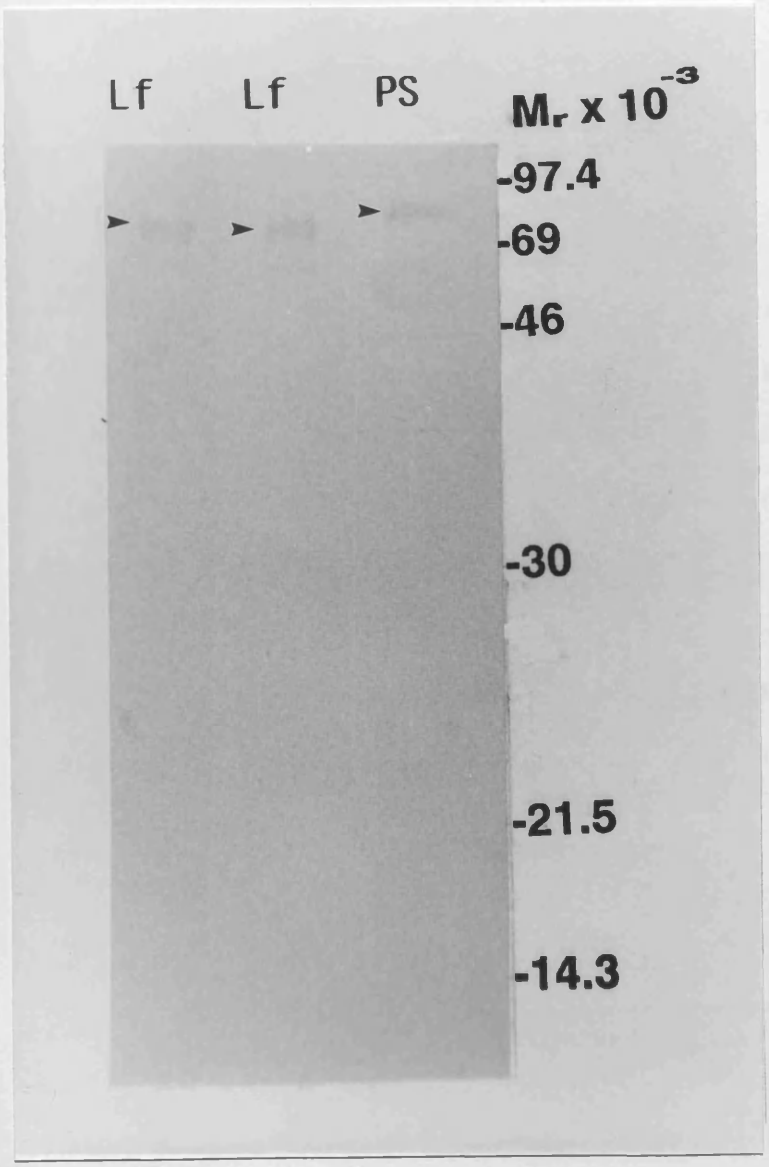


(continued on following page/)

(/continued from previous page)

Figure 3.10

(b)



with CBB R250 (Figs. 11 (a) and (b)).

Parotid salivary proteins from normal subjects, RA patients and SS patients were also fractionated by SDS-PAGE in a 5-20 %T linear gradient polyacrylamide gel which was silver stained (Fig. 3.12).

Except for one SS patient, a female approximately 50 years of age, who displayed a broad blue-staining band of M_r approximately 90,000 and a fainter blue-staining band of M_r approximately 35,000 (shown in Fig. 3.11(a)), no significant differences could be noted between the SDS-PAGE band patterns of the patients with those of normal healthy controls. The other band, of M_r 35,000, was not detected in other patients or normal subjects. No obvious differences in the PRP band patterns could be associated with these disorders.

3.2.6. Discussion

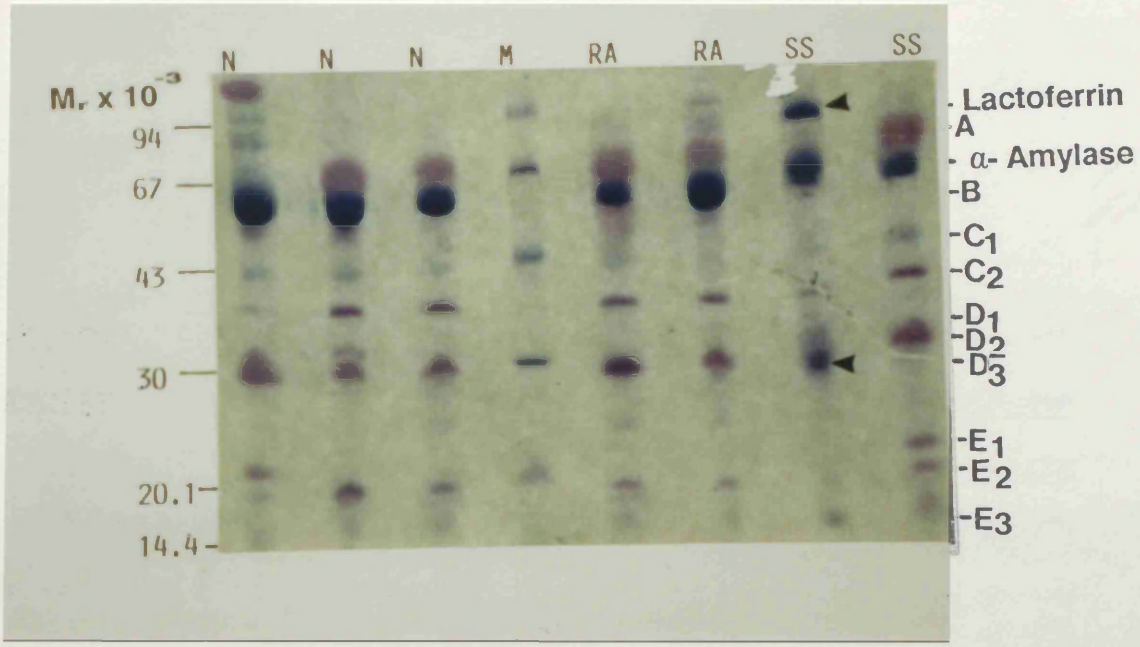
In most previous studies of human salivary proteins by SDS-PAGE followed by staining with CBB R250, the pink-staining bands in the gels which correspond to PRP's have not been well characterised. The main reason for this is the inclusion of alcohol in the destaining solution, which causes the PRP's to stain poorly or not at all. Thus Marshall and Williams (1987) and Marshall *et al*. (1989) who included methanol in the destaining solution, reported that the pink-staining components (approximately 11 in all) visualised by SDS-PAGE of whole human saliva were diffuse

Fig. 3.11. SDS-PAGE of parotid salivary proteins from normal healthy individuals, RA patients and SS patients followed by CBB R250 stain

The tracks labelled "N" were each loaded with 40 μg of parotid salivary protein from normal healthy subjects, while the tracks labelled "RA" and "SS" were each loaded with similar loads of parotid salivary proteins from different RA and SS patients, respectively. The tracks labelled "M" were loaded with M_r marker proteins. The concentration of the gel in panel (a) was 12.5 %T, while the concentration of the gel in panel (b) was 20 %T. The arrowheads in (a) indicate the bands in an SS sample which were either weakly stained or not detectable in the normal samples.

Figure 3.11

(a)



(b)

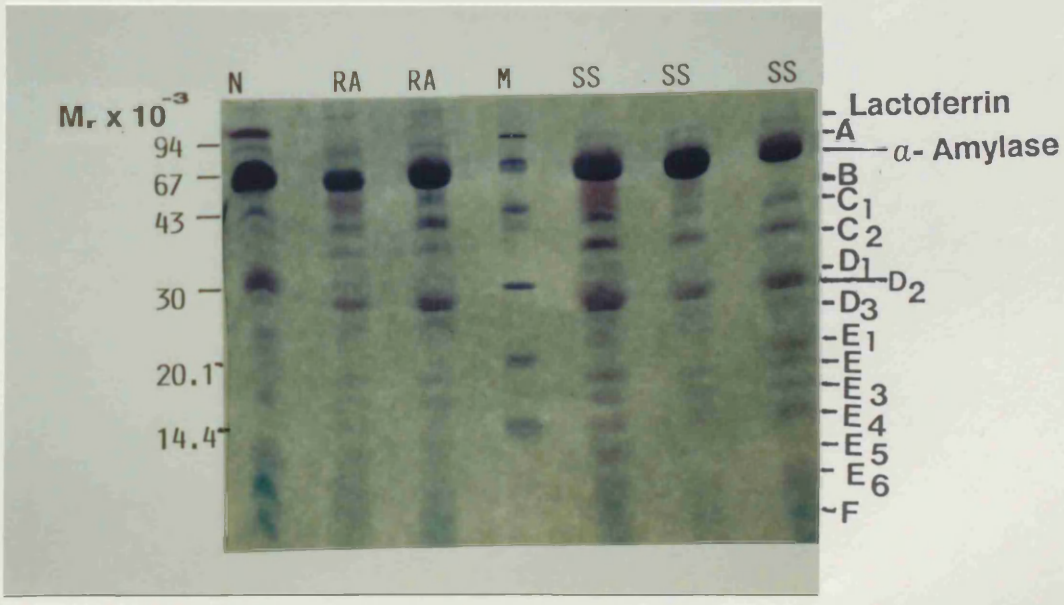
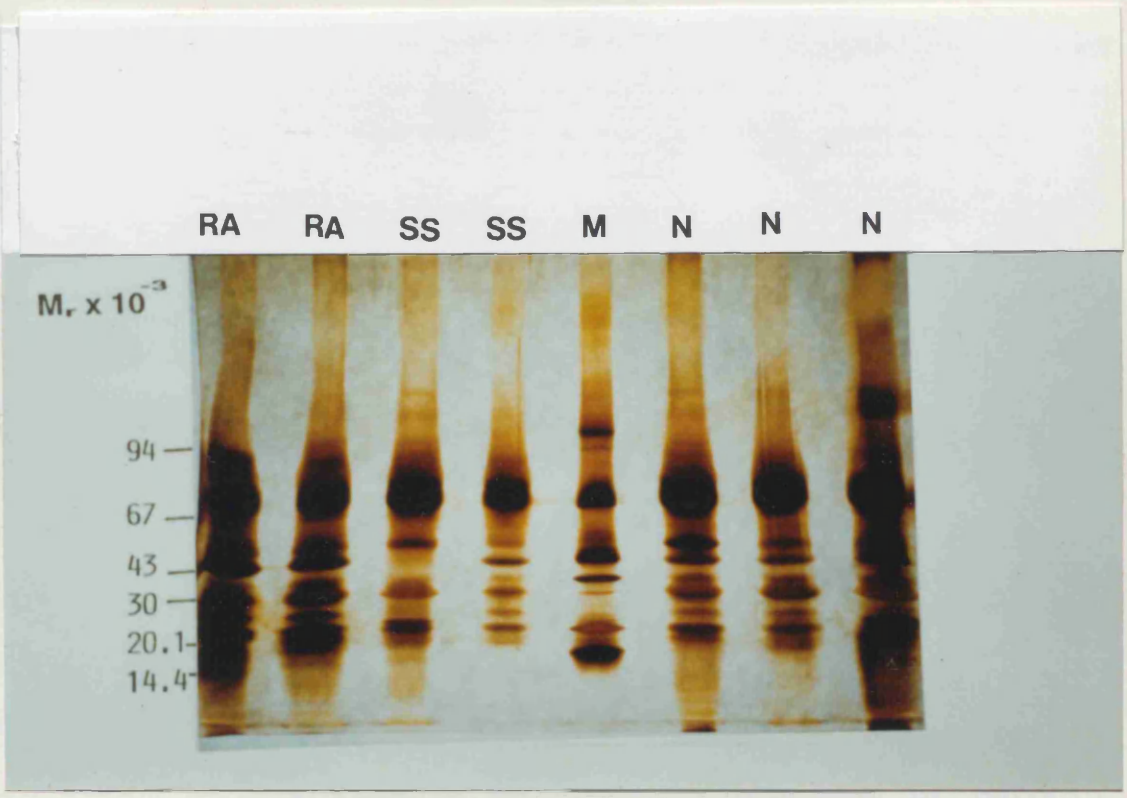


Fig. 3.12. SDS-PAGE of parotid salivary proteins from normal healthy individuals, RA patients and SS patients followed by silver stain

The tracks labelled "N" were each loaded with 5μg of parotid salivary proteins from different normal healthy subjects, while the tracks labelled "RA" and "SS" were each loaded with equal amounts of parotid salivary protein from different RA and SS patients, respectively. The track labelled "M" was loaded with M_r marker proteins.

The gel was a 5-20 %T linear concentration gradient gel.

Figure 3.12



zones. Shiba et al. (1986) performed SDS-PAGE on human whole, parotid, submandibular and palatine saliva followed by staining with CBB R250, and found approximately 16 bands in parotid saliva but did not give any experimental details on the destaining procedure they used. Nor did they make any mention of the pink-staining components. Lindsay and Beeley (1986) found that the pink-staining components were most strongly stained when alcohol was omitted from the destaining solution and recommended the use of 10% (v/v) acetic acid for the destaining of gels.

Most studies carried out on human salivary proteins have utilised whole human saliva, presumably because it is easier to collect than glandular secretions. Whole saliva contains many extra-salivary components; for example, bacteria, food debris and sloughed-off cells from the oral cavity. Thus for diagnostic purposes, glandular saliva, such as the secretion of the parotid gland, is preferable as it is relatively free of such components.

Based on the results obtained by SDS-PAGE of parotid salivary proteins that have been isolated and characterised by other research groups, such as the acidic PRP's described by Bennick and Connell (1971), the glycosylated PRP described by Levine et al. (1969) and the basic non-glycosylated PRP's described by Kauffman et al. (1986) and by SDS-PAGE of parotid salivary proteins followed by immuno- or lectin- blotting, or staining of the gels with CBB R250 or PAS stains, a generalised one-dimensional map of human

parotid salivary proteins in which some of the components have been identified, has been obtained (Fig. 3.13). In this map, 3 of the 6 bands which are stained blue by CBB R250, and 9 of the 11 pink-staining bands have been identified. Thus with the exception of bands C_1, C_2 and D_1 (M_r's approximately 45,000, 38,000 and 31,000, respectively) the other pink-staining bands have been correlated with those which have been previously isolated and partially characterised.

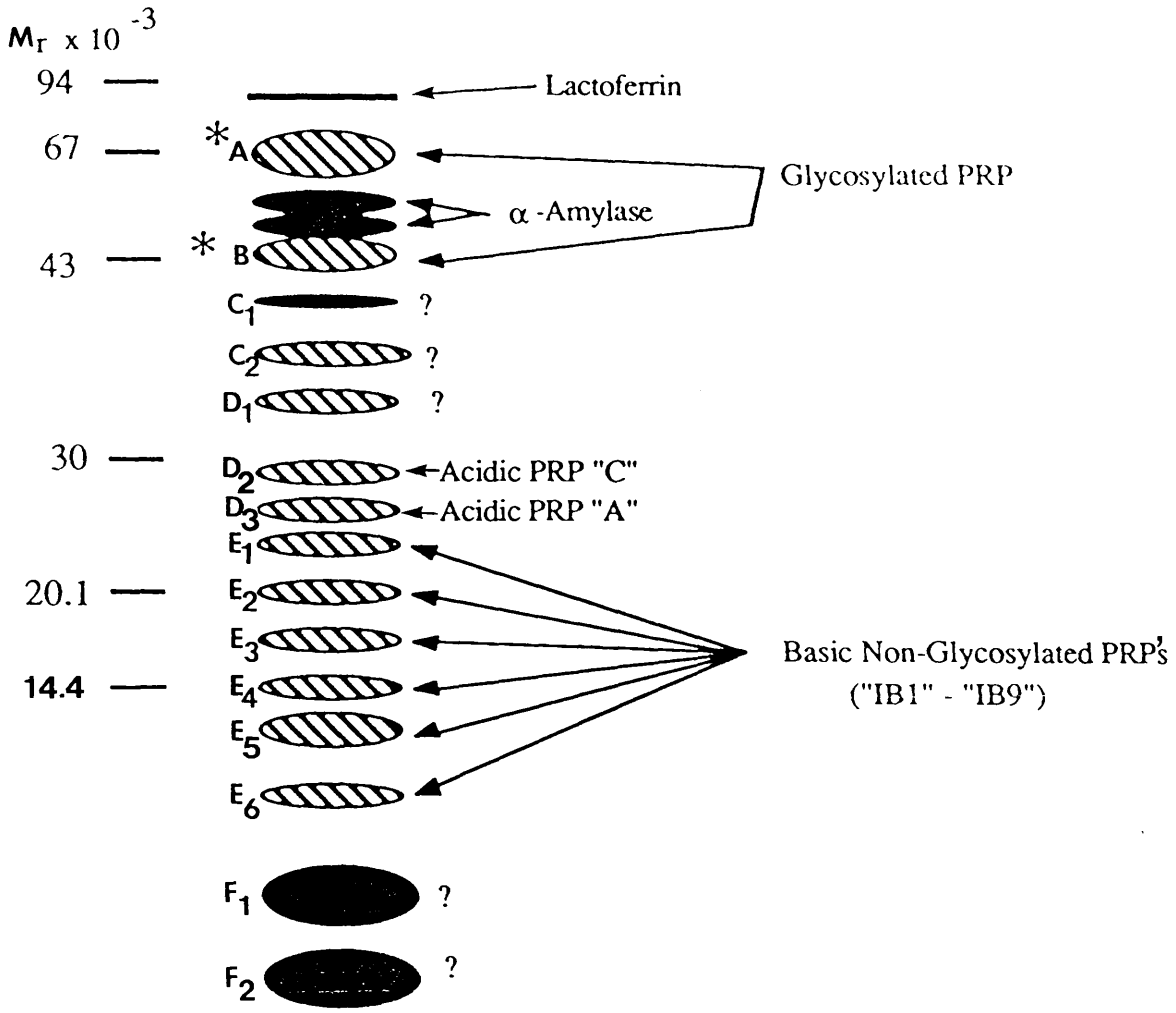
The M_r's of the purified PRP's which were supplied by other workers were determined by SDS-PAGE and found to be significantly higher than the values determined by other methods such as gel filtration, ultracentrifugation and amino acid sequencing.

Many proteins are known to exhibit anomalous M_r's in SDS-PAGE gels. For example, glycoproteins with > 10% carbohydrates will bind less SDS per unit weight than other proteins, thus giving different apparent M_r's than those determined by using other methods (Hames, 1981). The unusual amino acid sequence of proteins with a high proline content may alter the charge and conformation of the SDS-polypeptide complex, thus producing abnormally high M_r's in SDS-PAGE gels (See and Jackowski, 1989). SDS-PAGE of collagen, which has a high content of proline and hydroxyproline, has been shown by Freytag et al. (1979) to yield anomalously high M_r values for the polypeptides when typical globular proteins were used as the standards.

Fig. 3.13. Generalised band pattern of parotid salivary proteins revealed by SDS-PAGE

The darkened zones represent the bands which were stained blue by CBB R250, while the shaded zones represent the bands which were stained pink. The pink-staining bands were assigned the nonmenclature used in Fig. 3.3. The bands which have been identified, either by immuno- and lectin affinity-blotting or co-electrophoresis with PRP's which have been purified and partially characterised by other researchers, are indicated.

Figure 3.13


M_r x 10^{-3}
94
67
43
30
20.1
14.4
Lactoferrin
*A
Glycosylated PRP
α -Amylase
* B
C_1 ?
C_2 ?
D_1 ?
D_2 ←Acidic PRP "C"
D_3 ←Acidic PRP "A"
E_1
E_2
E_3
E_4
E_5
E_6
Basic Non-Glycosylated PRP's
("IB1" - "IB9")
F_1 ?
F_2 ?
Con A-binding and
* PAS-positive bands
? Unidentified Bands
Stained pink by CBB R250
Stained blue by CBB R250

The band patterns of the PRP's exhibited considerable individual-to-individual variation, which has been attributed to differential mRNA-splicing and post-translational modification (Maeda et al., 1985).

Of the RA and SS patients whose parotid salivary proteins were fractionated on SDS-PAGE gels, only one exhibited additional bands. These blue-staining bands of M_r's approximately 90,000 and 35,000 could correspond to the additional proteins, of high and low R_f values respectively, found in the parotid saliva of SS patients by Benedak-Spät et al. (1980). The blue-staining band of M_r 90,000, which has been shown by immunoblotting to be lactoferrin (Fig. 3.10 (b)), was present in all the other salivary samples analysed by SDS-PAGE, but is more intensely stained in the SS patient previously mentioned. The level of salivary lactoferrin has been reported to be elevated in SS patients (Jezequel et al., 1989).

Two possible reasons why SDS-PAGE did not reveal substantial differences between the patients and normal subjects, despite differences being apparent on isoelectric focusing, are:

(a) The considerable degree of individual-to-individual variation, especially in the PRP's, tended to complicate the comparison of the band patterns between different individuals.

(b) The anionic salivary proteins associated with RA and SS could have M_r's which are equal or very close to that of

another protein and therefore were not adequately resolved by a one-dimensional SDS-PAGE system.

Accordingly isoelectric focusing and 2-D PAGE must be carried out to in order to establish their pI's and M_r's.

Rhodus et al. (1989) subjected salivary proteins from primary and secondary SS patients to SDS-PAGE followed by silver stain. They found that PRP's of lower M_r were deficient in all SS patients compared to those of normal healthy controls, and also that the primary SS patients demonstrated more missing bands than the secondary SS patients. Although PRP's, in particular the acidic ones, are known to be poorly stained by the silver stain technique (Friedman, 1982), this was the staining procedure used. Their findings, however, could not be confirmed in this study. Indeed, no PRP band pattern which was charactersitic of either RA or SS could be detected. It is therefore highly probable that the differences that were reported by Rhodus et al. (1989) were due to individual-to-individual variation in the band pattern of the PRP's, and to the lack of sensitivity of the silver stain technique with respect to PRP's.

3.3. Isoelectric Focusing of Human Parotid Salivary Proteins From Normal Healthy Individuals in Hybrid Carrier Ampholyte-Immobilised pH Gradient Polyacrylamide gels

3.3.1. Determination of the pH Gradient

IPG's of a broad pH range, i.e. pH 4-9, were used to analyse human parotid salivary proteins. The pH profiles of pH 4-9 IPG's were determined using coloured pI marker proteins. The pH profile of the IPG's was determined under non-denaturing conditions (i.e. in the absence of urea) as the pI's of the marker proteins in urea was not supplied by the manufacturer. In the presence of urea, changes in the pI's of many proteins are known to occur (Andrews, 1986). The pH profile of a pH 4-9 IPG, shown Fig. 3.14, appears to be approximately linear.

3.3.2. Effect of Sample Application Position on the Focused Pattern

Samples of parotid saliva were applied at various positions on a pH 4-9 IPG in order to determine the application point which resulted in the best resolution of the band pattern.

Optimum results were obtained when the samples were applied at a distance of 2-3 cm from the cathode, where the local pH is 6.5-7.0 (Fig. 3.15). When the samples were applied at other points along the gel, some degree of precipitation and streaking is observed. Application of the sample close to the cathode resulted in the most severe

Fig. 3.14. pH profile of a pH 4-9 IPG

The IPG was rehydrated with 20% (w/v) glycerol containing 0.5% (w/v) pH 3.5-10 Ampholine. BDH broad range (pH 4.7-10.6) coloured pI markers were used.

All the protein markers were coloured and could be visualised without prior staining.

Figure 3.14.

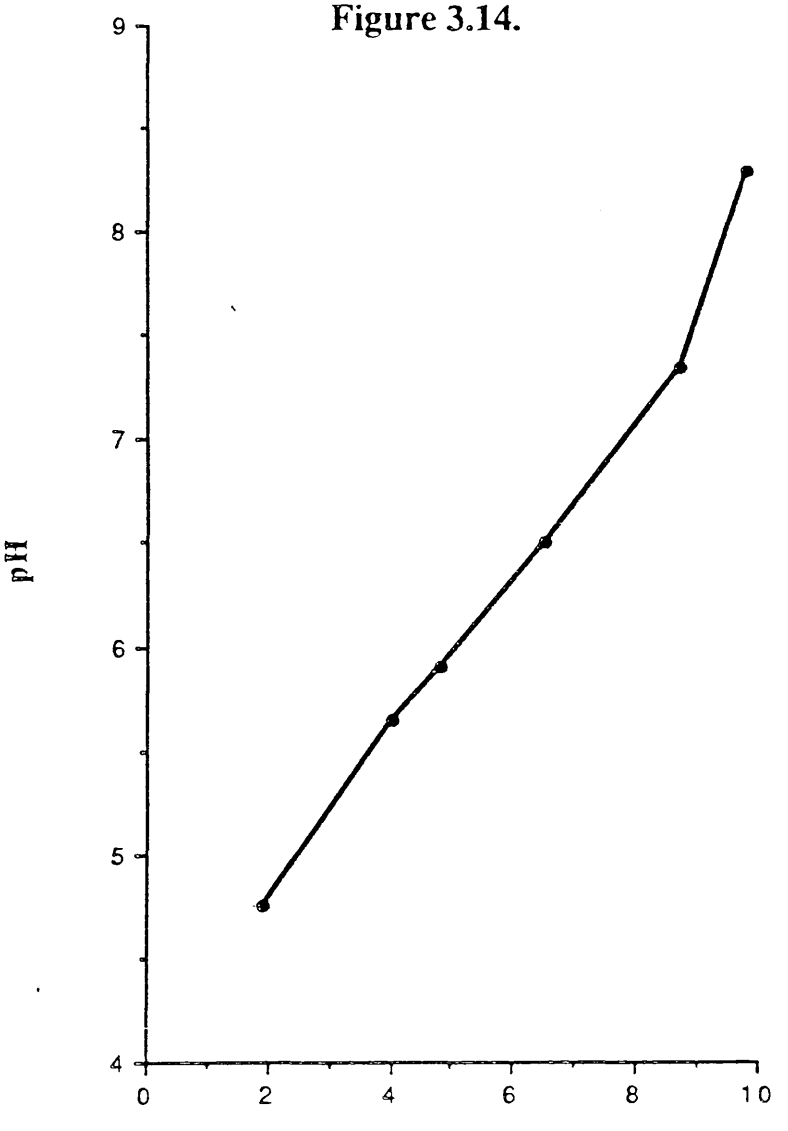
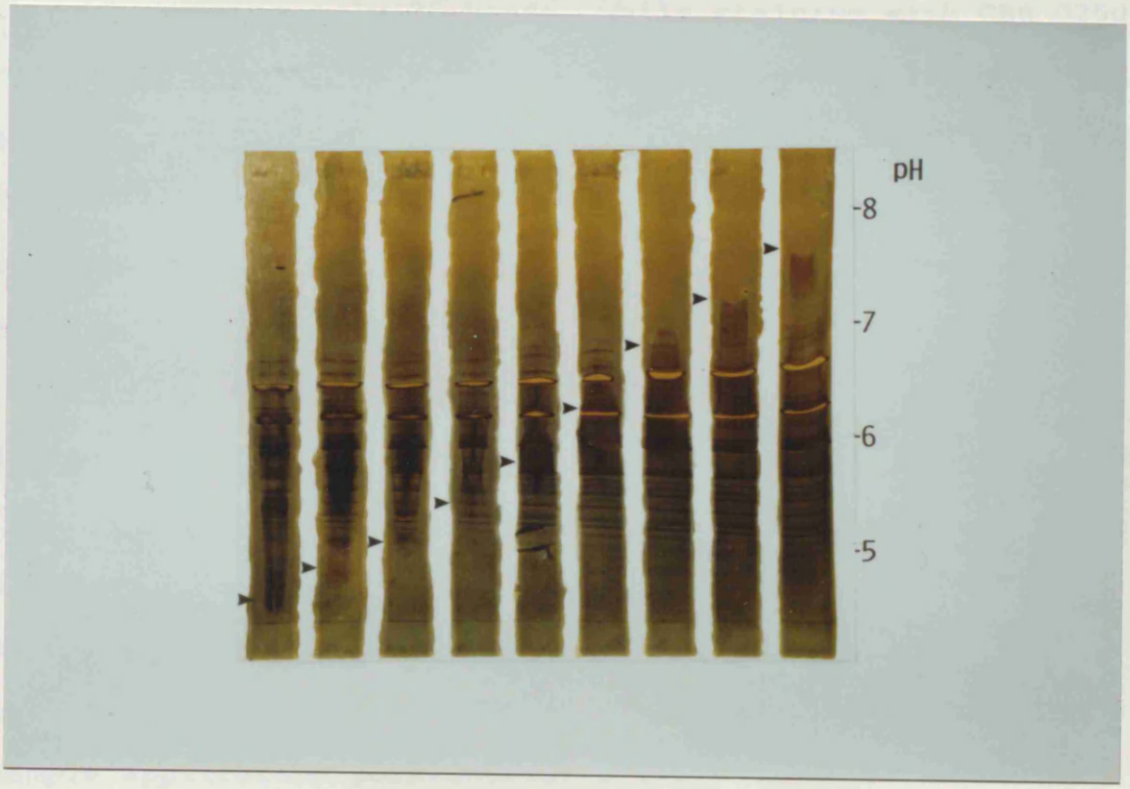


Fig. 3.15. IEF of parotid salivary proteins from a single individual in a pH 4-9 IPG, with sample application made at various points along the gel

Twenty five μg of protein was applied to each track and the gel was silver stained. The sample application points are indicated by arrowheads. The IPG was rehydrated with 20% (w/v) glycerol containing 0.5% (w/v) pH 3.5-10 Ampholine. The gel was silver stained.

Figure 3.15



streaking.

3.3.3. Comparison of Coomassie and Silver Staining

A range of protein loads, from 10-250μg, was applied to a pH 4-9 IPG which was subsequently stained by CBB G250 (Fig.3.16 (a)) followed by silver stain (Fig. 3.16 (b)).

By silver staining, 20μg of protein allowed visualization of approximately 25 bands, while staining with CBB G250 required 250μg in order to give similar results. The band patterns, however, were similar using both procedures, and the differences were only at the level of sensitivity rather than additional components being stained by the silver procedure.

3.3.4. Effect of Salt Levels on the Band Patterns

Upon adding known levels of sodium chloride to a sample of parotid saliva, it was observed that denaturation occurred at sodium chloride concentrations of 200mM and above, the effect being more pronounced on the cathodal side of the sample application points (Fig. 3.17).

No differences were observed between lyophilisates of parotid saliva, either dialysed or undialysed, which have been concentrated up to sevenfold (Fig. 3.18). The sodium, potassium and calcium concentrations of the unconcentrated parotid saliva prior to dialysis were determined by flame photometry and found to be 18mM, 16mM and 0.2mM respectively.

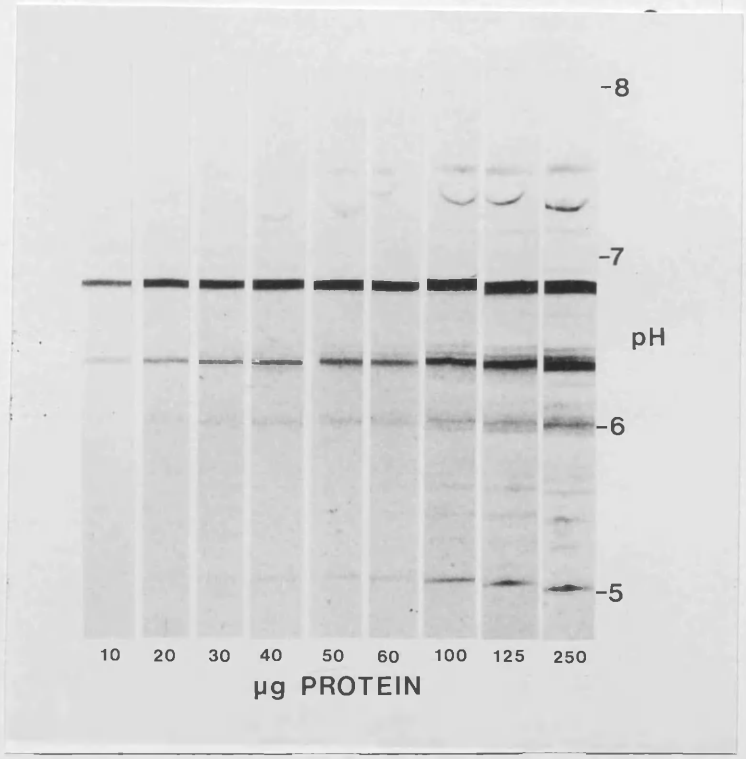
Figure 3.16. Comparison of Coomassie Brilliant Blue and silver staining of parotid salivary proteins fractionated in a pH 4-9 IPG

Panel (a) shows the IEF of human parotid salivary proteins from a single individual on a pH 4-9 IPG followed by CBB G250 staining. The protein loads are as indicated.

Panel (b) shows the same gel after silver staining.

Figure 3.16

(a)



(b)

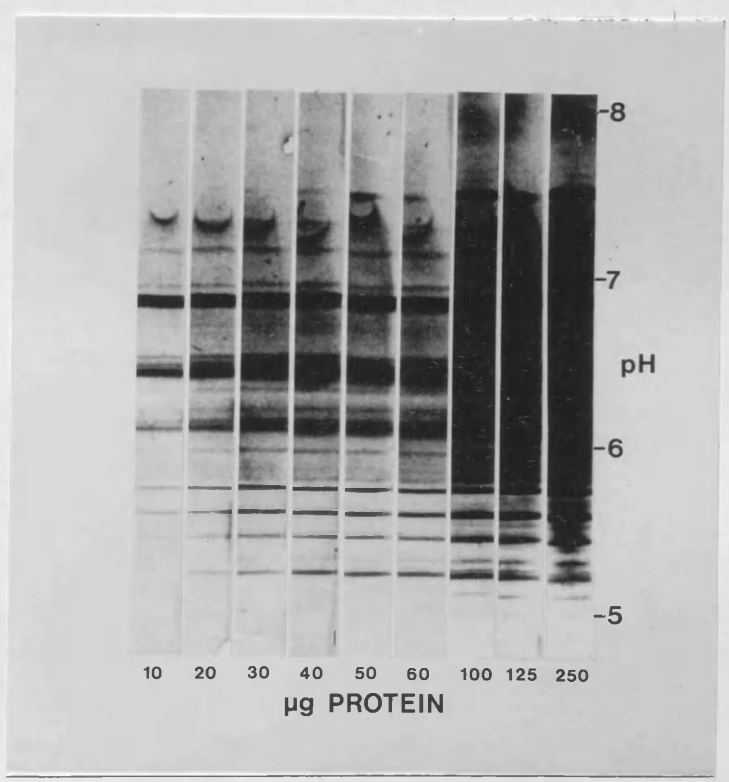


Figure 3.17. IEF, in a pH 4-9 IPG, of human parotid salivary protein samples containing increasing concentrations of sodium chloride

The samples, containing 25μg of protein in 50μl of solution, were from the same individual. Arrowheads indicate precipitation. The concentrations of sodium chloride are as indicated. The IPG was rehydrated with 20% (w/v) glycerol containing 0.5% (w/v) pH 3.5-10 Ampholine. The gel was silver stained.

Figure 3.17

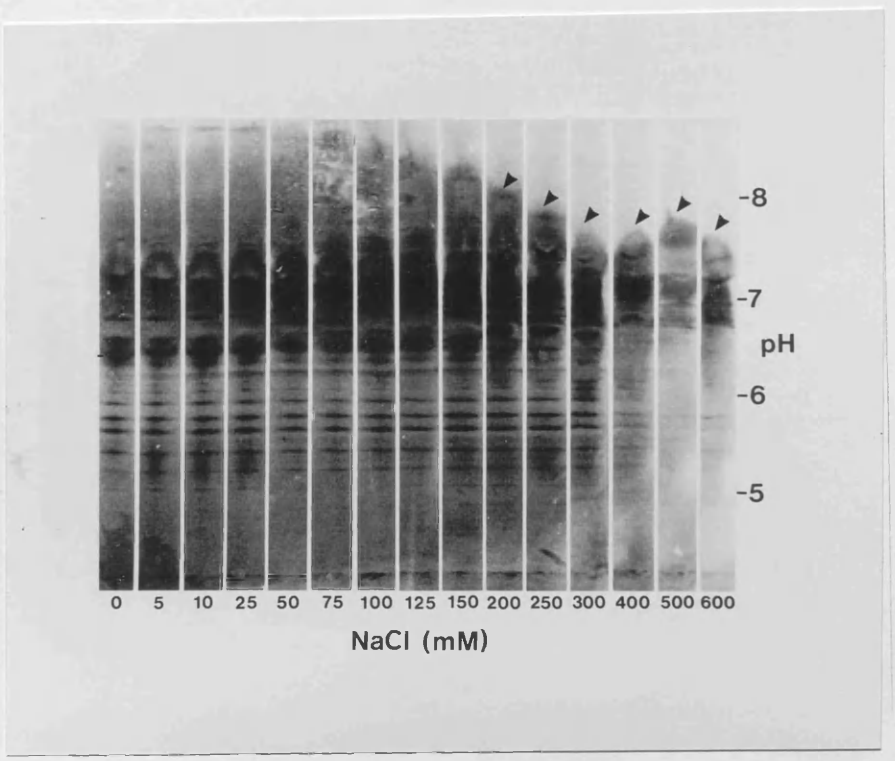
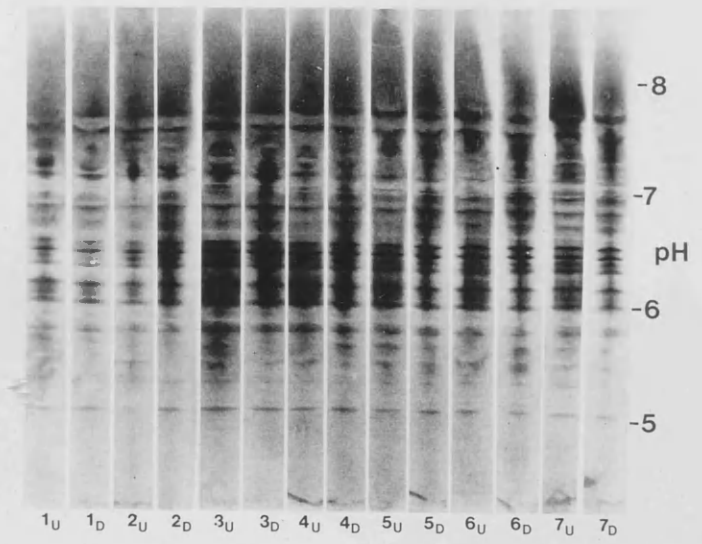


Figure 3.18. IEF, in a pH 4-9 IPG, of dialysed and undialysed lyophilised concentrates of human parotid saliva

1 to 7-fold concentrates were applied to tracks 1 to 7, respectively. The tracks with subscript "U" were loaded with undialysed samples while tracks with subscript "D" were loaded with dialysed samples. 25 μg of protein were applied to each track. The IPG was rehydrated with 20% (w/v) glycerol containing 0.5% (w/v) pH 3.5-10 Ampholine. The gel was silver stained.

Figure 3.18



3.3.5. Effect of Urea on the Band Patterns

In a pH 4-9 IPG, a urea gradient (0-8 M urea) perpendicular to the pH gradient was found to increase the resolution of the protein bands, especially above a urea concentration of 5M (Fig. 3.19). Most of the proteins focused as continuous bands throughout the length of the IPG. Some components exhibited a slight pI shift towards the anode, which is probably due to the exposure of charged groups in the proteins when they were enfolded by urea (Creighton, 1979).

3.3.6. Comparison of Band Patterns of Different Individuals

Parotid saliva samples from 6 different individuals were fractionated in a pH 4-9 IPG (Fig. 3.20). There were few major but several minor variations from individual to individual. The technique appears to give better resolution than that of Eckersall *et al*. (1981) who performed IEF in carrier ampholyte gels and also reported that the band patterns of different individuals were essentially similar with only minor differences.

3.3.7. Discussion

In order to further the understanding of the functions of salivary proteins and to develop the potential for the analysis of this fluid in clinical diagnosis, improved analytical electrophoretic procedures are needed. Whilst

Figure 3.19. IEF, in a pH 4-9 IPG, of a continuous zone of normal human parotid salivary proteins applied 2cm from the cathode throughout the length of a gel superimposed with a transverse 0-8 M urea gradient

One mg of protein was applied to a gel containing a urea gradient perpendicular to its pH gradient. The gel was silver stained.

Figure 3.19

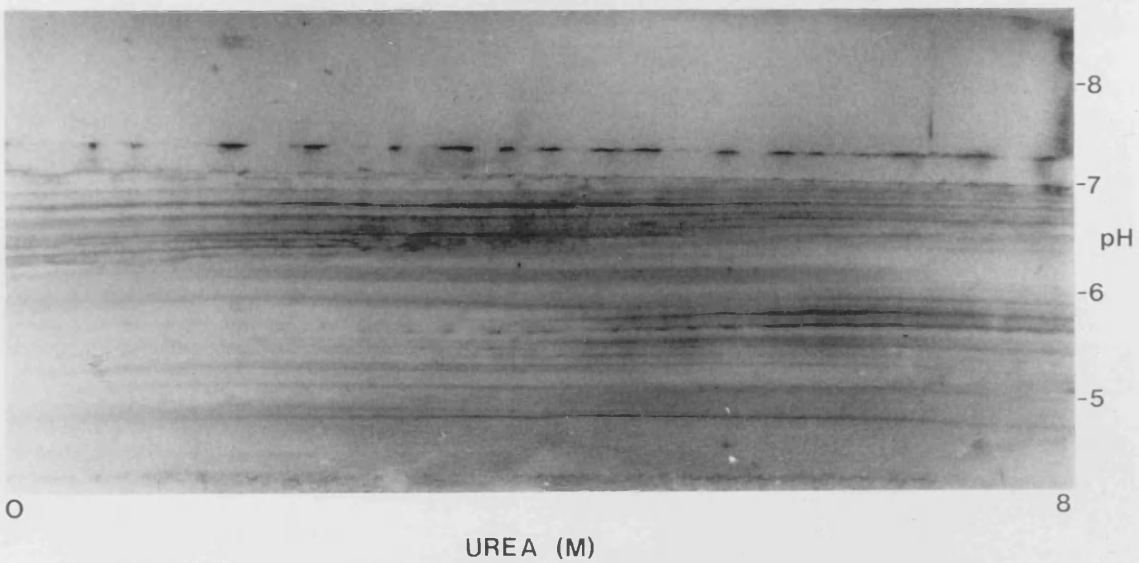
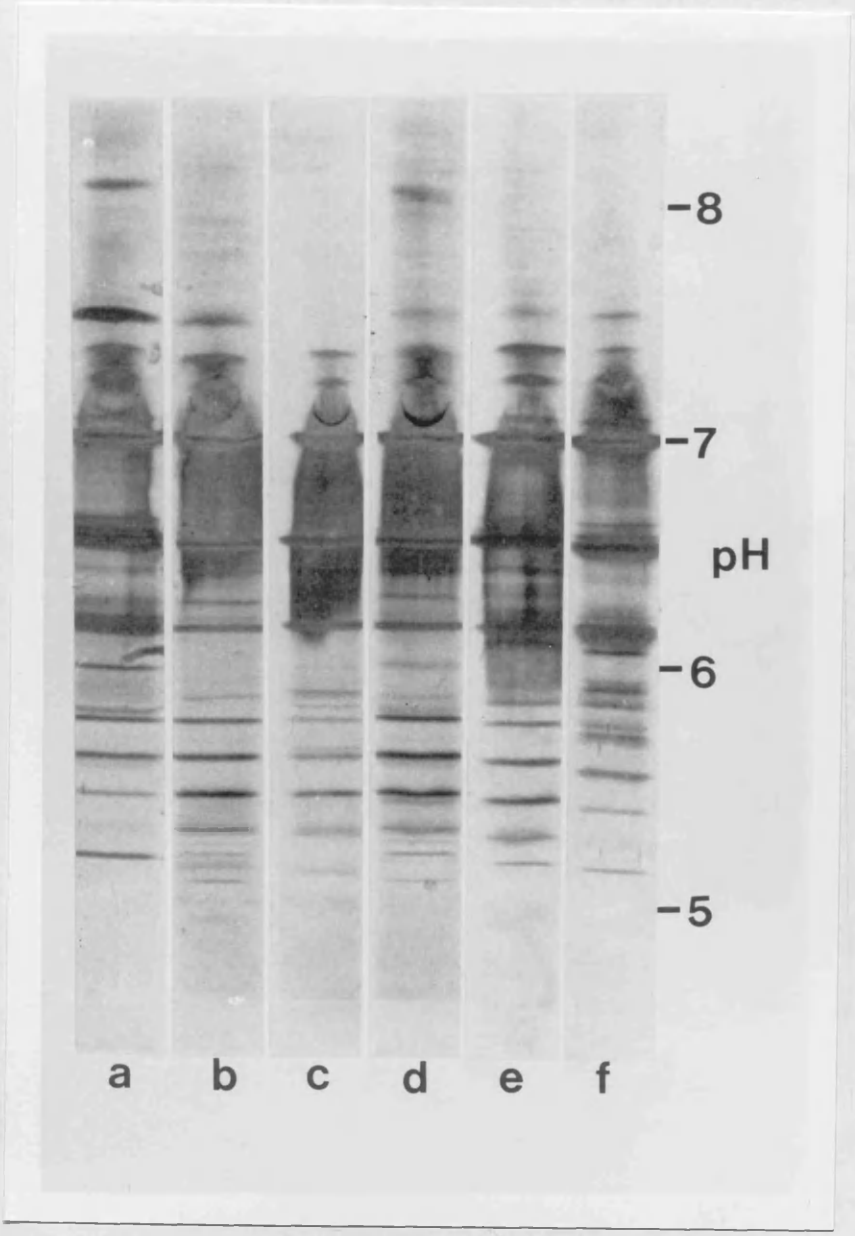


Figure 3.20. IEF, in a pH 4-9 IPG, of parotid salivary proteins from 6 diferent normal healthy individuals in a pH 4-9 IPG

The protein load was 25μg per sample. The IPG was rehydrated with 20% (w/v) glycerol containing 0.5% (w/v) pH 3.5-10 Ampholine. The gel was silver stained.

Figure 3.20



isoelectric focusing of human parotid saliva using carrier ampholytes has given improved resolution, as compared to other electrophoretic procedures, and revealed further information about the nature of proteins present in both normal healthy individuals (Eckersall et al., 1981) and in disorders such as rheumatoid arthritis (Mairs and Beeley, 1985), use of the technique has been impaired by the need for prior desalting of samples because of the high electrolyte to protein levels. This procedure is time-consuming and leads to protein loss; furthermore the sample sizes necessary for manipulation (> 2ml) are frequently not available from diseased glands. The technique is also subject to problems such as cathodic drift together with the fact that the formation of "tailor-made" gradients necessary for the proteins under investigation can be difficult or impossible.

IPG's overcame most of these problems (Righetti, 1984) but did occasionally give rise to streaking and precipitation at the sample application site. Addition of carrier ampholytes to the IPG's to form hybrid carrier ampholyte-IPG's (Fawcett and Chrambach, 1986a) resolved this problem and the procedure described is based on this methodology.

Incorporation of urea into the gel gave improved resolution in pH 4-9 IPG's. Urea is often used as a solubilising agent in electrophoresis (Andrews, 1986) and IEF in gels containing urea gradients perpendicular to the pH axis (Hobart, 1975; Altland et al., 1984) may yield im-

portant clues about protein structure. Alterations in the pI's of proteins will occur if unfolding by urea results in the exposure of charged groups (Creighton, 1979). In IPG's of both pH ranges, urea was found to cause shifts in the pI's of most protein bands but results in better resolution.

Rehydrating the gels with 20% (w/v) glycerol was found to be advantageous as it prevented liquid exudation (Altland et al., 1986). When liquid exudation or "sweating" occurs, the resulting droplets on the gel surface may after prolonged focusing adversely affect the focused pattern.

A limitation of IPG's is their inherently low conductivity which causes difficulties such as slow sample entry, lateral zone spreading and the formation of salt fronts which halt protein migration. A hybrid carrier ampholyte-immobilised pH gradient IEF system was employed to circumvent this problem (Fawcett and Chrambach, 1986a; Altland et al., 1987). This was achieved by reswelling dried IPGs in carrier ampholyte-containing rehydration solutions.

The presence of salts in the samples is capable of causing protein denaturation because acidic and alkaline boundaries are produced at application points upon the application of an electric field across the gel. Thus even low levels of salts (5mM) have been reported to induce modification of haemoglobin patterns and higher levels to cause denaturation (Righetti et al., 1988). In order to investigate whether the levels of electrolytes present in saliva would have any adverse effects, a study was performed to

determine the salt level which would lead to protein precipitation and denaturation. We have found salivary proteins to be less affected by salts, and satisfactory patterns were obtained from samples containing up to 200mM sodium chloride. This is well above the electrolyte levels in parotid saliva. No precipitation was observed when lyophilisates concentrated up to sevenfold without desalting were fractionated on IPG's.

pH 4-9 IPG's of parotid salivary proteins produced highly resolved focused patterns. With samples containing as little as 20μg of proteins, 25-30 components were evident on silver staining. Interestingly, IPG's, like carrier ampholyte-based IEF, do not reveal the individual-to-individual differences in protein pattern in parotid saliva as is the case with SDS-PAGE (Lindsay and Beeley, 1986).

The IPG technique gives superior resolution to carrier ampholyte IEF of human salivary proteins (Eckersall et al., 1981). Analysis of salivary proteins by focusing on IPG's also removes the need to desalt samples prior to analysis and can be used with sample volumes as small as 50μl.

Accordingly, it is now possible to analyse rapidly large numbers of parotid saliva samples as a function of pI. This development should lead to considerable progress in the analysis of human salivary proteins.

The commercially-available Immobilines allow gels of pH range between 4 and 10 to be prepared. While such a range is sufficient for many applications, it may not produce optimal

resolution of highly basic or acidic proteins. Thus at present, a major disadvantage of the IPG system is that it is not possible, using commercial Immobilines, to prepare IPG's covering pH extremes (i.e. < 4 and > 10) (Altland, 1990). Although acidic (pK 0.8 and 3.1) and basic (pK 10.3) Immobiline-type acrylamide derivatives have been synthesized by various laboratories in order to produce IPG's spanning the pH range between 2.5 and 11 (Gianazza et al., 1989; Altland and Altland, 1990), they are not yet commercially available. When these are finally available, IPG's of pH range such as 2.5-10.5 could be prepared. An IPG of such a pH range would allow the fractionation of almost all the salivary proteins in a single run. IPG's covering the acidic (e.g. pH 2.5-5.0) or basic (e.g. pH 8-11) pH ranges could be prepared, which could be very useful in studying, for example, the acidic and basic PRP's.

The problem of cathode drift, which often makes it difficult or even impossible to fractionate basic proteins (pI's > 8) in carrier-ampholyte IEF gels, does not occur in IPG's. Thus the advantages that IPG's offer over conventional carrier ampholyte-based IEF make them especially well-suited for the fractionation of salivary proteins. IPG's may also be used for the separation of salivary proteins in the first dimension of 2-D PAGE.

3.4. 2-D PAGE of Human Parotid Salivary Proteins from Normal Healthy Individuals

3.4.1. 2-D PAGE with Carrier Ampholyte-Containing Polyacrylamide Gels in the First Dimension

Parotid salivary protein (100μg) from a normal healthy subject was fractionated using a modification of the 2-D PAGE method of O'Farrell (1975). A carrier ampholyte-containing rod gel was used in the first dimension and a 5-20 %T linear gradient polyacrylamide gel of 1.5mm thickness in the second. Staining of the 2-D PAGE gel with CBB R250 revealed 7 pink-staining and one blue-staining components (Fig. 3.21 (a)). With the exception of a blue-staining component of low M_r (approximately 14,000) and a pink-staining component of M_r approximately 28,000, which were located near the anodic end of the gel, most of the other components were located near the cathodic end. Silver-staining revealed an additional 5 spots of M_r aproximately 45,000 which were not visualised by Coomassie staining (Fig. 3.21 (b)). Considerable horizontal streaking was evident in the silver-stained 2-D PAGE gel. The resolution was poor and it appears that many constituents may have been lost as a result of cathode drift.

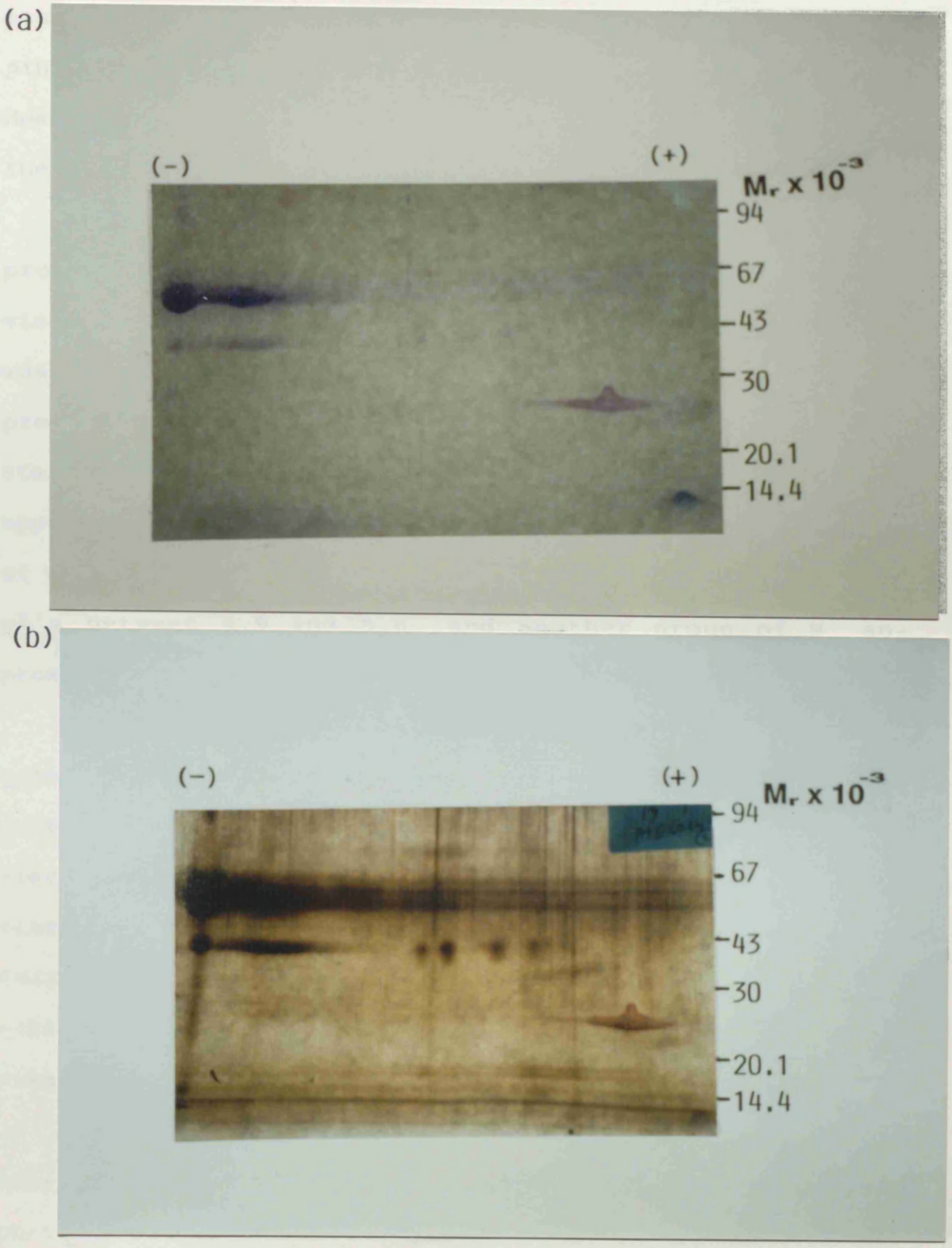
3.4.2. 2-D PAGE with Immobilised pH Gradients in the First Dimension

Parotid salivary protein (100μg) from a normal healthy subject was fractionated by 2-D PAGE, using a pH 4-9 IPG in

Fig. 3.21. O'Farrell-type 2-D PAGE of parotid salivary proteins

One hundred μg of protein was applied to the first dimension gel. The gel in panel (a) was stained by CBB R250. Panel (b) shows the same gel after silver staining. The anodic and cathodic ends of the first dimension gel are denoted by "+" and "-" signs, respectively.

Figure 3.21


(a)
(-)
(+)
$M_r \times 10^{-3}$
94
67
43
30
20.1
14.4
(b)
(-)
(+)
$M_r \times 10^{-3}$
94
67
43
30
20.1
14.4

the first dimension and a 3-15 %T linear gradient polyacrylamide gel in the second. The gel was subsequently stained with CBB R250, which revealed approximately 10 pink-staining and 12 blue-staining spots (Fig. 3.22 (a)). Most of the pink-staining components were located close to the cathodic end of the gel.

Silver staining of a gel which contained a similar protein load revealed many additional components not visualised by CBB R250 (Fig. 3.22(b)). Excellent resolution was obtained and about 50 spots could be detected. The protein spots which were stained pink by CBB R250 were stained a yellowish-brown colour by the silver procedure and appear to be PRP's. Silver staining also revealed two groups of multiple spots; one group of M_r approximately 45,000 and pI's between 4.9 and 5.6, and another group of M_r approximately 28,000 and pI's between 4.8 and 5.3.

In order to identify some of the spots in the 2-D PAGE gels of human parotid salivary proteins and thus construct a normalised 2-D map of these proteins, the 2-D gels were electroblotted and probed with a variety of specific antisera to various proteins. After incubation in solutions containing the appropriate anti-species immunoglobulin-alkaline phosphatase conjugates, the blots were developed in alkaline phosphatase substrate solution.

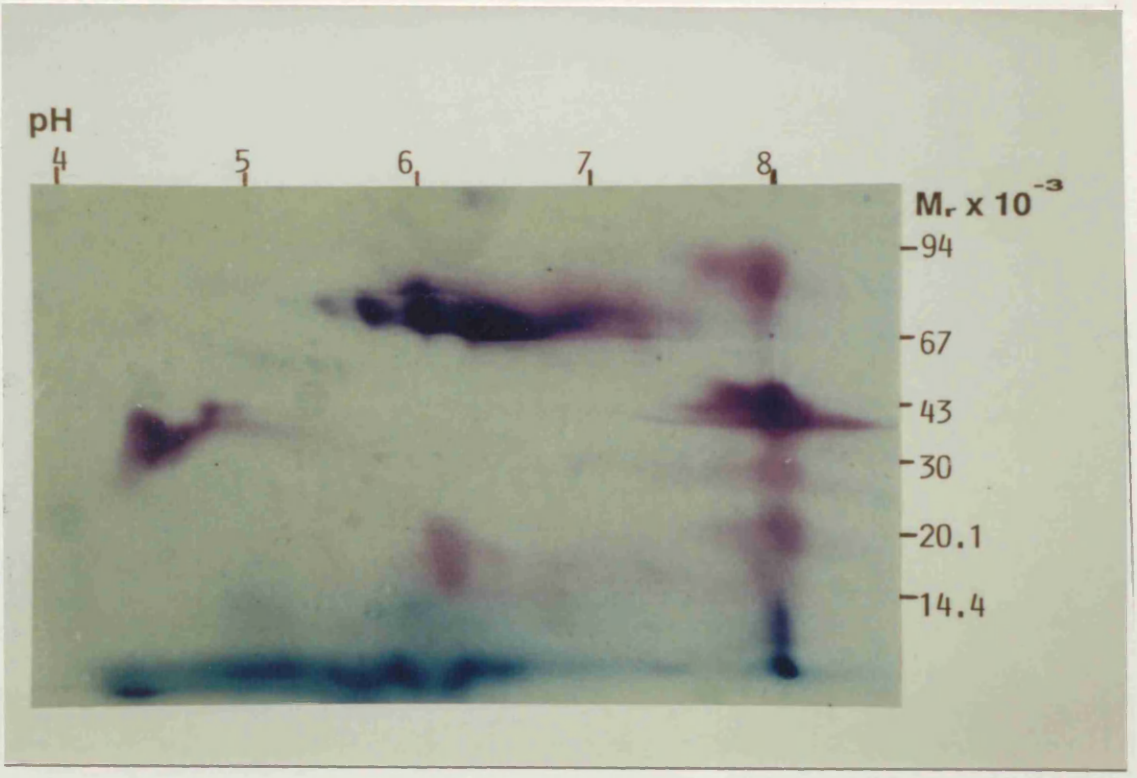
Two different sets of control experiments were carried out. In the first, following 2-D PAGE of parotid salivary proteins and electroblotting, the blots were probed with the

Fig. 3.22. 2-D PAGE, with pH 4-9 IPG in the first dimension, of parotid salivary proteins

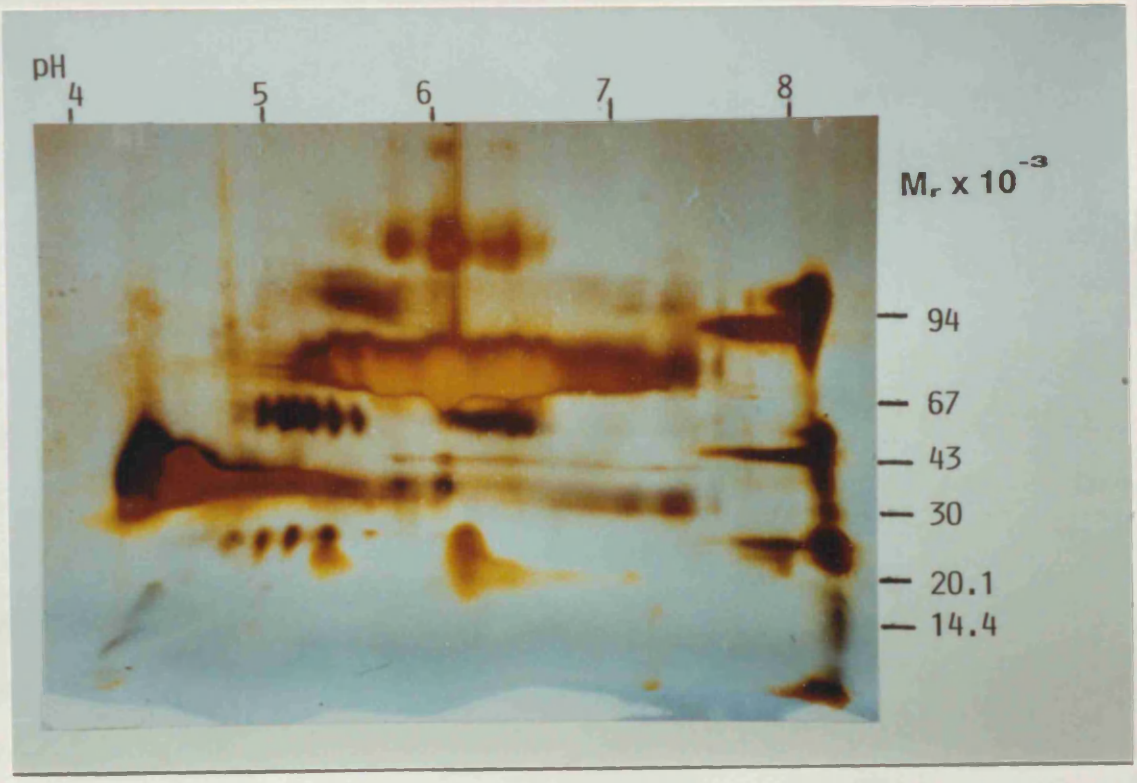
One hundred μg of protein from different normal healthy subjects was applied to each gel. The gel in panel (a) was stained with CBB R250, while the gel in panel (b) was silver stained.

Figure 3.22

(a)



(b)



anti-species immunoglobulin-alkaline phosphatase conjugate, i.e. omitting the primary antibody and developed in the substrate solution for alkaline phosphatase. Another control experiment involved 2-D PAGE of parotid salivary proteins followed by electroblotting, after which the blots were incubated in PBS containing non-immune rabbit serum (1:200 dilution), followed by incubation in a solution containing anti-rabbit immunoglobulin-alkaline phosphatase conjugate, and finally by development of the blot in the substrate solution for alkaline phosphatase. None of the control experiments, however, resulted in any alkaline phophatase signal in the blots after they were developed.

By immunoblotting, the locations of α-amylase (Fig. 3.23(a)), lactoferrin (Fig. 3.23(b)), secretory component (Fig. 3.23(c)), α-heavy chain of IgA (Fig. 3.23(d)), κ-light immunoglobulin chain (Fig. 3.23(e)), λ-light immunoglobulin chain (Fig. 3.23(f)), lysozyme (Fig. 3.23(g)) and albumin (Fig. 3.23(h)) were determined. The antisera to lactoferrin, secretory component, lysozyme and albumin exhibited non-specific binding to α-amylase. In addition, the antiserum to secretory component showed non-specific binding to 5 protein spots of M_r approximately 45,000.

Colostral sIgA was fractionated in a 2-D PAGE gel, with a pH 4-9 IPG in the first dimension, which was eventually silver stained (Fig. 3.23(i)). This revealed components corresponding to secretory component, α-heavy chain and κ- and λ-light chains; which correspond well with the immunoblots

Fig. 3.23. 2-D PAGE, with pH 4-9 IPG in the first dimension, of parotid salivary proteins followed by immunoblots for α-amylase, lactoferrin, secretory component, α-heavy chain, κ-light chain, λ-light chain, lysozyme and albumin; and 2-D PAGE of human colostral sIgA followed by silver stain

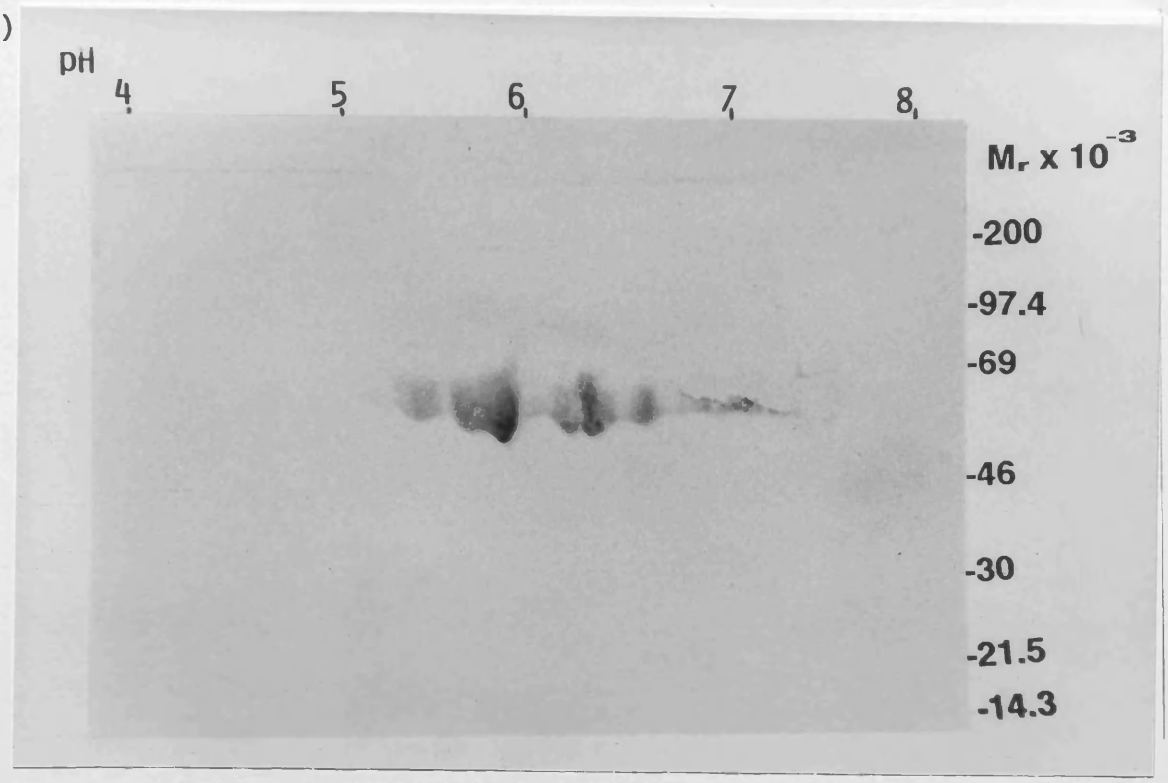
Fifty μg of parotid salivary protein was applied to each gel. Panel (a) shows an immunoblot for α-amylase. The primary antibody was sheep antiserum to human α-amylase, and the detecting antibody was anti-sheep immunoglobulin-alkaline phosphatase conjugate.

Panel (b) shows an immunoblot for lactoferrin. The primary antibody was rabbit antiserum to human lactoferrin, and the detecting antibody was anti-rabbit immunoglobulin-alkaline phosphatase conjugate. The arrowheads indicates the spot arising from non-specific binding of the primary antibody to α-amylase.

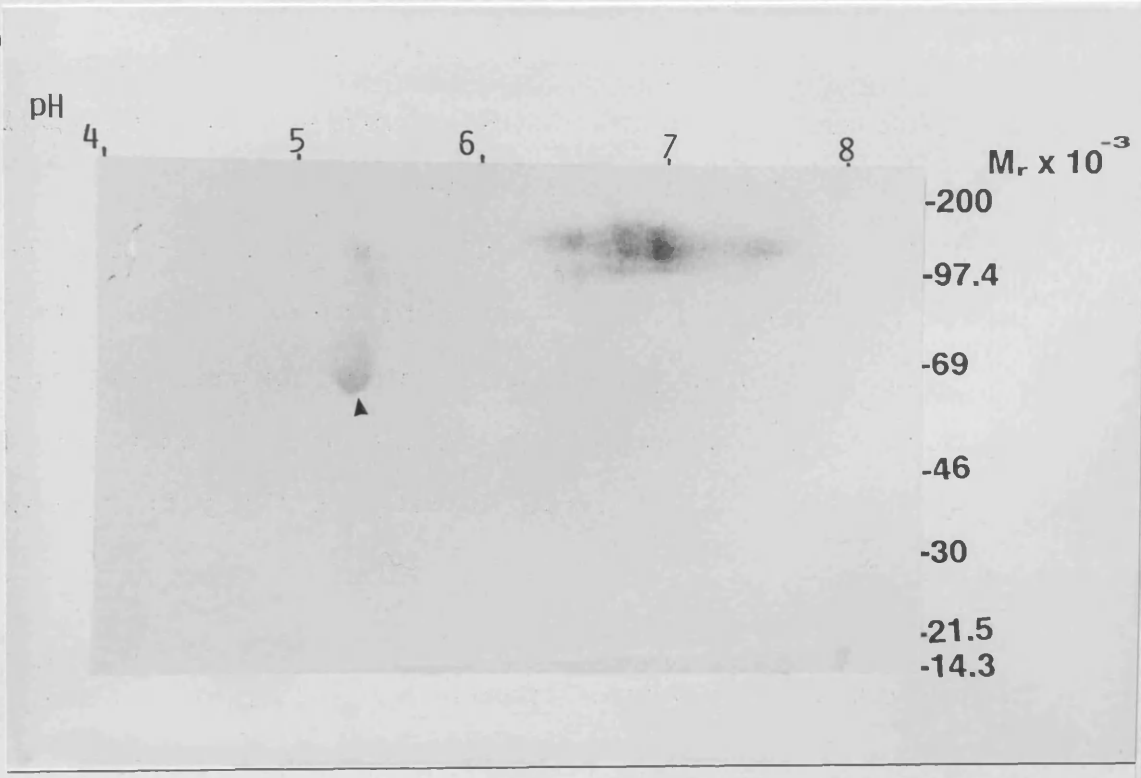
(Continued on following page/)

Figure 3.23

(a)



(b)



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Panel (c) shows an immunoblot for secretory component. The primary antibody was rabbit antiserum to human secretory component, and the detecting antibody was anti-rabbit immunoglobulin-alkaline phosphatase conjugate.

The arrowheads indicate spots arising from non-specific binding of the primary antibody to α-amylase and components of M_r approximately 45,000.

Panel (d) shows an immunoblot for α-heavy chain. The primary antibody was goat antiserum to human α-heavy chain, and the detecting antibody was anti-goat immunoglobulin-alkaline phosphatase conjugate.

Panel (e) shows an immunoblot for κ-light chain. The primary antibody was rabbit antiserum to human κ-light chain, and the detecting antibody was anti-rabbit immunoglobulin-alkaline phosphatase conjugate.

Panel (f) shows an immunoblot for λ-light chain. The primary antibody was rabbit antiserum to human λ-light chain, and the detecting antibody was anti-rabbit immunoglobulin-alkaline phosphatase conjugate.

Panel (g) shows an immunoblot for lysozyme. The primary antibody was rabbit antiserum to human lysozyme, and the detecting antibody was anti-rabbit immunoglobulin-alkaline phosphatase conjugate. The arrowheads indicates the spot arising from non-specific binding of the primary antibody to α-amylase.(Continued on following page/)

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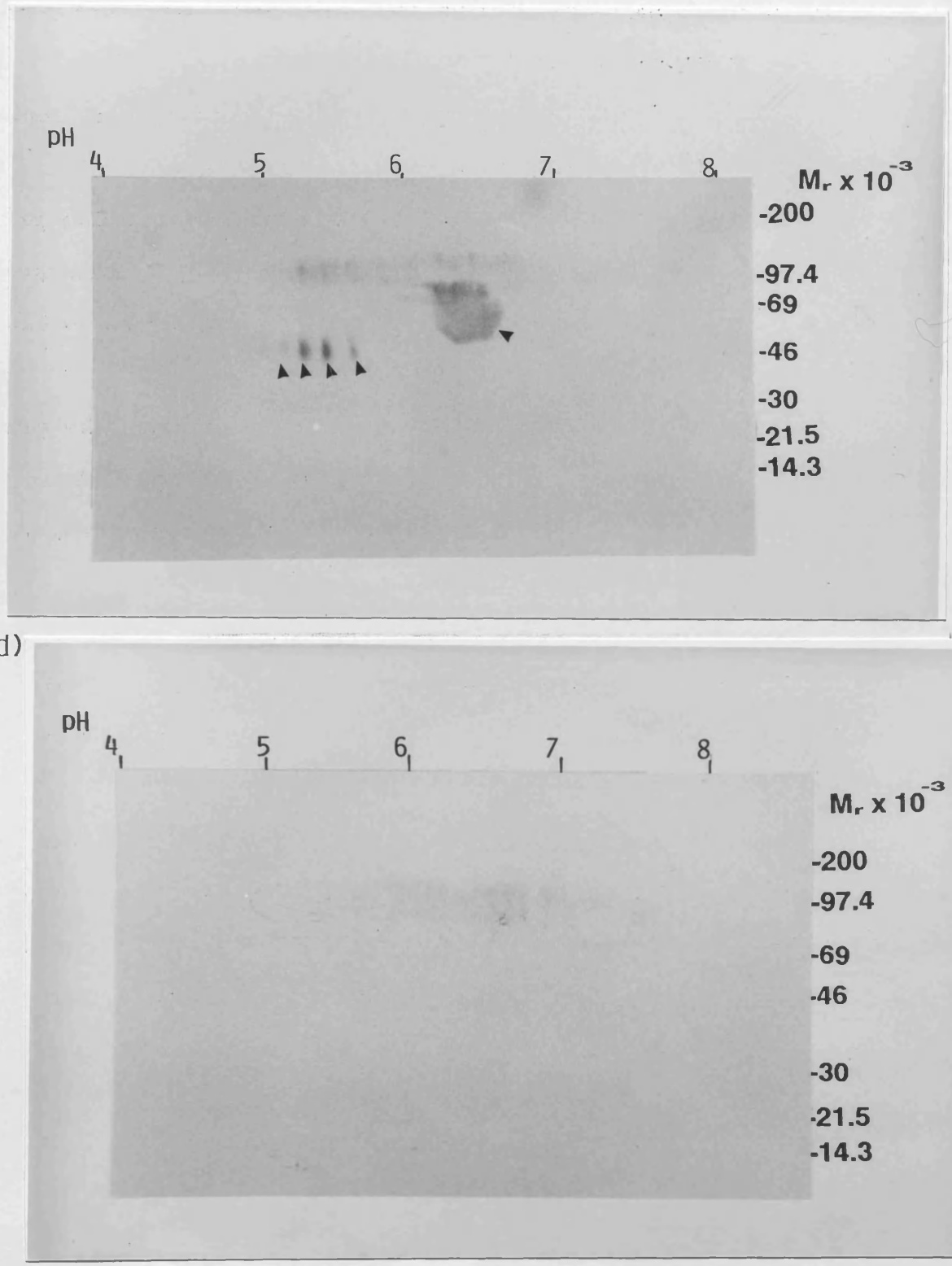
Figure 3.23



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Panel (h) shows an immunoblot for albumin. The primary antibody was goat antiserum to human serum albumin, and the detecting antibody was anti-goat immunoglobulin-alkaline phosphatase conjugate. The arrowhead indicates the spot arising from non-specific binding of the primary antibody to α-amylase.

Panel (i) shows a 2-D PAGE gel, using pH 4-9 IPG in the first dimension, of human colostral sIgA. 4μg of human colostral sIgA was applied, and the gel was silver stained. The components in the gel corresponding to secretory component (SC), α-heavy chain (α) and κ- or λ-light chains (κ/λ) are indicated.

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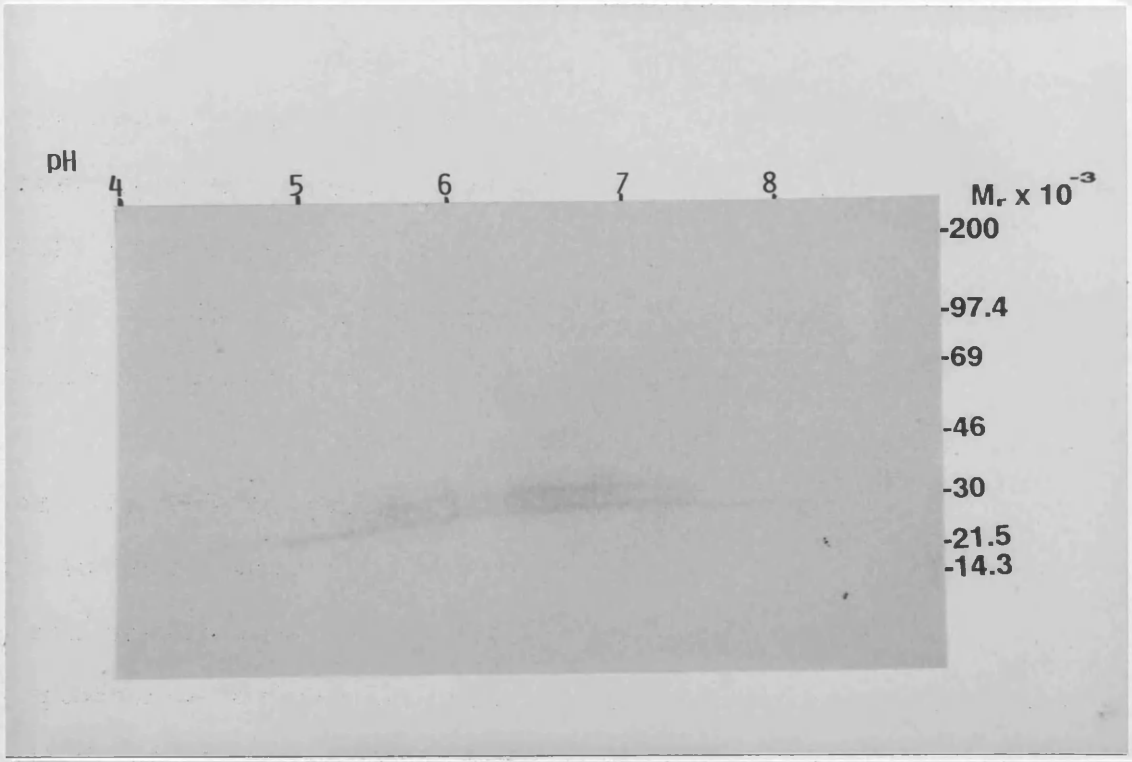
Figure 3.23

(e)



(f)

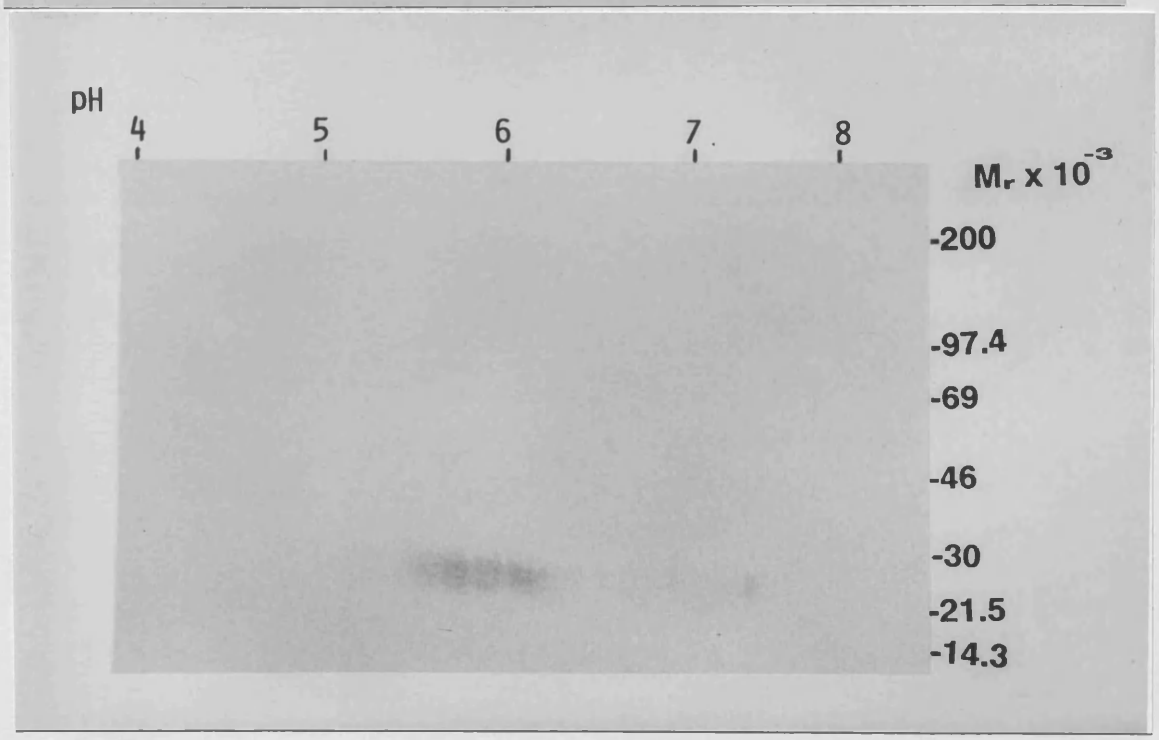


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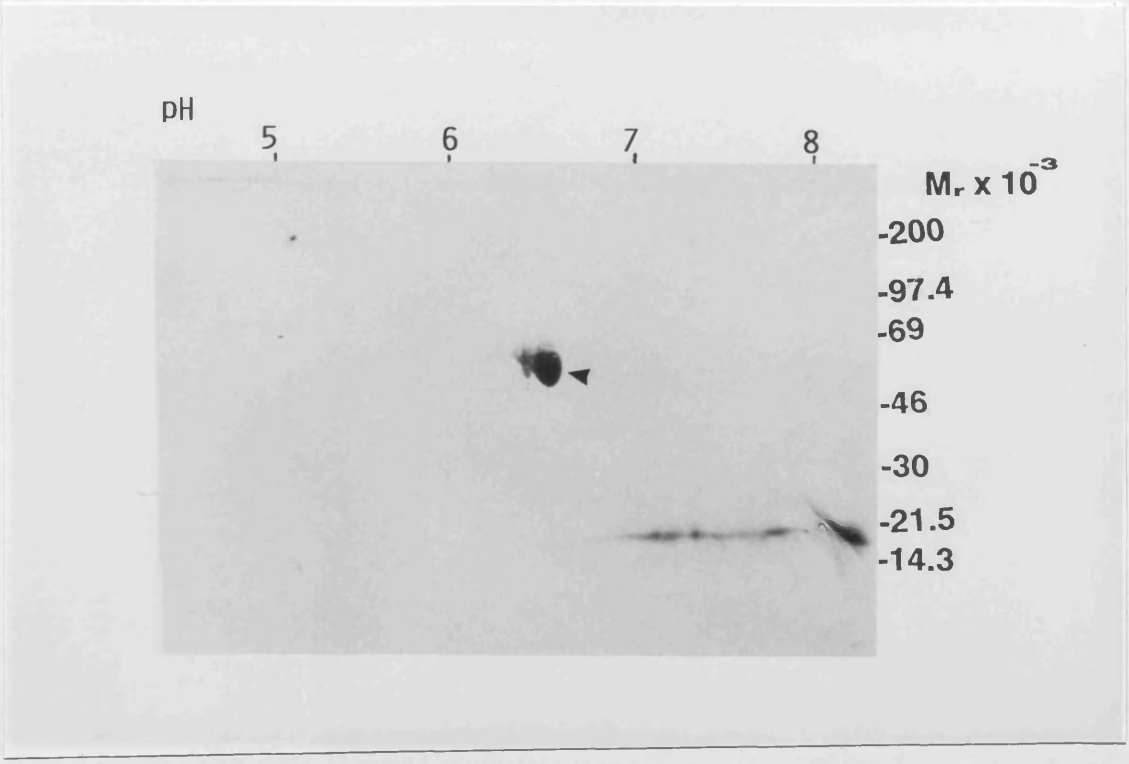
Figure 3.23

(g)



(h)

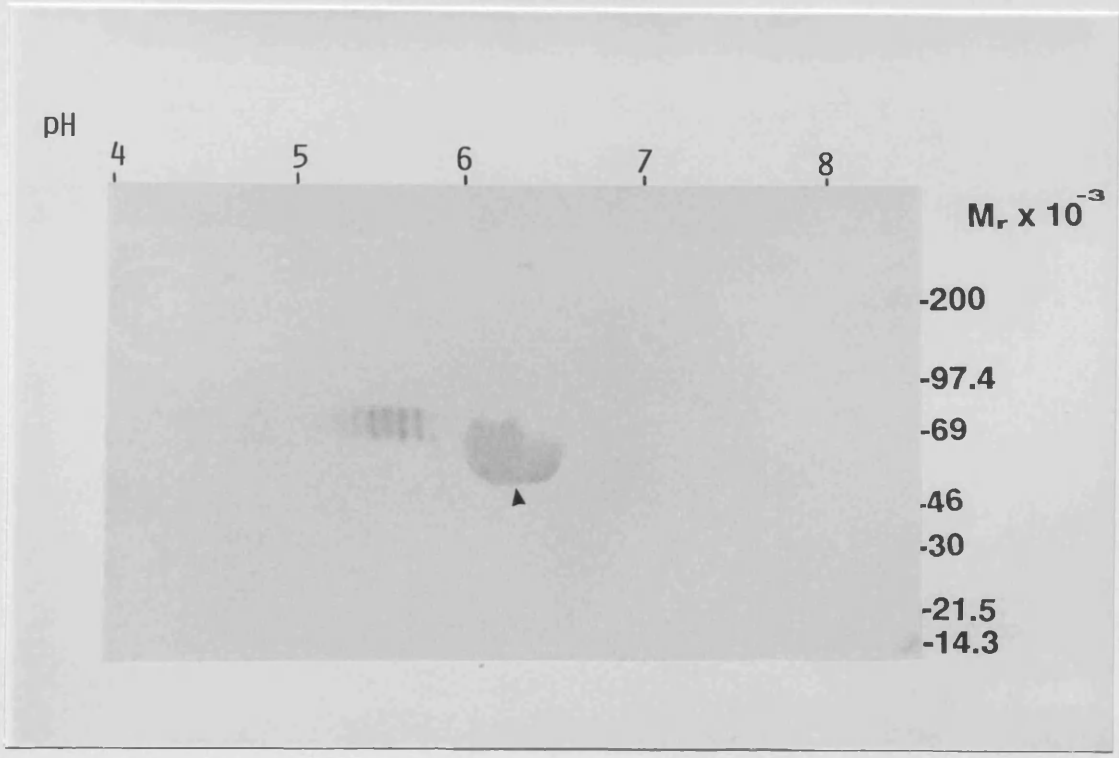


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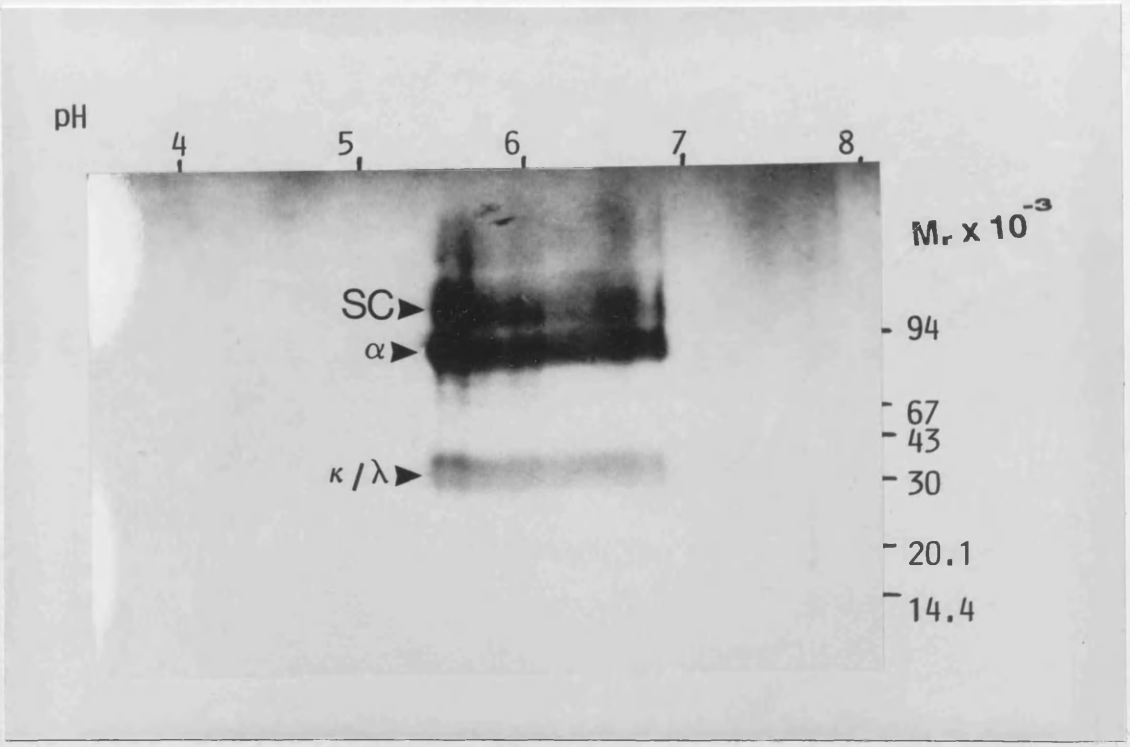
Figure 3.23

(i)



for similar components in parotid saliva in Figs. 3.23 (c)-(f).

3.4.2. Discussion

All previous attempts to study human salivary proteins by 2-D PAGE have used modifications of the method of O'Farrell (1975) which involved IEF in carrier ampholyte-containing rod gels in the first dimension of fractionation and vertical SDS-PAGE gels in the second (Giometti and Anderson, 1980; Bustos and Fung, 1981; Marshall, 1984). Mogi et al. (1986) performed 2-D PAGE of human salivary proteins under non-denaturing conditions in which the second dimension gels were polyacrylamide gradient gels which did not contain SDS. The prolonged focusing time, usually overnight, necessary when performing IEF in rod gels makes cathode drift unavoidable, resulting in the loss of most basic proteins. A number of salivary proteins have high pI values, among them the 9 basic PRP's isolated from parotid saliva by Kauffman and Keller (1979) and Kauffman et al. (1986) and the glycosylated basic PRP of Levine et al. (1969).

As a result of cathode drift, many of the protein bands which were revealed by SDS-PAGE, such as the basic proline-rich proteins, could not be located on the O'Farrell-type 2-D PAGE gels. Some of the protein spots in these gels exhibited considerable horizontal streaking, impairing the resolution of the spots and possibly masking other spots. The streaking effect is probably due to the

fact that when IEF is performed using carrier ampholyte-containing rod gels, the salivary samples were applied at the cathodic end; thus, upon application of an electric field, the carrier ampholytes, being smaller molecules than most of the salivary proteins, would migrate more quickly towards their respective pI's than the salivary proteins. This means that the pH gradient is already partially formed when most of the salivary proteins are still in the process of migrating towards their respective pI's. In the process of doing so, the salivary proteins are exposed to the high pH values at the cathodic end of the IEF gel, resulting in the precipitation and denaturation of some proteins.

The use of IPG's for the first dimension of 2-D PAGE avoids the occurrence of cathode drift and thus allows basic proteins to be focused. Other advantages of using IPG's include the option of fractionating saliva samples without prior desalting. By contrast, when using O'Farrell-type 2-D PAGE, the saliva samples had to be extensively dialysed prior to IEF. As IPG's are horizontal, flat-bed gels, the samples may be applied at the point on the gel that results in the best resolution of the protein bands. This is not possible when performing O'Farrell-type 2-D PAGE as the first dimension involves IEF in carrier ampholyte-containing rod gels, and therefore the samples must be applied at the anodic or cathodic ends of the gels. IPG's also offer the possibility of designing the pH profile to provide optimum resolution of the proteins of interest. Thus IPG's are

well-suited for use in 2-D PAGE. The superiority of IPG's over carrier ampholytes is illustrated by the fact that whereas none of the basic PRP's could be visualised on O'Farrell-type 2-D gels (Figs. 3.21(a) and (b)), about 10 spots of pI's > 8 are evident in a 2-D PAGE gel in which a pH 4-9 IPG was used in the first dimension (Figs. 3.22(a) and (b)). Also, the severe horizontal streaking seen on O'Farrell-type 2-D gels did not occur in the 2-D PAGE gels with IPG's in the first dimension.

As the IPG's and the linear polyacrylamide gradient gels were cast on GelBond plastic backing, reproducible 2-D patterns could be obtained as the gels do not change in size during the staining and destaining procedures. More accurate comparisons of the protein spots between different gels is thus made possible. The second dimension separation was performed using horizontal SDS-PAGE gels, which facilitates easier handling and optimum surface-to surface contact between the first- and second-dimension gels. In addition, the practice of casting the second dimension gels on plastic backing allows fairly robust thin-layer (0.5mm thickness) polyacrylamide gradient gels to be prepared (Görg et al., 1986). The improved cooling of such gels allows the application of higher field strengths. This improves the resolution of the protein zones and makes short separation times possible, thus the time required for the second dimensional run is approximately 3 hours.

Prior to this study, the only previous attempts to

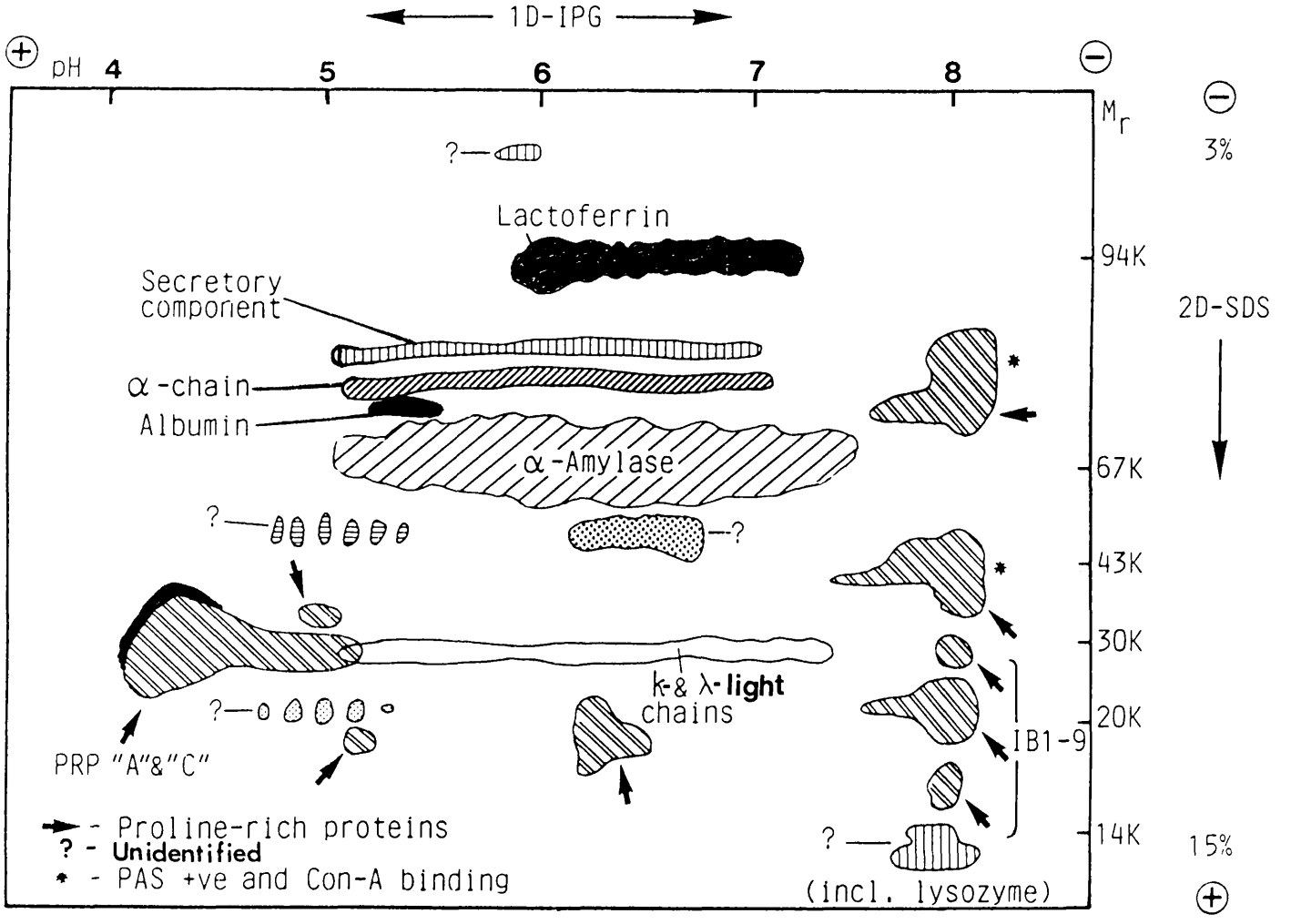
identify some of the components on the 2-D PAGE gels of human salivary proteins were made by Giometti and Anderson (1980) and Mogi et al. (1984). Giometti and Anderson (1980) performed O'Farrell-type SDS-PAGE of human whole and parotid saliva, and identified components such as α-amylase, albumin and immunoglobulin light chains by co-electrophoresis of the purified proteins with human salivary proteins. Mogi et al. (1984) performed microscale 2-D PAGE of human sublingual and submandibular saliva, and by immunoblotting located the positions of secretory IgA, α-amylase, albumin and acid phosphatase in the 2-D gels. The positions of the PRP's could not be located on either 2-D map, however, as most of these proteins were almost certainly lost as a result of cathode drift.

2-D PAGE was performed using IPG's in the first dimension and SDS-PAGE in the second, and by immunoblotting of the gels, a 2-D map of human parotid salivary proteins in which most of the major spots have been identified has been constructed (Fig. 3.24). In this map, the PRP's are identified by their characteristic pink-staining with CBB R250 and by comparison of their M_r's with those of purified PRP standards analysed by one-dimensional SDS-PAGE (Fig. 3.7).

The idea of a "human protein index" was first put forward by Anderson and Anderson (1982). In such an index, all the different proteins from human cells, tissues and body fluids are catalogued by establishing their standard 2-D PAGE patterns, assigning a specific designation to each

Fig. 3.24. Generalised 2-D map of human parotid salivary proteins

PRP's were identified by their pink-staining characteristic with CBB R250, and by correlation with results from Section 3.2. The locations in the 2-D map of α-amylase, lactoferrin, secretory component, α-heavy chain, κ- and λ- light chains, lysozyme and albumin were determined by immunoblotting; and that of the ConA-binding and PAS-positive components by correlation with results from Section 3.2.


1D-IPG
pH 4
5
6
7
8
Mr
3%
2D-SDS
94K
67K
43K
30K
20K
14K
15%
Lactoferrin
Secretory component
α-chain
Albumin
α-Amylase
k-& λ-light chains
IB1-9
PRP "A"&"C"
(incl. lysozyme)
- Proline-rich proteins
? - Unidentified
* - PAS +ve and Con-A binding

protein and characterising the individual protein. The proteins, together with their properties, could be recorded in a database which will allow easy access to a large amount of data from which the information accumulated may be retrieved (Klose, 1989). Manual or computerised databases may be constructed, although databases of the latter type will certainly prove to be more useful for the long-term, world-wide classification of human proteins. Catalogues of human proteins from body fluids such as cerebrospinal fluid, amniotic fluid, urine and milk have been partially established (Celis et al., 1989). The 2-D map described in this study is the first catalogue of proteins in human parotid saliva in which most of the proteins, including the PRP's, have been listed.

Even after silver staining, the 2-D PAGE gel of human parotid salivary proteins contained considerably less components than the 2-D gels of Marshall (1984), in which human whole saliva was fractionated and over 600 components were detected in a single gel after silver staining. As whole saliva consists of the secretions from several glands and contains many substances such as food debris, bacteria and sloughed-off cells from the oral cavity, it is likely that most of these components in the 2-D gels of Marshall (1984) were not of glandular origin.

3.5. Purification and Properties of the Anionic Parotid Salivary Proteins Associated with Rheumatoid Arthritis and Sjögren's Syndrome

In order to investigate the nature of anionic salivary proteins associated with RA and SS by electrophoretic analysis, acidic range IPG's of pH ranges 3.5-5.0 and 2.8-4.5, were prepared and used in both one- and two-dimensional analytical systems. Fast Protein Liquid Chromatography (FPLC) was also used.

3.5.1. Isoelectric Focusing in pH 3.5-5.0 IPG's

3.5.1.(i) pH Profile

The pH profile of a pH 3.5-5.0 IPG, determined by means of coloured pI markers, is shown in Fig. 3.25. The gradient appears to be approximately linear.

3.5.1.(ii) Isoelectric Focusing under Non-Denaturing Conditions

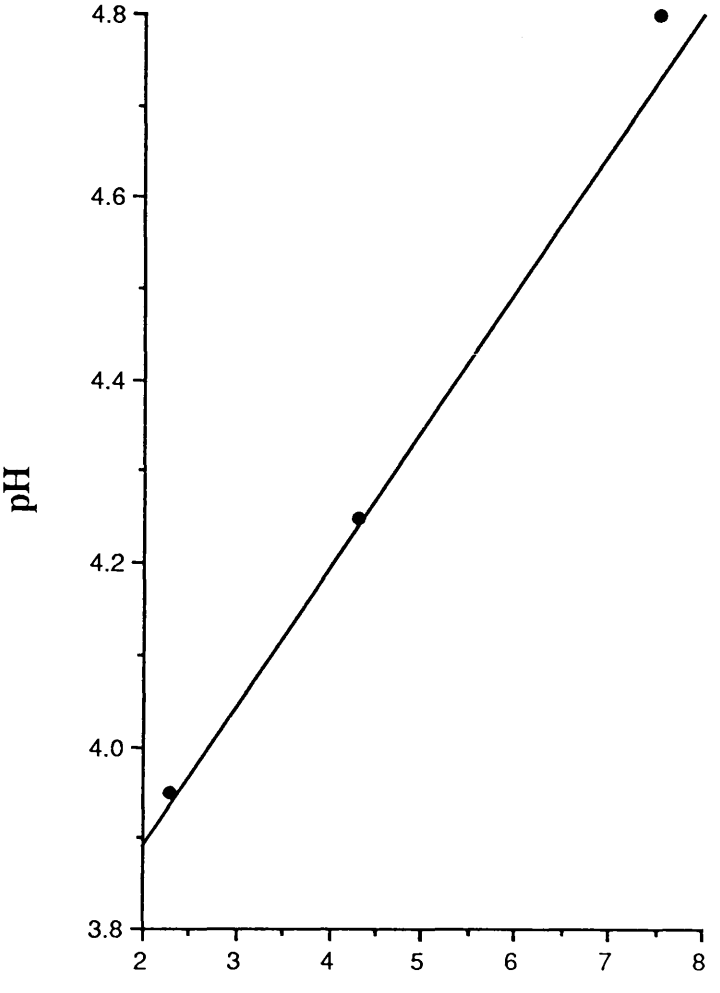
Parotid salivary proteins from RA patients, SS patients and normal healthy individuals were fractionated in pH 3.5-5.0 IPG's containing 0.5% (w/v) pH 3.5-5 Ampholine in 20% (w/v) glycerol, i.e. under non-denaturing conditions. Silver staining of the gels revealed multiple additional bands of pI's between 4.2 and 4.8 in the RA and SS patients, which were not detectable, or present at only very low levels, in the normal controls. These bands, however, were not well-resolved (Fig. 3.26).

Fig. 3.25. pH profile of a pH 3.5-5.0 IPG

The IPG was rehydrated with 20% (w/v) glycerol containing 0.5% (w/v) pH 3.5-5 Ampholine. BDH narrow range (pH 2.4-5.65) coloured pI markers were used.

All the protein markers indicated were coloured and could be visualised without prior staining.

Figure 3.25

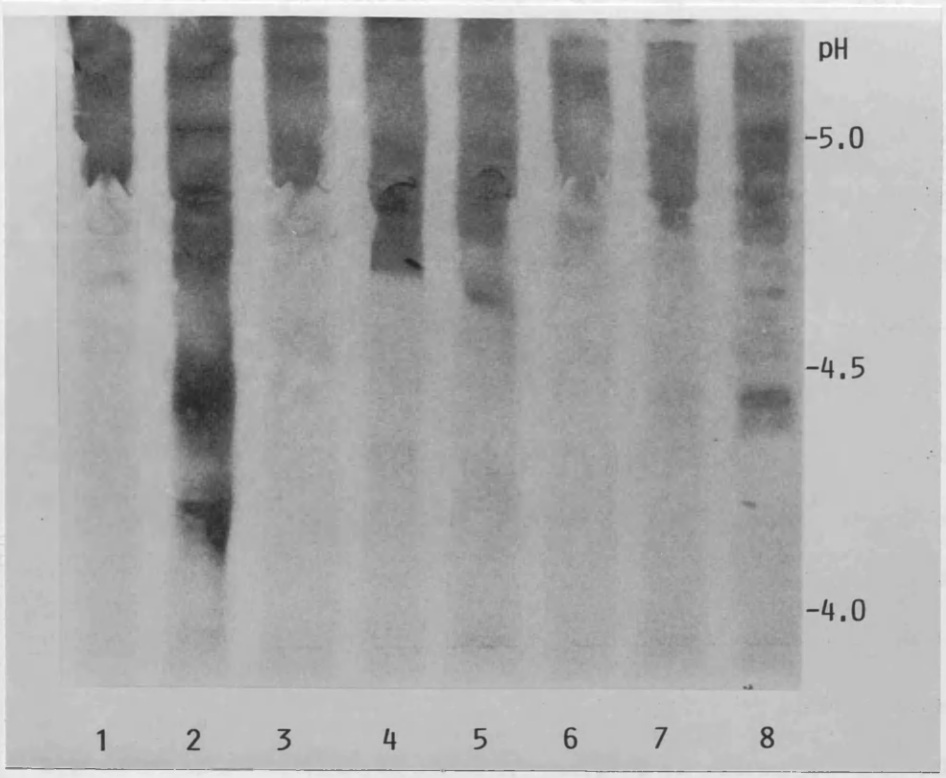


Distance from Anodic End (cm).

Fig. 3.26. IEF, under non-denaturing conditions, of parotid salivary proteins in a pH 3.5-5.0 IPG

The gel was rehydrated with 0.5% (w/v) pH 3.5-5 Ampholine in 20% (w/v) glycerol. One hundred μg of parotid salivary protein, which had previously been concentrated by lyophilisation, was applied to each track. Samples from normal individuals were applied to the odd-numbered tracks, while samples from RA patients were applied to the even-numbered tracks. The gel was silver stained.

Figure 3.26



3.5.1.(iii) Effect of Urea on the Band Patterns

In order to investigate whether the addition of urea to IPG's of pH range 3.5-5.0 would improve the resolution of the parotid salivary proteins, an IPG of this pH range was prepared which contained a 0-8 M urea gradient perpendicular to the pH gradient. A continuous zone of parotid salivary protein from a normal healthy subject, which had previously been concentration by lyophilisation followed by reconstitution with distilled water, was applied along the length of the gel at a distance of 2cm from the cathodic strip. After IEF and silver staining, it was found that the resolution of the protein bands improved with increasing urea concentration, and all the bands displayed a slight pI shift towards the anode with increasing urea concentration (Fig. 3.27). Below 3M urea, most of the bands were poorly resolved, but above that concentration, the resolution steadily improved and was optimum between 6-8 M urea. Based on this observation, 8M urea was included in the rehydration solution for all subsequent IPG's of pH range 3.5-5.0.

3.5.1.(iv) Isoelectric Focusing Under Denaturing Conditions

When 100μg (Fig. 3.28(a)) and 50μg (Fig. 3.28(b)) loads of protein from RA patients, SS patients and normal controls were fractionated in pH 3.5-5.0 IPG's containing 8M urea, silver staining revealed that the anionic salivary proteins associated with RA and SS were resolved in 20 or more well-

Fig. 3.27. IEF of a continuous zone of parotid salivary proteins applied 2cm from the cathode throughout the length of a gel in a pH 3.5-5.0 IPG superimposed with a transverse 0-8 M urea gradient

Protein (10.5mg) from a normal healthy individual was applied to a gel containing a urea gradient perpendicular to its pH gradient. The gel was silver stained.

Arrowheads indicate bands the resolution of which improve with increasing urea concentration.

Figure 3.27

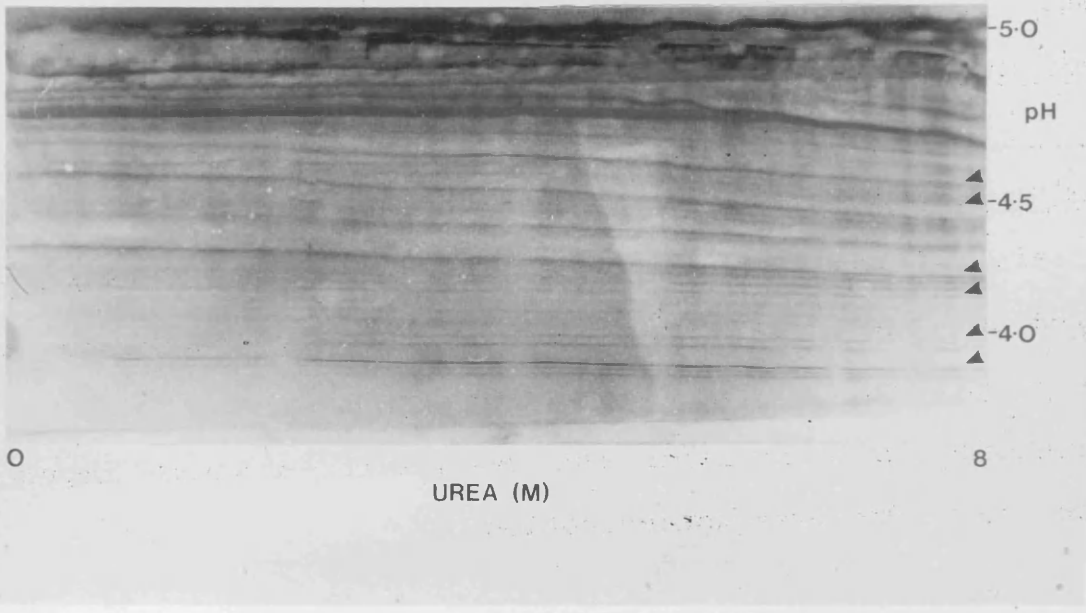


Fig. 3.28. IEF in a pH 3.5-5.0 IPG of parotid salivary proteins

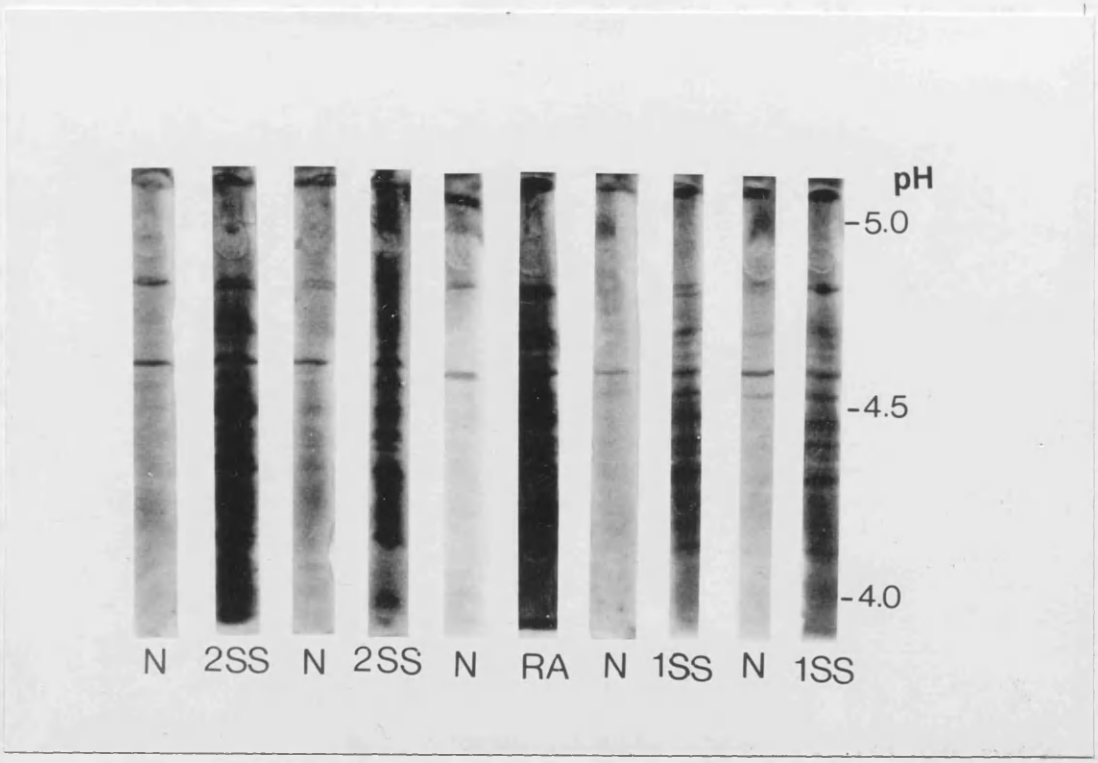
Both gels were rehydrated with 8M urea in 20% (w/v) glycerol and 0.5% (w/v) pH 3.5-5 Ampholine.

In panel (a) the protein load on each track was 100 μg. Before application to the gel, the saliva samples were concentrated by lyophilisation followed by reconstitution with distilled water. The tracks in panels (a) and (b) marked "N", "RA", "1SS" and "2SS" were loaded with samples from normal healthy individuals, RA patients, primary SS patients and secondary SS patients, respectively.

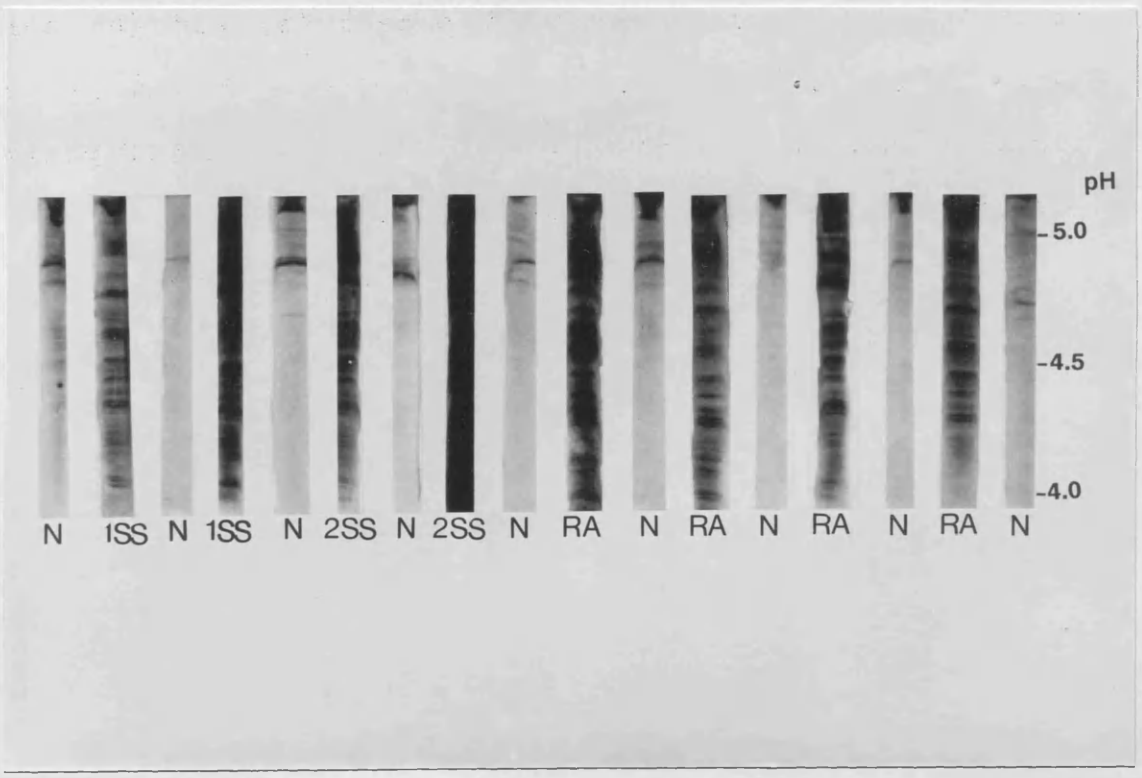
In panel (b), the protein load on each track was 50 μg. The gels were silver stained.

Figure 3.28

(a)



(b)



focused components of pI's between 3.65 and 4.75. In contrast, the normal subjects had few or no detectable proteins focusing in this pH range. In order to apply 100μg loads of protein, it was necessary to concentrate the parotid saliva samples by lyophilisation followed by reconstitution in a smaller volume of distilled water. For the application of 50 μg loads, no prior treatment was necessary, and the samples could be applied directly onto the gels after centrifugation to remove any insoluble material.

Laser densitometry was carried out on silver-stained pH 3.5-5.0 IPG's. Scans of samples from a normal healthy individual and a secondary SS patient are shown in Fig. 3.29. The SS patient displayed multiple peaks which were not detectable in the normal individual. However, as silver stain is non-monochromatic and non-stoichiometric (Poehling and Neuhoff, 1981), the densitometric scans are at best a semi-quantitative evaluation of the amount of the proteins present in the gel.

In total, parotid saliva samples from 32 normal healthy individuals (16 male and 16 female), 34 RA patients (11 male and 23 female), 20 primary SS patients (3 male and 17 female), 18 secondary SS patients (all female), 26 patients with xerostomia (2 male and 24 female), 6 patients with sialadenitis (all female) and 4 patients with infection of the parotid salivary gland were analysed using pH 3.5-5.0 IPG's followed by silver staining. The results are summarised in Fig. 3.30.

Fig. 3.29. Laser densitometry of silver-stained pH 3.5-5.0 IPG's

The gels were rehydrated with 8M urea in 20% (w/v) glycerol and 0.5% (w/v) pH 3.5-5 Ampholine.

Panel (a) shows the densitometric scan of an IPG loaded with 100μg of parotid salivary protein from an normal healthy individual.

Panel (b) shows the densitometric scan of an IPG loaded with 100 μg of parotid salivary protein from a secondary SS patient.

The profiles shown were obtained with backgound subtraction but without filtering, enhancement or boosting.

Figure 3.29

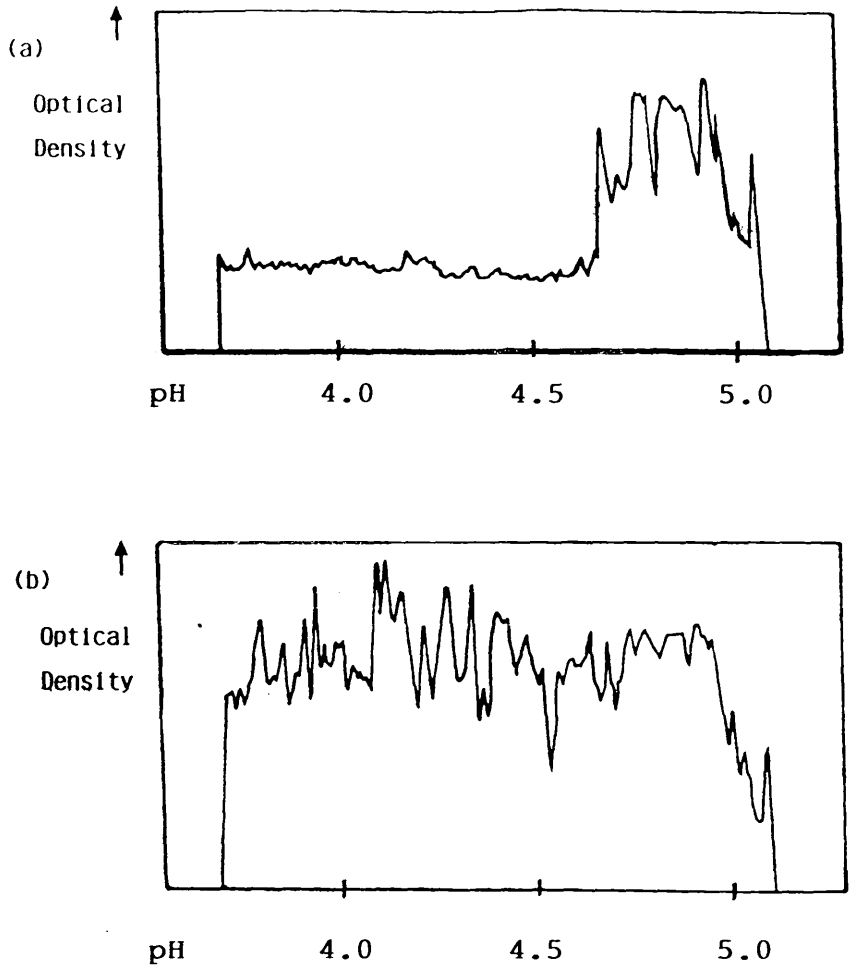
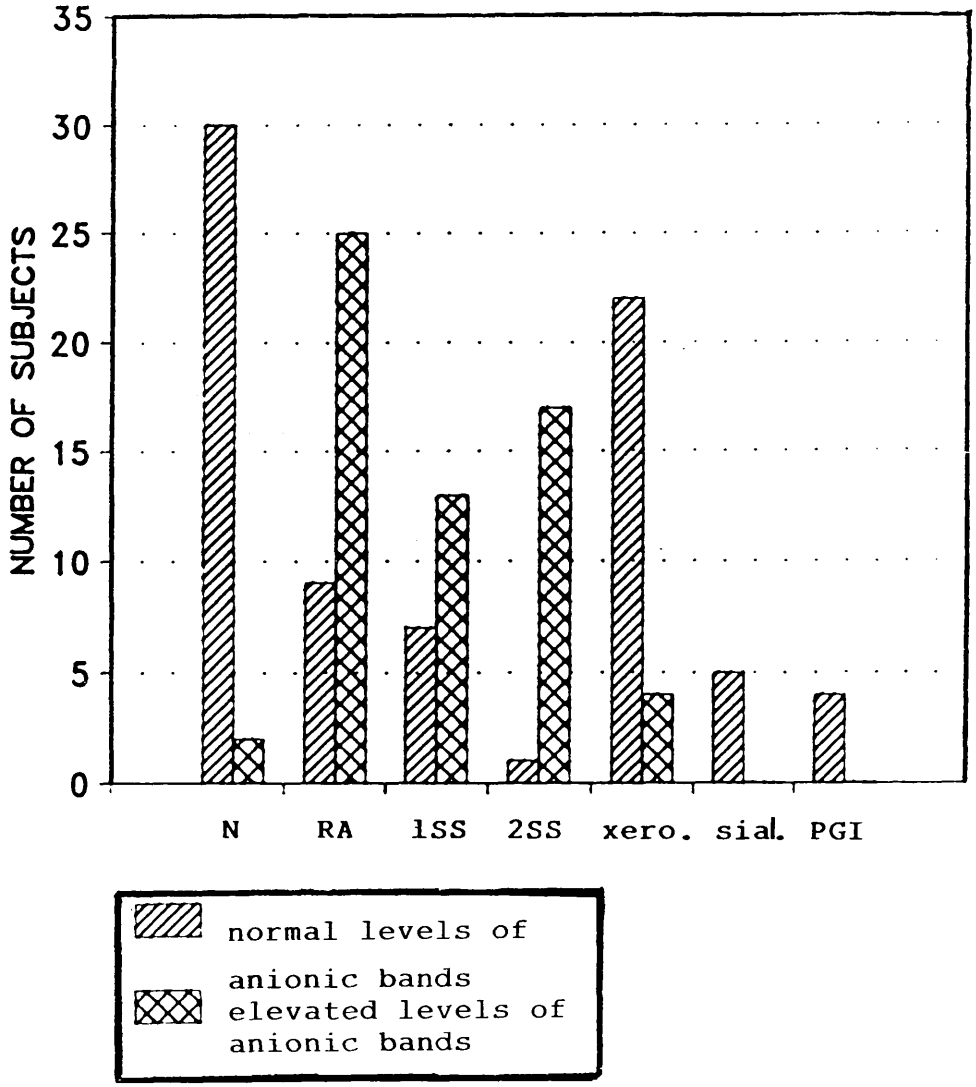


Fig. 3.30. Histogram showing results of isoelectric focusing in pH 3.5-5.0 IPG's

Fiftyμg loads of parotid salivary protein from normal healthy individuals, and patients with RA, primary SS, secondary SS, xerostomia, sialadenitis and parotid gland infection were fractionated in pH 3.5-5.0 IPG's which were silver stained. In the histogram, "N", "RA", "1SS", "2SS", "xero.", "sial." and "PGI" refers to normal subjects, and patients with RA, primary SS, secondary SS, xerostomia, sialadenitis and parotid gland infection, respectively.

The gels had previously been rehydrated with 8M urea in 20% (w/v) glycerol and 0.5% (w/v) pH 3.5-5 Ampholine. After staining, the samples were classified into two groups; those who had visually detectable elevated levels of anionic bands and those in which such bands were only present at normal low levels and difficult to detect.

Figure 3.30


35
30
25
20
15
10
5
0
NUMBER OF SUBJECTS
N
RA
1SS
2SS
xero.
sial.
PGI
normal levels of anionic bands
elevated levels of anionic bands

Whilst a few of the normal controls and patients with disorders other than RA or SS were observed to contain elevated levels of the anionic salivary proteins, they were of a lesser intensity than in most of the RA or SS patients. An example of a normal individual with elevated levels of the anionic bands is shown in Fig. 3.37. Most of the RA and primary SS patients, and all of the secondary SS patients but one exhibited substantially increased levels of these proteins.

3.5.2. Isoelectric Focusing in pH 2.8-4.5 IPG's

The pH profile of a pH 2.8-4.5 IPG, determined using coloured pI markers, is shown in Fig. 3.31. The gradient appears to be approximately linear.

Urea was not added to IPG's of this pH range; the addition of urea was not possible because after the gels had been polymerised, they were used without the further washing, drying and rehydration procedures to which pH 3.5-5.0 and pH 4-9 IPG's were subjected. These steps were omitted for pH 2.8-4.5 IPG's so that the gels would retain their glycerol density gradient which helps to prevent gel-burning in strongly acidic IPG's (Righetti et al., 1988).

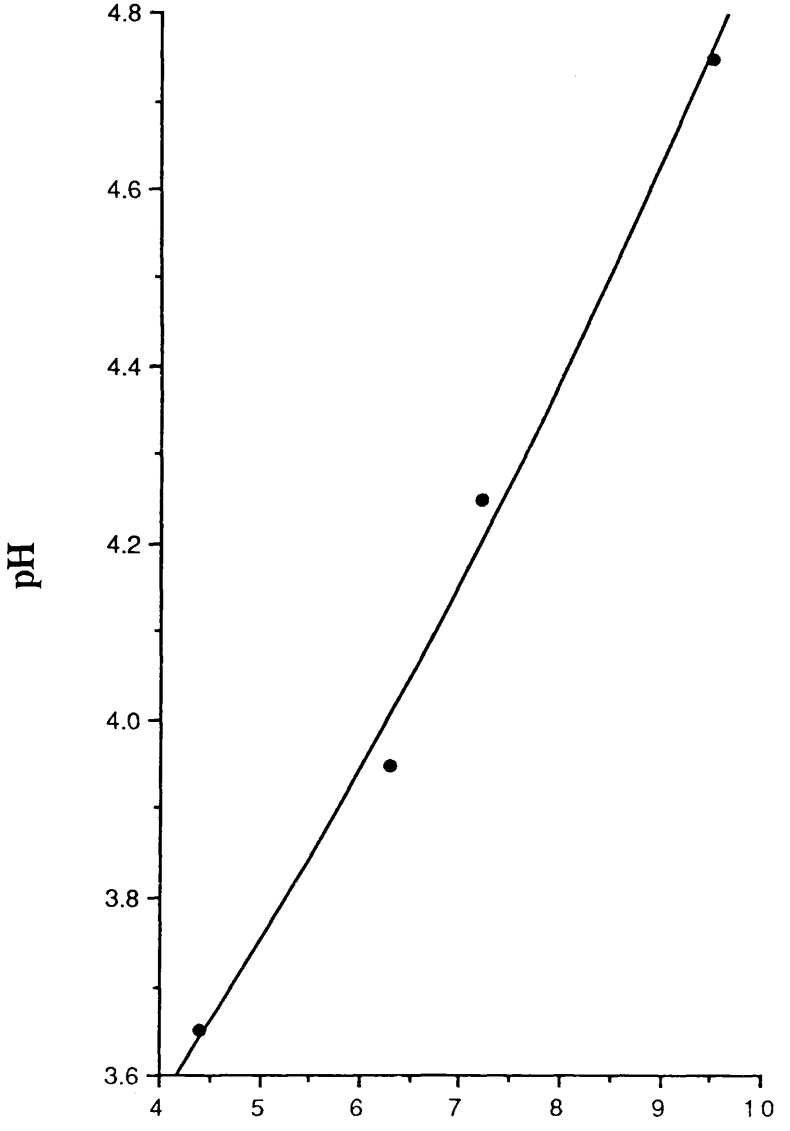
Parotid salivary proteins from SS patients and normal healthy individuals were fractionated in pH 2.8-4.5 IPG's. The saliva samples from the SS patients had previously been analysed using pH 3.5-5.0 IPG's, and were found to contain strongly-staining anionic bands. Silver staining of the pH

Fig. 3.31. pH profile of a pH 2.8-4.5 IPG

The IPG was run without the washing, drying and rehydration steps to which pH 4-9 and pH 3.5-5.0 IPG's were subjected. BDH narrow range (pH 2.4-5.65) coloured pI markers were used.

The protein markers of pI's 3.95 and 4.75 were coloured and could be visualised without prior staining, while the markers of pI's 3.65 and 4.25 were visualised after staining with CBB G250.

Figure 3.31



2.8-4.5 IPG revealed no difference between the band patterns of the two groups, and no protein bands focused at a pH < 4.1 (Fig. 3.32). It is unlikely that the paucity of protein bands in this pH region is due to precipitation of the salivary proteins, as the pI marker proteins were well-focused.

3.5.3. Analysis by 2-D PAGE

As pH 3.5-5.0 IPG's have been found to give optimum resolution of the the anionic salivary proteins associated with RA and SS, IPG's of this pH range were used for the first dimension of 2-D PAGE.

3.5.3.(i) 2-D PAGE of Parotid Salivary Proteins from Normal Healthy Individuals

2-D PAGE of parotid salivary proteins from normal healthy individuals, with pH 3.5-5.0 IPG's in the first dimension, followed by staining of the gels with CBB R250 revealed only pink-staining spots (Fig. 3.33(a) and (b)). Their M_r's were approximately 26,000 to 30,000. The pink-staining components of M_r's approximately 26,000 and 28,000 have been shown by SDS-PAGE to correspond to the acidic PRP's "A" and "C", respectively, of Bennick and Connell (1971) (Fig. 3.7(a)). These proteins were stained with a yellowish-brown colour by the silver staining procedure (Fig. 3.34(a) and (b)). 2-D PAGE revealed that there is considerable individual-to-individual variation in these

Fig. 3.32. IEF in a pH 2.8-4.5 IPG of parotid salivary proteins

The IPG was run without the washing, drying and rehydration steps to which pH 4-9 and pH 3.5-5.0 IPG's were subjected. The tracks marked "N", "1SS" and "2SS" were loaded with 50μg loads of parotid salivary proteins from normal healthy individuals, primary SS patients and secondary SS patients, respectively. The track marked "pI" was loaded with pI marker proteins. The gel was silver stained.

Figure 3.32

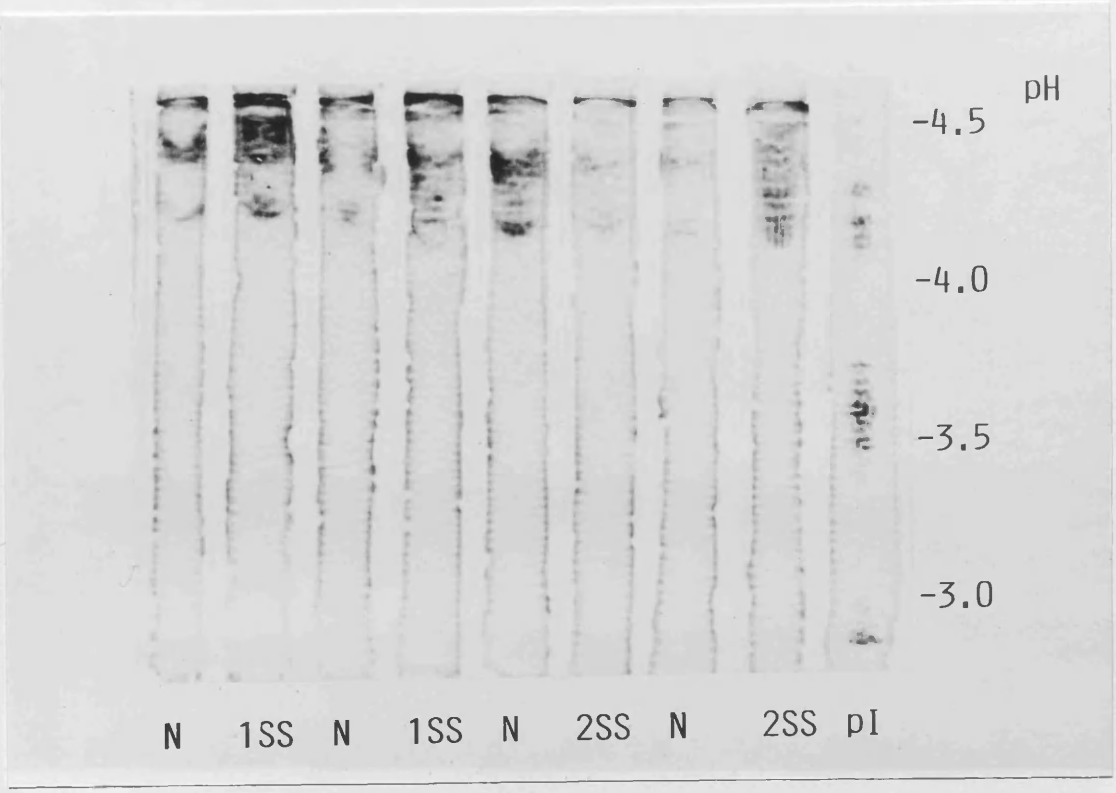
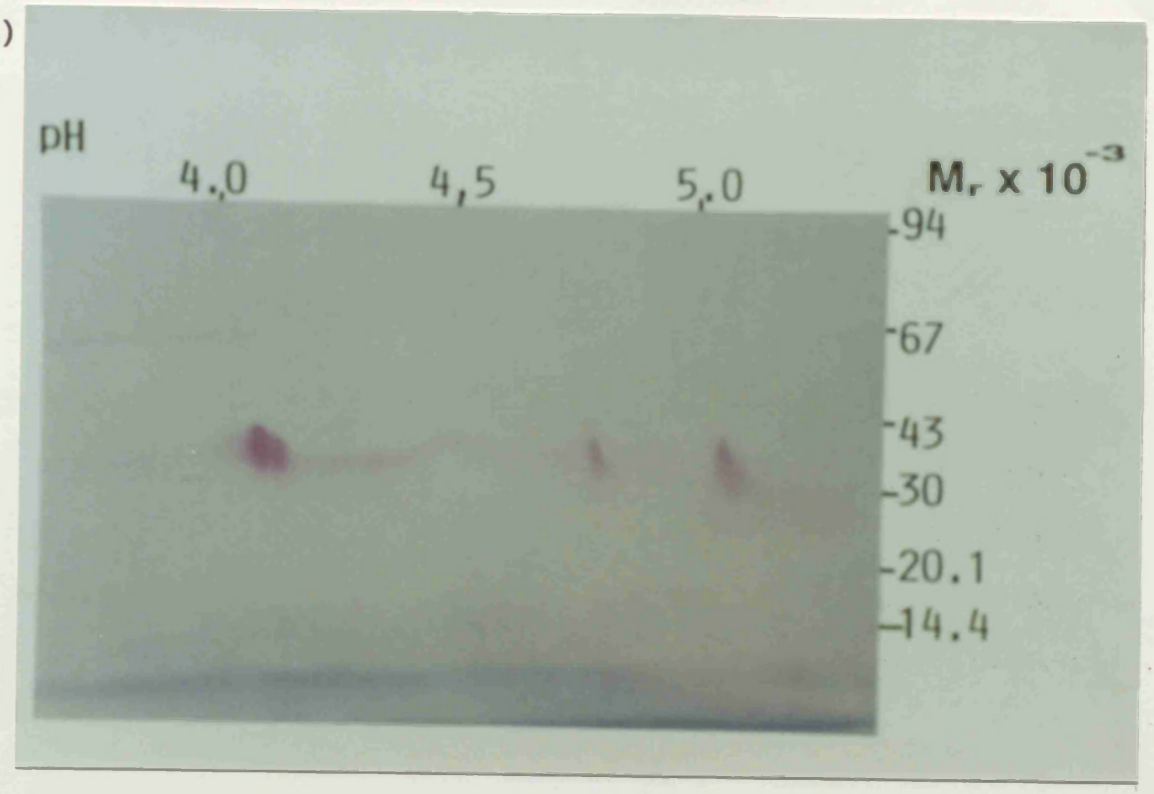


Fig. 3.33. 2-D PAGE, with pH 3.5-5.0 IPG in the first dimension, of parotid salivary proteins followed by staining with CBB R250

One hundred μg of protein from two different normal healthy individuals was applied to each gel (panel (a) and (b)).

Figure 3.33

(a)



(b)

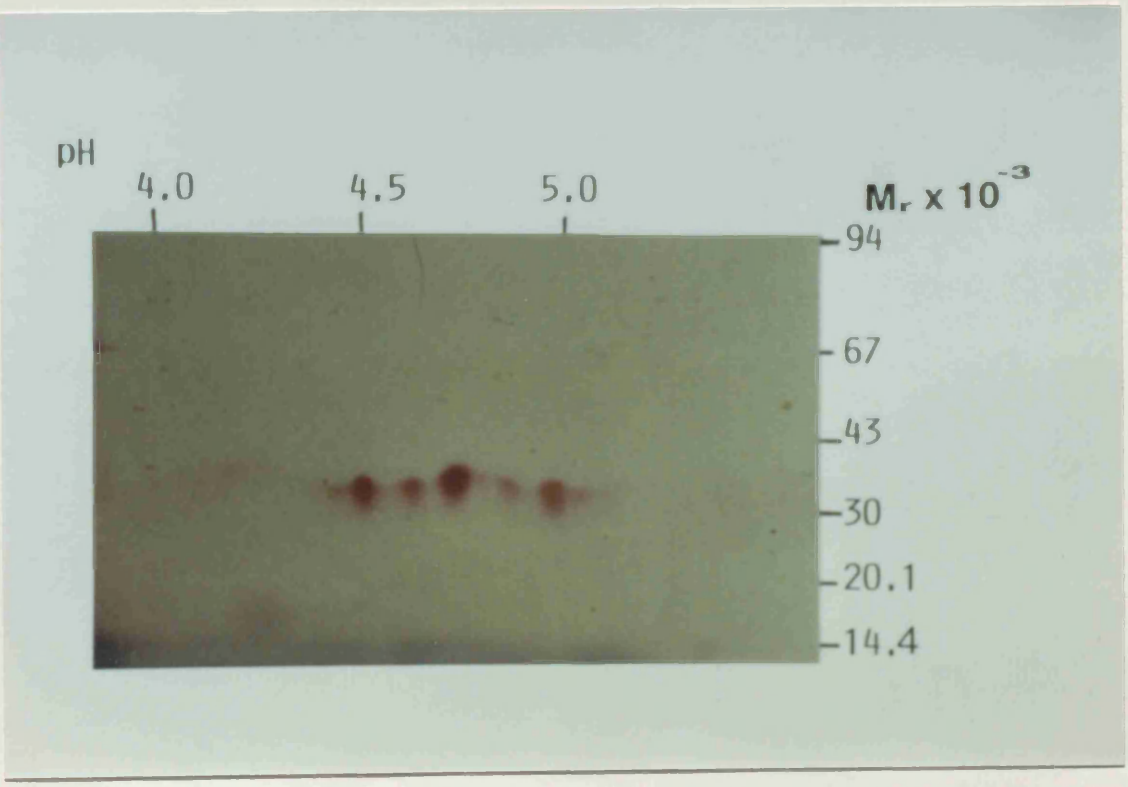
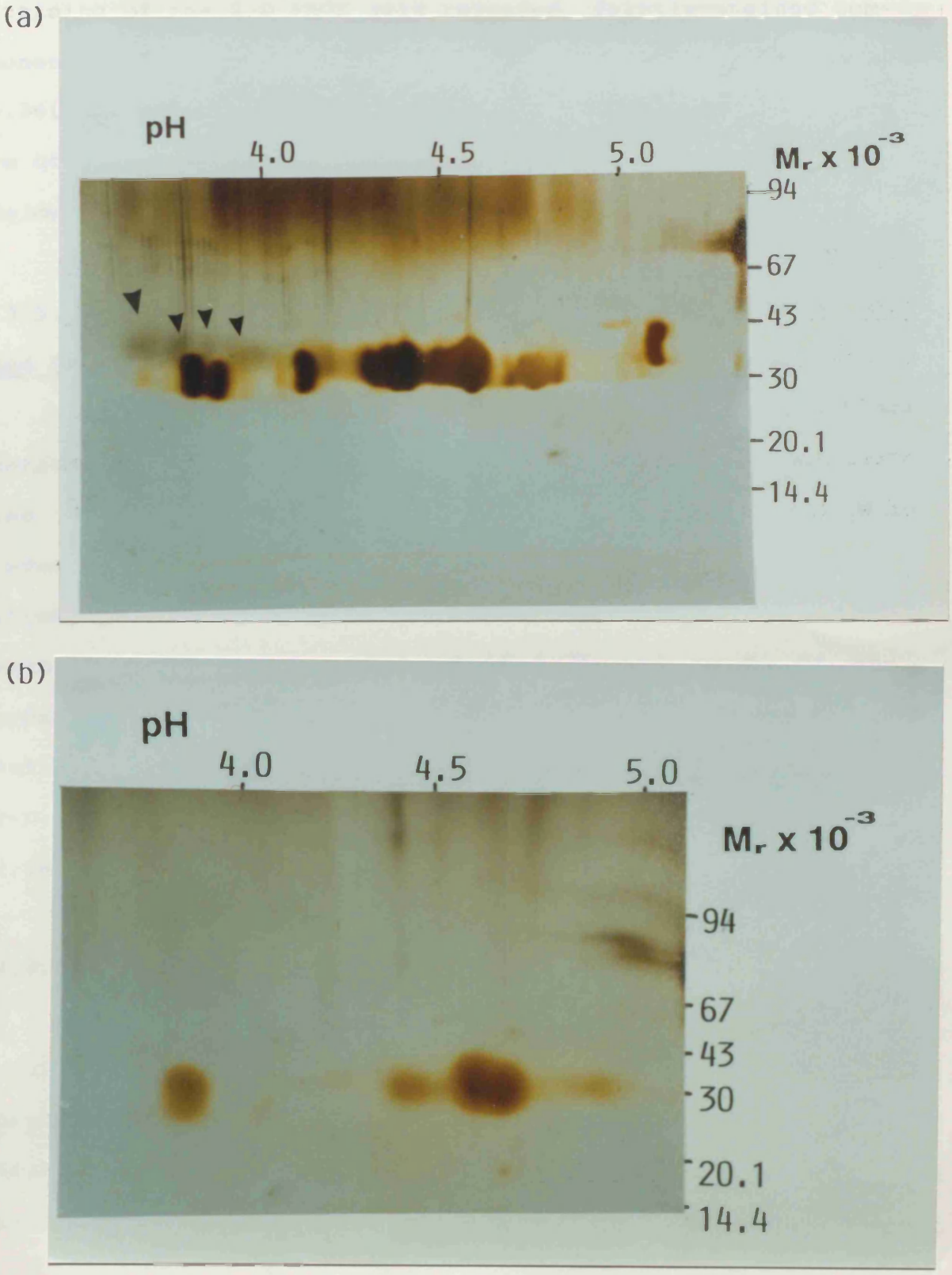


Fig. 3.34. 2-D PAGE, with pH 3.5-5.0 IPG in the first dimension, of parotid salivary proteins followed by silver stain

One hundred μg of protein from two different normal healthy individuals was applied to each gel (panel (a) and (b)). The arrowheads in panel (a) indicate faintly-stained components of M_r approximately 32,000 and pI's approximately 3.65-4.25.

Figure 3.34


(a)
pH
4.0
4.5
5.0
Mr x 10-3
94
67
43
30
20.1
14.4
(b)
pH
4.0
4.5
5.0
Mr x 10-3
94
67
43
30
20.1
14.4

proteins, a phenomenon which Azen (1978) has attributed to genetic variation. Other than the acidic PRP's, silver staining of the 2-D PAGE gels revealed faintly-stained components of pI's 3.65-4.25 and M_r approximately 32,000 (Fig. 3.34(a)). These components, however, could not be detected in other samples (Fig. 3.34(b)); possibly because they were below the detection limits in these samples.

3.5.3.(ii) 2-D PAGE of Parotid Salivary Proteins from RA and SS patients

Parotid salivary proteins from an RA and a secondary SS patient were analysed by 2-D PAGE, with pH 3.5-5.0 IPG's in the first dimension. Silver staining of the 2-D PAGE gels revealed that in addition to the acidic PRP's, there were strongly-staining components which were microheterogeneous with pI's of approximately 3.65-4.75 and an M_r of approximately 32,000 (Fig. 3.35(a) and (b)). These anionic salivary proteins were also visualised in a silver-stained 2-D PAGE gel, with a pH 4-9 IPG in the first dimension (Fig. 3.36).

3.5.4. Identification by Immunoblotting

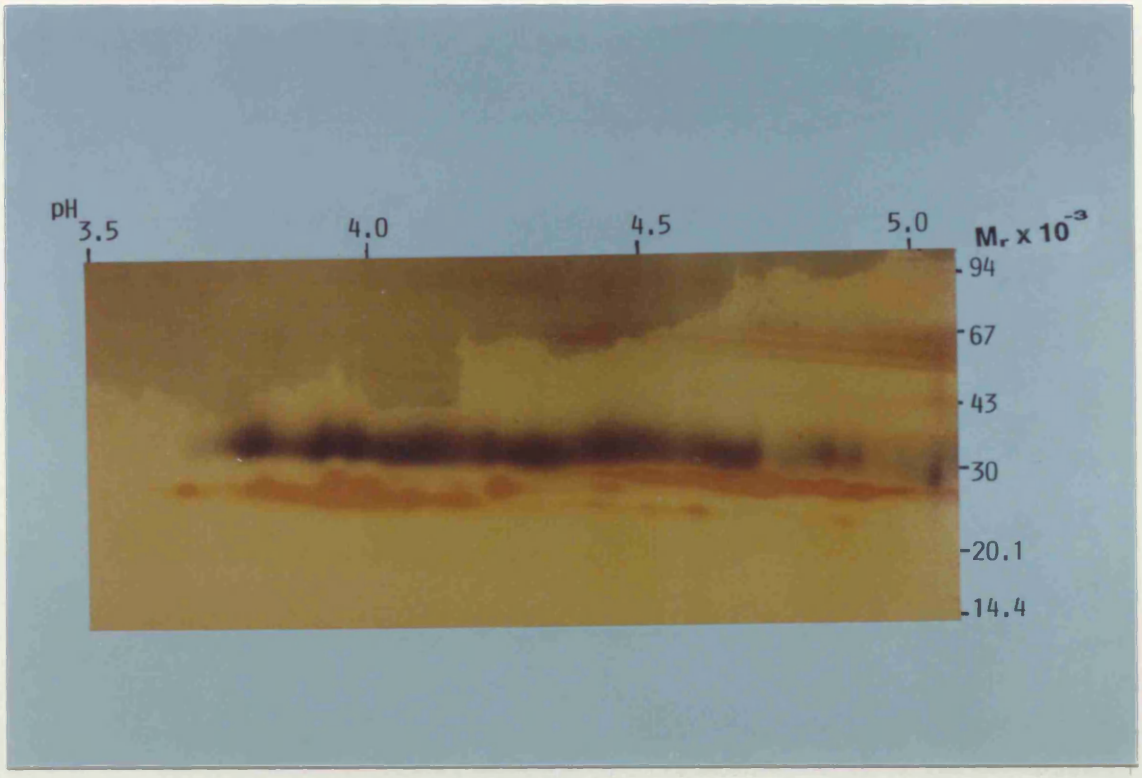
In order to identify the anionic salivary proteins associated with RA and SS, parotid salivary proteins from RA patients, SS patients and normal healthy individuals were fractionated in pH 3.5-5.0 IPG's which were subsequently electroblotted onto nitrocellulose membranes. Immunoblotting

Fig. 3.35. 2-D PAGE, with pH 3.5-5.0 IPG in the first dimension, of parotid salivary proteins from RA and SS patients

100μg of protein from a secondary SS patient was applied to the gel in panel (a) while a similar load of protein from an RA patient was applied to the gel in panel (b). The gels were silver stained.

Figure 3.35

(a)



(b)

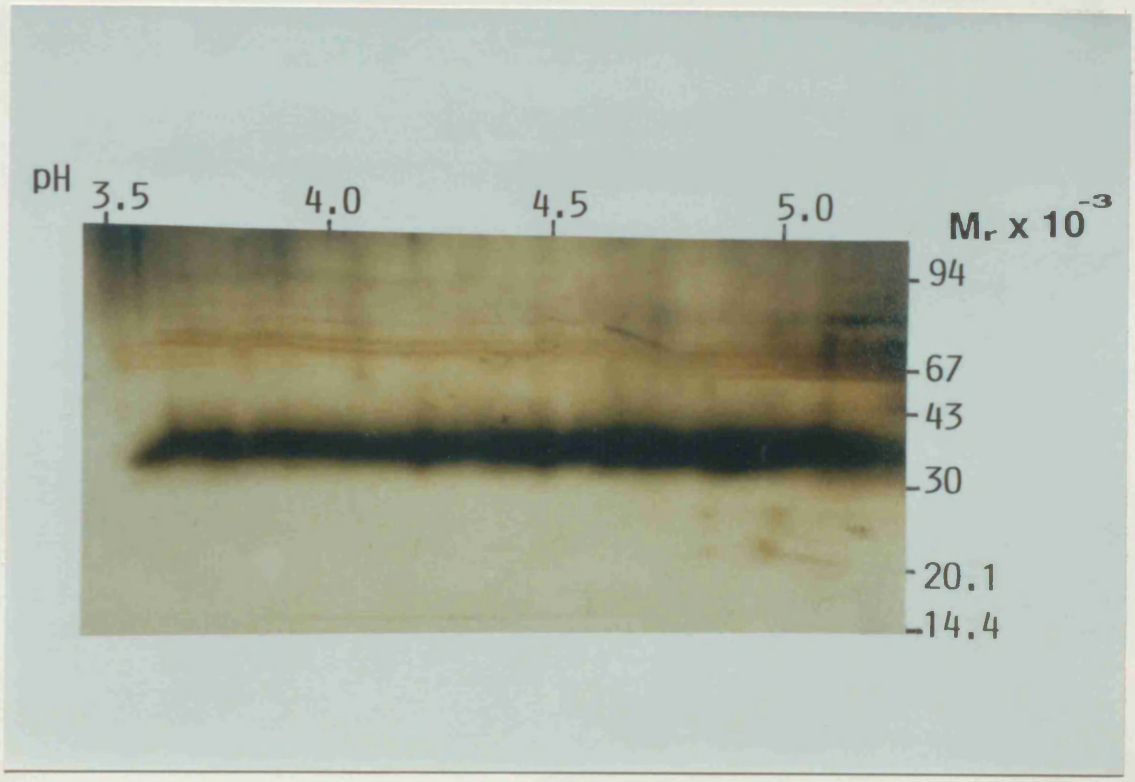
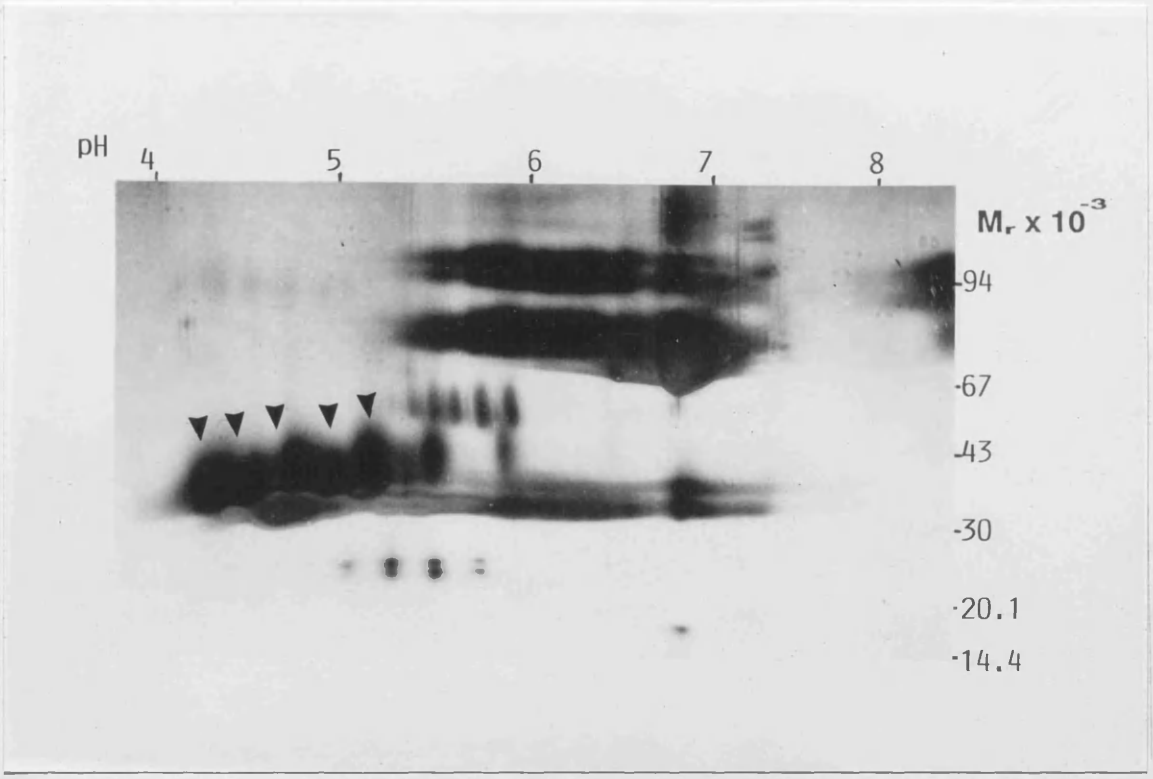


Fig. 3.36. 2-D PAGE, with pH 4-9 IPG in the first dimension, of parotid salivary proteins from a secondary SS patient

One hundredμg of protein was applied, and the gel was silver stained. The anionic proteins associated with RA and SS are indicated by arrowheads.

Figure 3.36



was performed using the alkaline phosphatase-conjugated second antibody method of Blake et al. (1984). These electroblotted membranes were first probed using the following antisera and antibodies: Rabbit antiserum to C-Reactive Protein, rabbit antiserum to κ-light chain, rabbit antiserum to λ-light chain, rabbit antiserum to secretory component, rabbit antiserum to human plasma kallikrein, sheep antiserum to α_1-acid glycoprotein (orosomucoid), sheep antiserum to IgG (γ-chain specific), goat antiserum to IgA (α-chain specific), goat antiserum to human urinary (tissue) kallikrein and mouse monoclonal antibody to β_2-microglobulin.

Following incubation of the membranes in the appropriate species-specific alkaline phosphatase-conjugated antibody solutions, the blots were developed in the substrate solution for alkaline phosphatase.

None of the immunoblots gave a positive response, except for the blot which had been incubated with commercial goat antiserum to human urinary kallikrein (HUK) which resulted in a weak staining reaction in a few of the tracks which had been loaded with RA and SS samples. These, however, were difficult to photograph. The use of laboratory-produced rabbit antiserum to HUK, however, did result in a strong staining reaction in RA and SS samples and a weaker staining reaction in the normal individuals (Fig. 3.37).

Two sets of control experiments were carried out. In

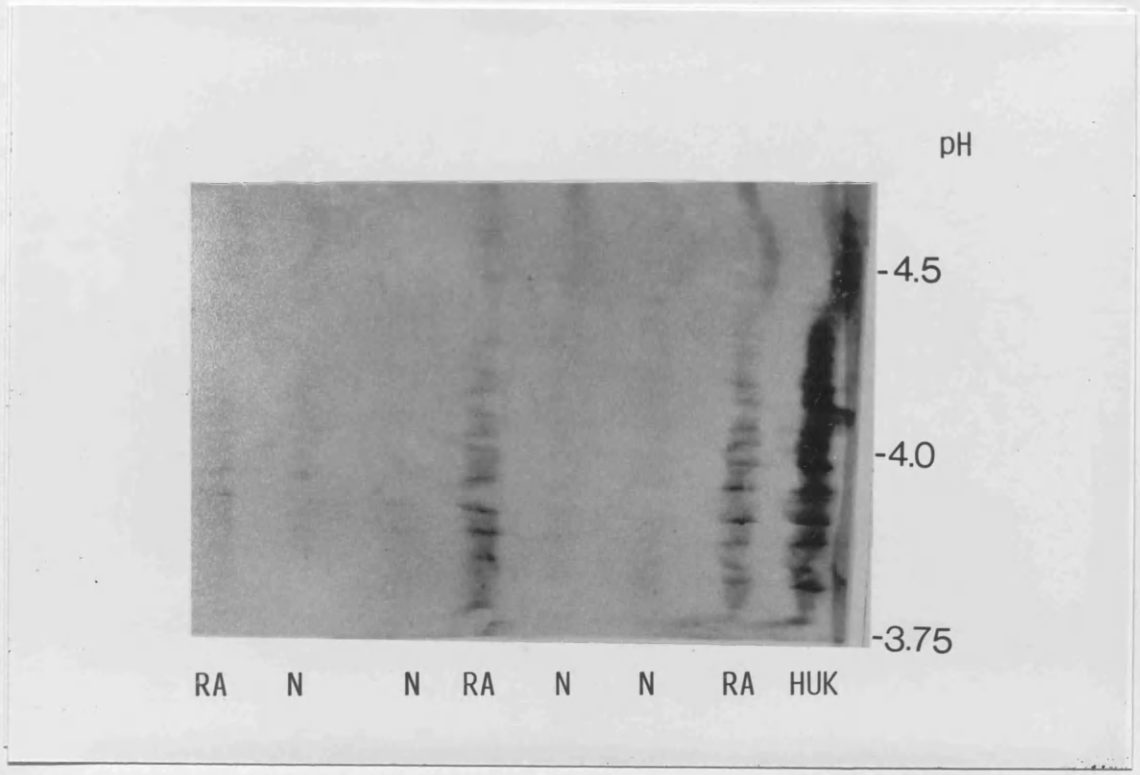
Fig. 3.37. IEF in a pH 3.5-5.0 IPG of parotid salivary proteins and human urinary kallikrein followed by an immunoblot for tissue kallikrein

The IPG was 1mm thick and cast on glass rather than on Gel-Bond film so as to facilitate easy removal prior to the electroblotting step. The gel was incorporated with 0.5% (w/v) pH 3.5-5 Ampholine and 4.46M urea.

100μg of parotid salivary protein was applied to each track. The tracks marked "N" and "RA" were loaded with parotid saliva from normal healthy individuals and RA patients, respectively. The track marked "HUK" was loaded with 2μg of human urinary kallikrein (HUK).

Rabbit antiserum to HUK was used as the primary antibody and anti-rabbit immunoglobulin-alkaline phosphatase-conjugate as the detecting antibody.

Figure 3.37



the first, after electroblotting, the membranes were incubated with the anti-rabbit immunoglobulin-alkaline phosphatase conjugate and developed in alkaline phosphatase substrate solution, omitting the stage where they were incubated with the primary antisera. This did not result in any staining reaction. Another control experiment involved incubation of the blots in PBS containing non-immune rabbit serum (1:200 dilution), followed by incubation in a solution containing anti-rabbit immunoglobulin-alkaline phosphatase conjugate, and finally development of the blot in the substrate solution for alkaline phosphatase. This procedure also did not result in any alkaline phosphatase signal in the blots after development.

Immunoblotting was also performed on RA, SS and normal parotid saliva which had been fractionated in 2-D PAGE gels, with pH 3.5-5.0 IPG's in the first dimension. Using the alkaline phosphatase-conjugated second antibody method of Blake et al. (1984), both the commercial goat antiserum to HUK and laboratory-produced rabbit antiserum to HUK produced either very weak or no staining reactions. This lack of sensitivity could be due to the loss of some proteins from the IPG strips during the equilibration period of 30 minutes before they were applied to the second dimension gels.

In order to increase the sensitivity of the staining reaction, an amplification procedure, involving an alkaline phosphatase / anti-alkaline phosphatase (APAAP) complex was used. The 2-D PAGE gels were first electroblotted, and the

blots then incubated in goat antiserum to HUK, followed by mouse monoclonal antibody to goat IgG, rabbit antiserum to mouse IgG and the APAAP complex. The second and third antibodies served as the link between the primary antibody (i.e. goat antiserum to HUK) and the APAAP complex. Amplification of the alkaline phosphatase signal using 3 stages of the APAAP amplification procedure (Cordell et al., 1984) resulted in more strongly-stained bands of pI's between 3.65-4.75 and M_r approximately 32,000 in the blots of the 2-D PAGE gels which had been loaded with salivary samples from a normal healthy individual and an RA patient (Figs. 3.38(a) and (b)). These parameters corresponded to those of the anionic salivary proteins associated with RA and SS, revealed after silver staining of 2-D PAGE gels (Figs. 3.35(a) and (b)). The control experiment, which consisted of first probing the membrane using goat antiserum to human serum albumin followed by mouse monoclonal antibody to goat IgG, rabbit antiserum to mouse IgG and amplification of the alkaline phosphatase signal using 3 stages of the APAAP amplification procedure, did not result in any staining.

3.5.5. Enzymic Degradation of Parotid Salivary Proteins with Exo- and Endo-Glycosidases

3.5.5.(i) Neuraminidase Treatment

In order to investigate whether differing amounts of sialic acid in the oligosaccharide side chain is a con-

Fig. 3.38. 2-D PAGE, with pH 3.5-5.0 IPG in the first dimension, of parotid salivary proteins followed by an immunoblot for tissue kallikrein

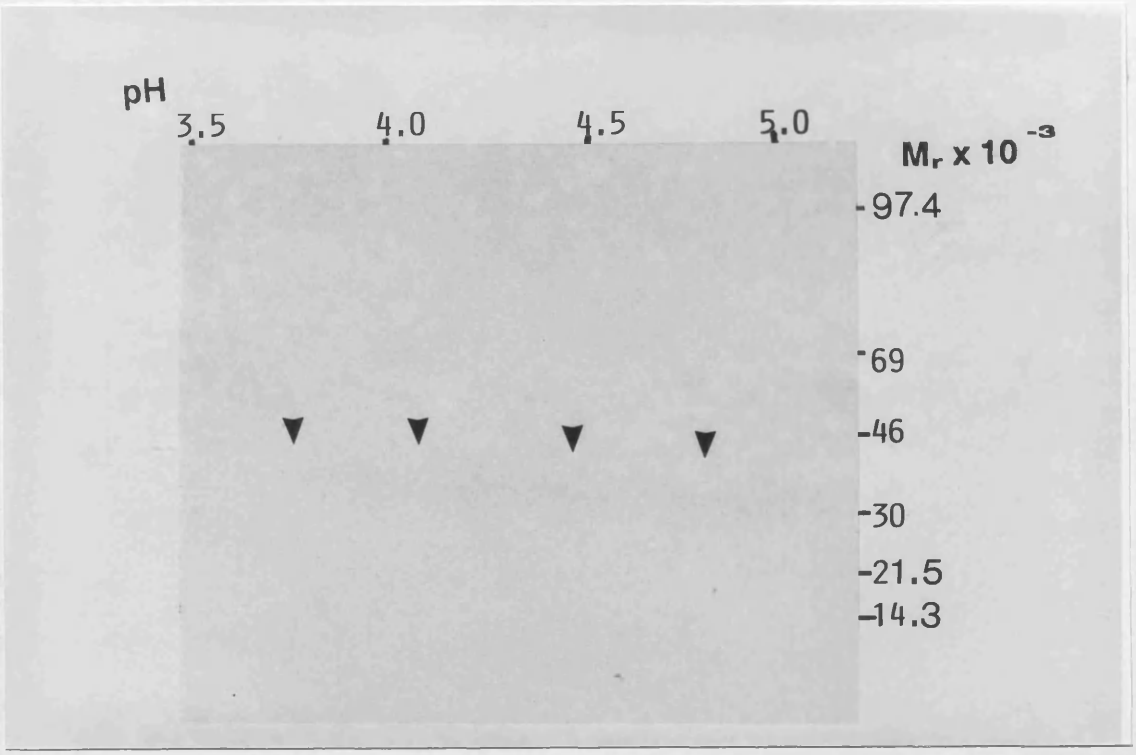
Fifty μg of proteins from a normal healthy individual was applied to the gel in panel (a), and a similar load from an RA patient to the gel in panel (b).

Goat antiserum to human urinary kallikrein was used as the primary antibody, followed by 3 stages of APAAP complex for detection.

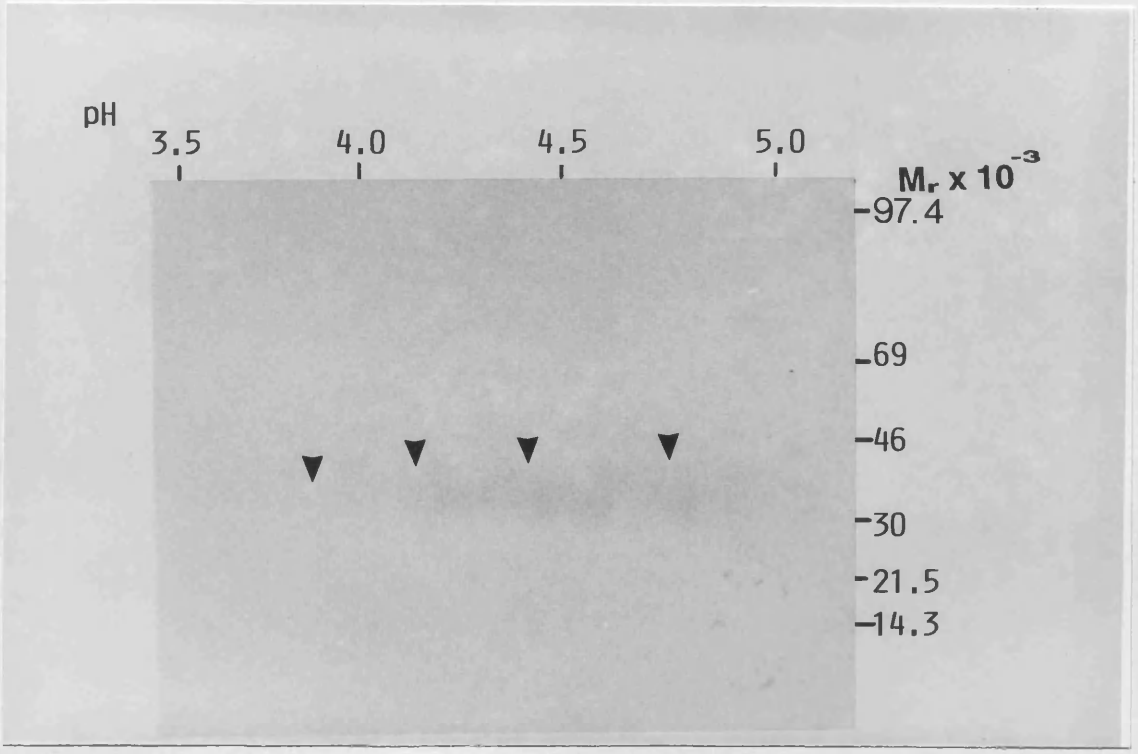
The components visualised are indicated by arrowheads.

Figure 3.38

(a)



(b)



tributory factor to the charge heterogeneity of the anionic proteins being studied, neuraminidase-treated and control parotid saliva samples from RA patients, SS patients and normal healthy individuals were fractionated in pH 3.5-5.0 IPG's and 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension. The neuraminidase incubations had previously been carried out for 18 hours. It was found that incubation with neuraminidase resulted in the loss of the anionic bands and also the bands which cross-reacted with antiserum to HUK (Fig. 3.39, Fig. 3.40). This finding was also confirmed by 2-D PAGE of parotid salivary proteins from an SS patient, with pH a 4-9 IPG in the first dimension (Fig. 3.41). Immunoblotting of the pH 3.5-5.0 IPG's using goat antiserum against urinary kallikrein revealed that the immunoreactive protein bands, both in an RA patient and in a normal healthy individual were removed by neuraminidase treatment (Fig. 3.42). Attempts to determine the pI's of the digested components by immunoblotting a pH 4-9 IPG were unsuccessful because of non-specific binding of the antiserum to the isozymes of α-amylase.

3.5.5. (ii) Endo F and PNGase F Treatment

The results from neuramindase treatment suggests that the anionic bands and the bands which cross-reacted with antiserum to HUK are sialated. If the sialic acid residues are located on oligosaccharide moieties which are suscep-

Fig. 3.39. IEF in a pH 3.5-5.0 IPG of control and neuraminidase-treated parotid salivary protein

The IPG was rehydrated with 8 M urea in 20% (w/v) glycerol and 0.5% (w/v) pH 3.5-5 Ampholine.

One hundred μg of parotid salivary protein was applied to each track. The tracks labelled "1" were loaded with parotid saliva from a normal healthy individual, the tracks labelled "2", "3" and "4" were loaded with parotid saliva from RA patients and the tracks labelled "5" and "6" were loaded with parotid saliva from secondary SS patients. The superscript "n" refers to neuraminidase-treated samples, while and the superscript "u" refers to control samples which had been treated under the same buffer conditions in the absence of the enzyme.

The gel was silver stained.

Figure 3.39

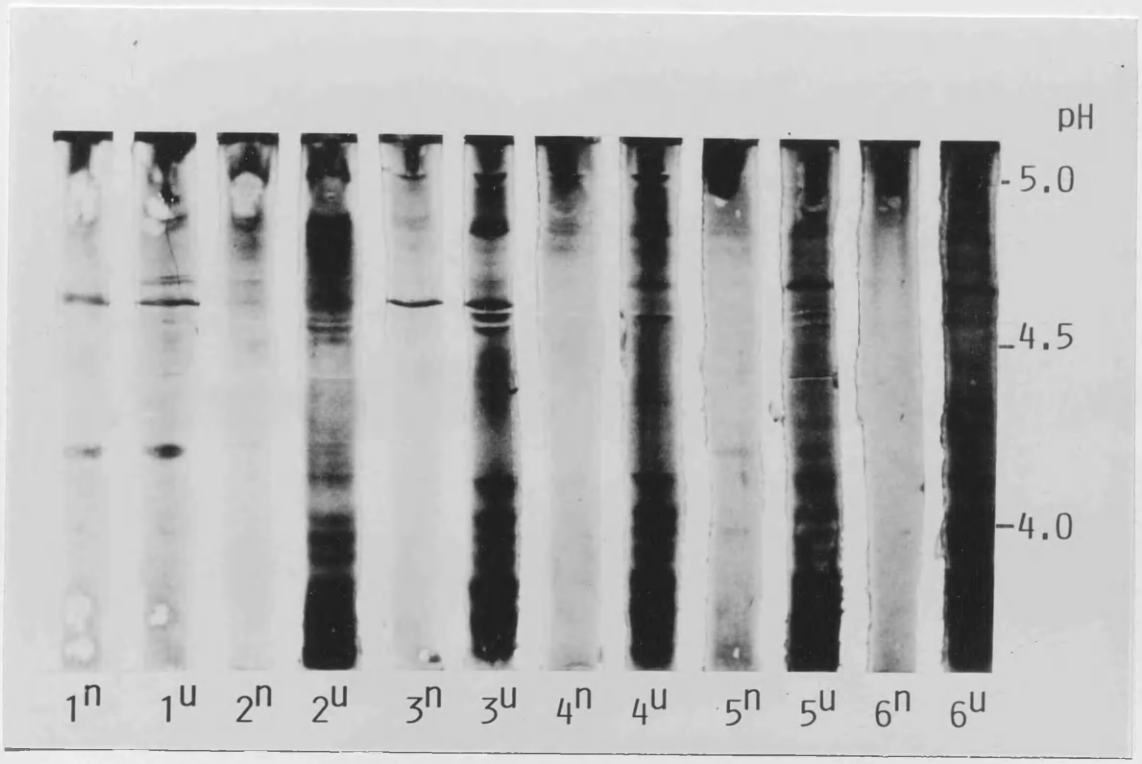


Fig. 3.40. 2-D PAGE, with pH 3.5-5.0 IPG in the first dimension, of native and neuraminidase-treated parotid salivary protein from a secondary SS patient

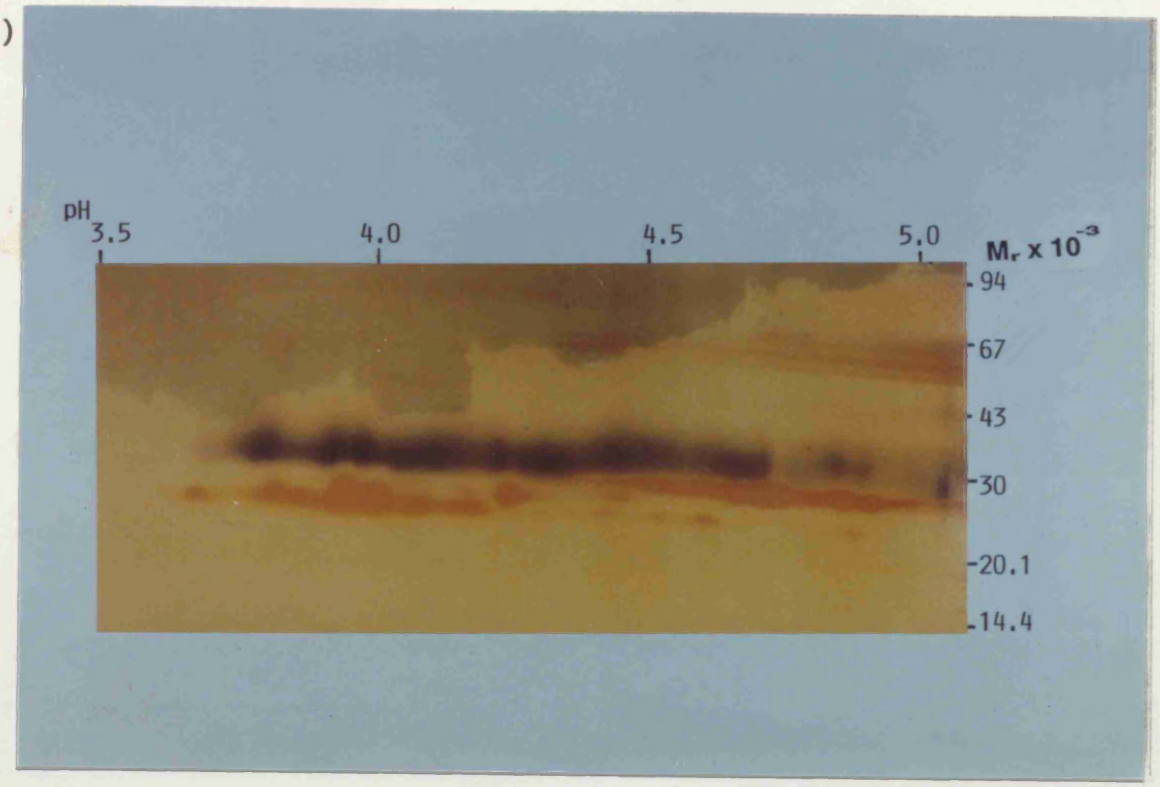
One hundredμg of protein was applied to each gel.

The gel in panel (a) was loaded with native parotid saliva while the gel in panel (b) was loaded with neuraminidase-treated parotid saliva.

The gels were silver stained.

Figure 3.40

(a)



(b)

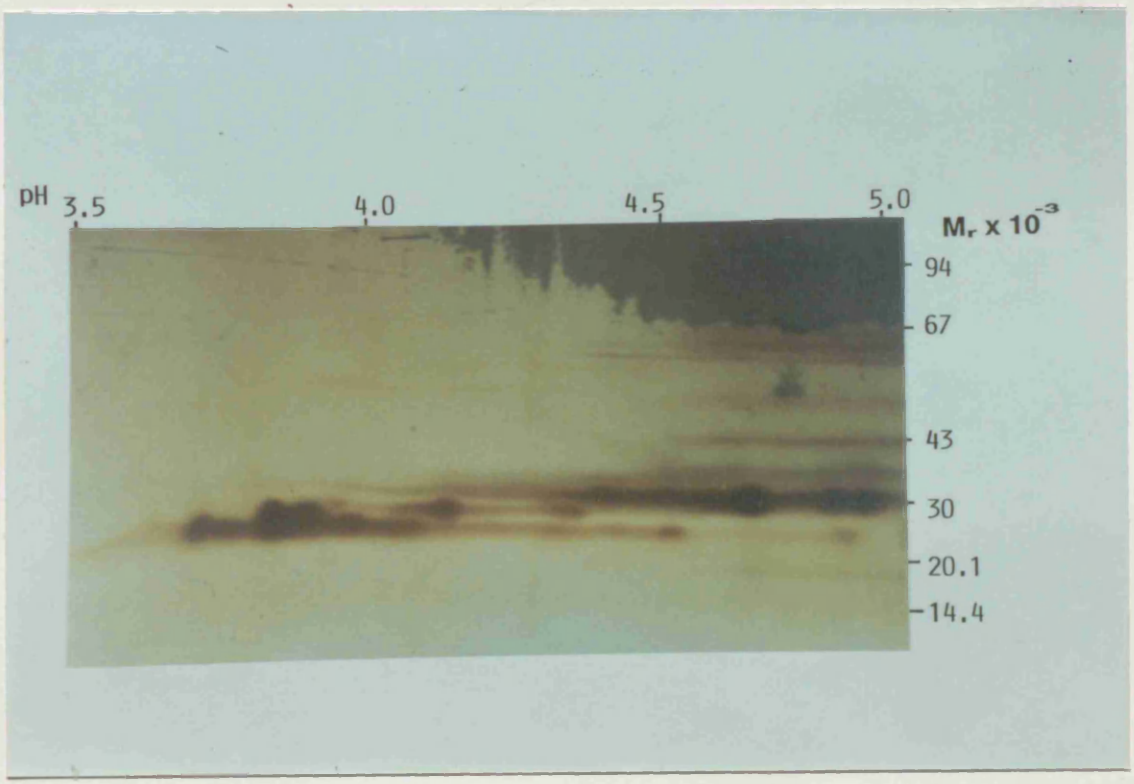


Fig. 3.41. 2-D PAGE, with pH 4-9 IPG in the first dimension, of native and neuraminidase-treated parotid salivary protein from a secondary SS patient

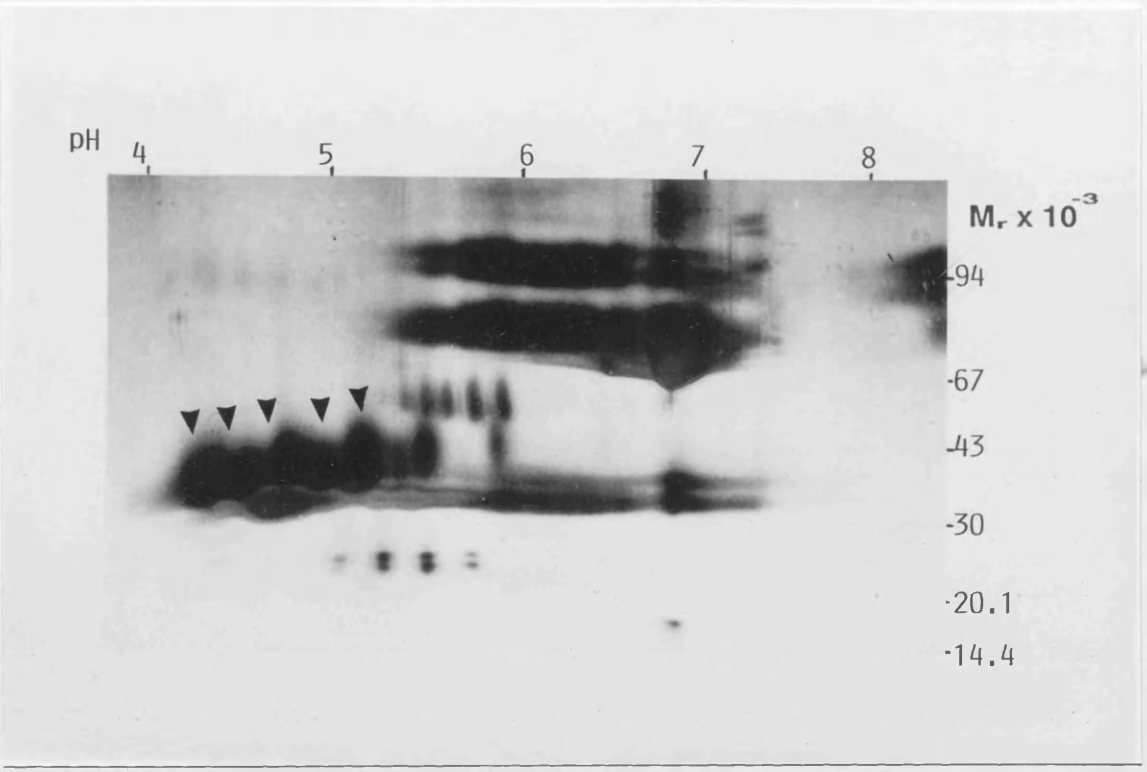
One hundredμg of protein was applied to each gel.

The gel in panel (a) was loaded with native parotid saliva while the gel in panel (b) was loaded with neuraminidase-treated parotid saliva.

The gels were silver stained.

Figure 3.41

(a)



(b)

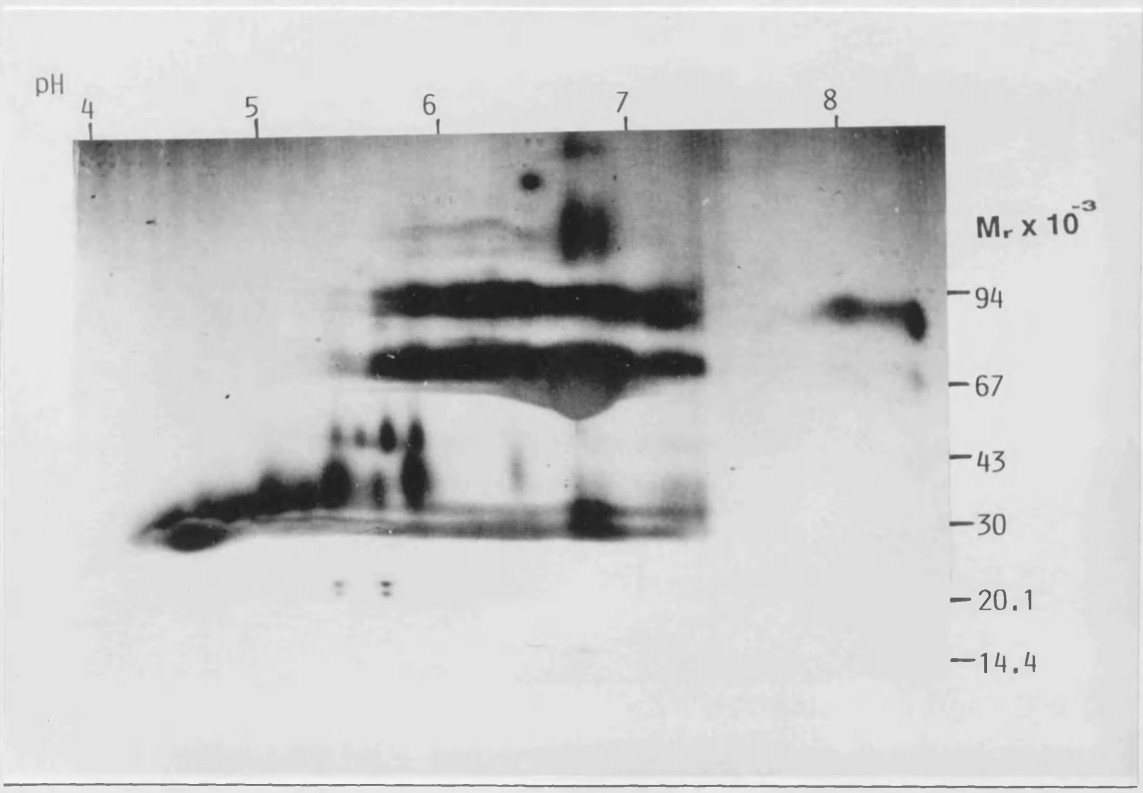


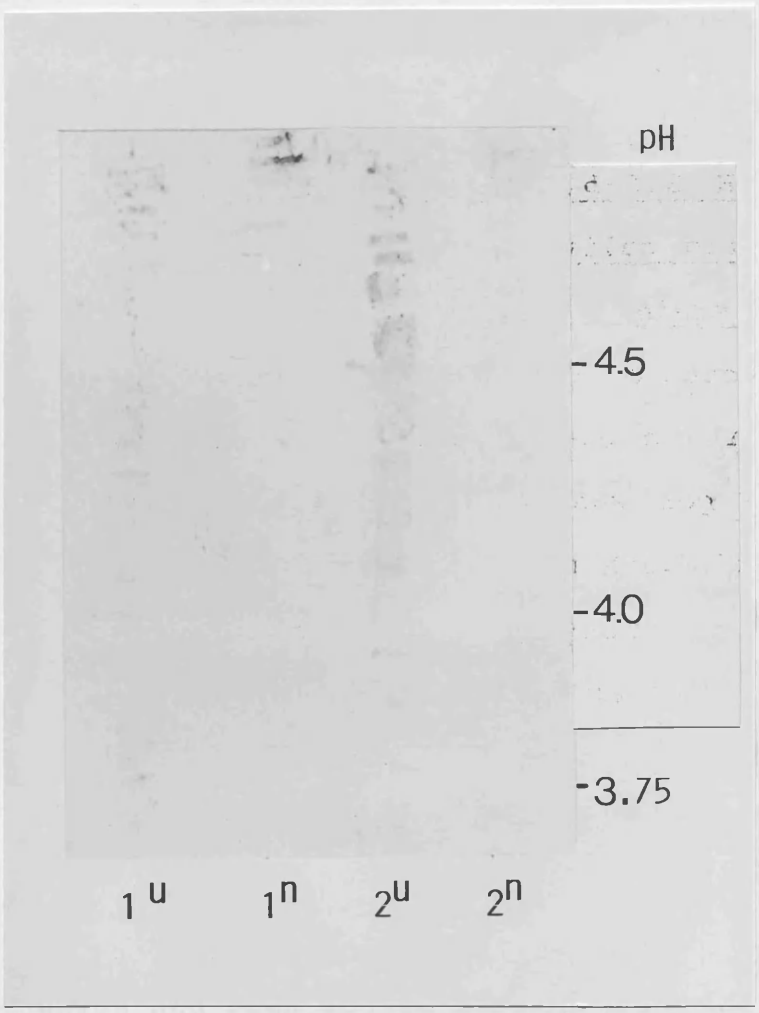
Fig. 3.42. IEF in a pH 3.5-5.0 IPG of control and neuraminidase-treated parotid salivary proteins followed by immunoblot for tissue kallikrein

The IPG was 1mm thick and cast on glass rather than on Gel-Bond film so as to facilitate easy removal prior to the electroblotting step. The gel was incorporated with 0.5% (w/v) pH 3.5-5 Ampholine and 4.46M urea.

Fifty μg of parotid salivary protein was applied to each track. The tracks marked "1" and "2" were loaded with parotid saliva from a normal healthy individual and an RA patient, respectively. The superscript "**n**" refers to neuraminidase-treated samples and the superscript "**u**" refers to control samples which had been treated under the same buffer conditions in the absence of the enzyme.

Goat antiserum to human urinary kallikrein was used as the primary antibody, followed by 3 stages of APAAP complex for detection.

Figure 3.42



tible to hydrolysis by endoglycosidases such as Endo F and PNGase F, then incubation of the salivary samples with these enzymes should also cause a shift in the pI's of the anionic bands and the bands which cross-reacted with antiserum to HUK. Although Endo F and PNGase F are more effective in deglycosylating N-linked oligosaccharides from SDS-denatured proteins (Elder and Alexander, 1982; Tarentino et al., 1985), there were insufficient amounts of the salivary samples for such an experiment to be carried out.

As Endo F and PNGase F have been reported, in some cases, to be able to effect the deglycosylation of N-linked oligosaccharides from native glycoproteins, an attempt was made to deglycosylate N-linked oligosaccharides from salivary proteins in their native states. For this purpose, control and Endo F- and PNGase F-treated parotid saliva from an RA patient and a normal healthy individual, together with HUK were fractionated in a pH 3.5-5.0 IPG which was then silver stained (Fig. 3.43). While incubation of the RA sample in the enzymes resulted in a substantial decrease in the intensity of the anionic bands, the process of deglycosylation did not appear to be exhaustive, even after an incubation time of 18 hours, as faintly-stained bands could be observed in the tracks which had been loaded with Endo F- and PNGase F-treated samples. Endo F did not appear to have any effect on the band patterns of HUK, while PNGase F appeared to decrease the intensity of some bands.

IEF in a pH 3.5-5.0 IPG of Endo F- or PNGase F-treated

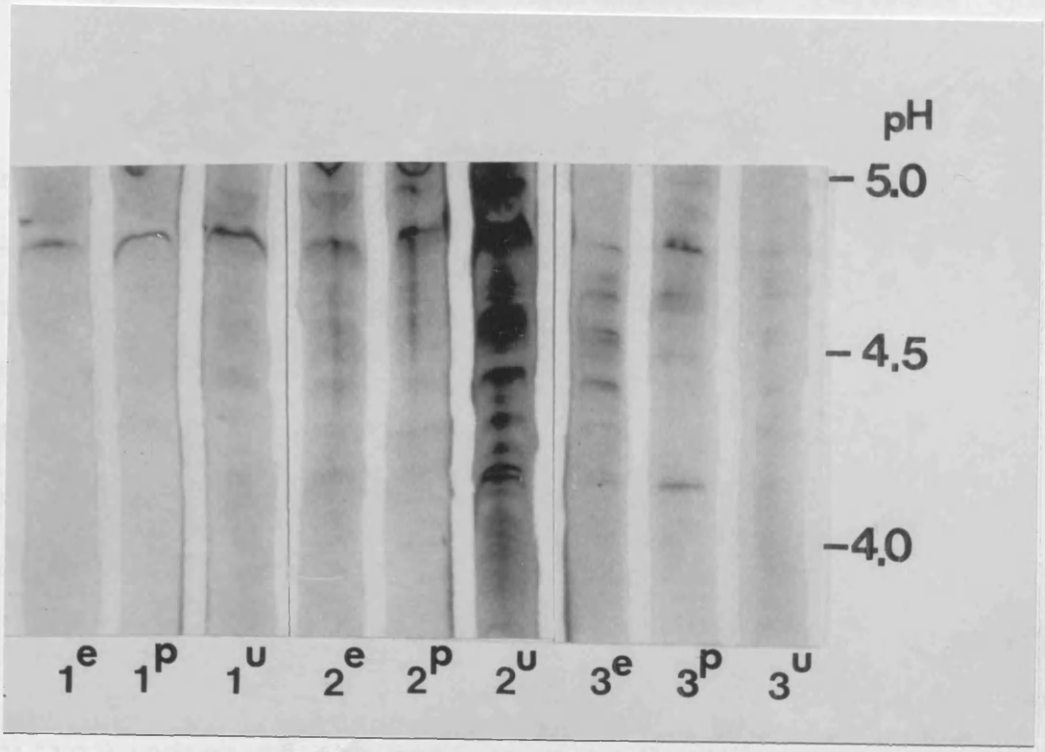
Fig. 3.43. IEF in a pH 3.5-5.0 IPG of control, Endo F-treated and PNGase F-treated parotid salivary proteins and urinary kallikrein followed by silver staining

The gel had previously been rehydrated with 8M urea in 20% (w/v) glycerol and 0.5% (w/v) pH 3.5-5 Ampholine.

The tracks labelled "1" were loaded with samples from a normal healthy individual and the tracks labelled "2" were loaded with samples from a secondary SS patient. The tracks labelled "3" were loaded with HUK. The superscripts "u", "e" and "p" refer to control, Endo F-treated and PNGase F-treated samples, respectively. The controls had been treated under the same buffer conditions in the absence of either enzyme.

Fifty μg of parotid salivary protein was loaded to each of tracks "1" and "2", and 2 μg of HUK to each of tracks "3".

Figure 3.43



and control parotid salivary protein from a normal healthy individual and an RA patient, followed by an immunoblot for tissue kallikrein, showed that the pI's of the immunoreactive bands were not affected by Endo F- or PNGase F treatment (Fig. 3.44) and unlike neuraminidase treatment there was no change in their intensity.

2-D PAGE, with a pH 4-9 IPG in the first dimension, was also used to compare control and Endo F-treated parotid salivary proteins from an RA patient. This revealed that pI's of most of the anionic components associated with connective tissue disorders were changed by incubation in Endo F (Fig. 3.45). Once again, attempts to immunoblot for the digested components, using goat antiserum to HUK and 3 stages of the APAAP complex, were unsuccessful because of the weak cross-reaction between the components of interest and the antiserum to HUK, and also the occurrence of non-specific binding of the antiserum to α-amylase.

One possible interpretation of these results is that the deglycosylation of the proteins which were immunoreactive with antiserum to HUK was not exhaustive or complete, and there may still have been sufficient levels of these proteins, even after deglycosylation, to be detected by immunoblotting. Another possible interpretation of the results involving Endo F and PNGase F is that since these two enzymes changed the pI's of the anionic proteins associated with RA and SS whilst those of the bands which were immunoreactive with antiserum to HUK remained unchanged, there

Fig. 3.44. IEF in a pH 3.5-5.0 IPG of control, Endo F- and PNGase F-treated parotid salivary protein followed by immunoblot for tissue kallikrein

The IPG was 1mm thick and cast on glass rather than on Gel-Bond film so as to facilitate easy removal prior to the electroblotting step. The gel was incorporated with 0.5% (w/v) pH 3.5-5 Ampholine and 4.46M urea.

Fifty μg of parotid salivary proteins was applied to each track. The tracks marked "1" and "2" were loaded with parotid saliva from an RA patient and a normal healthy individual, respectively. The superscript "e" refers to Endo F-treated samples, the superscript "p" refers to PNGase F-treated samples, and the superscript "u" refers to control samples. The controls had been treated under the same buffer conditions in the absence of either enzyme.

Rabbit antiserum to human urinary kallikrein was used as the primary antibody and anti-rabbit immunoglobulin-alkaline phosphatase-conjugate as the detecting antibody.

Figure 3.44

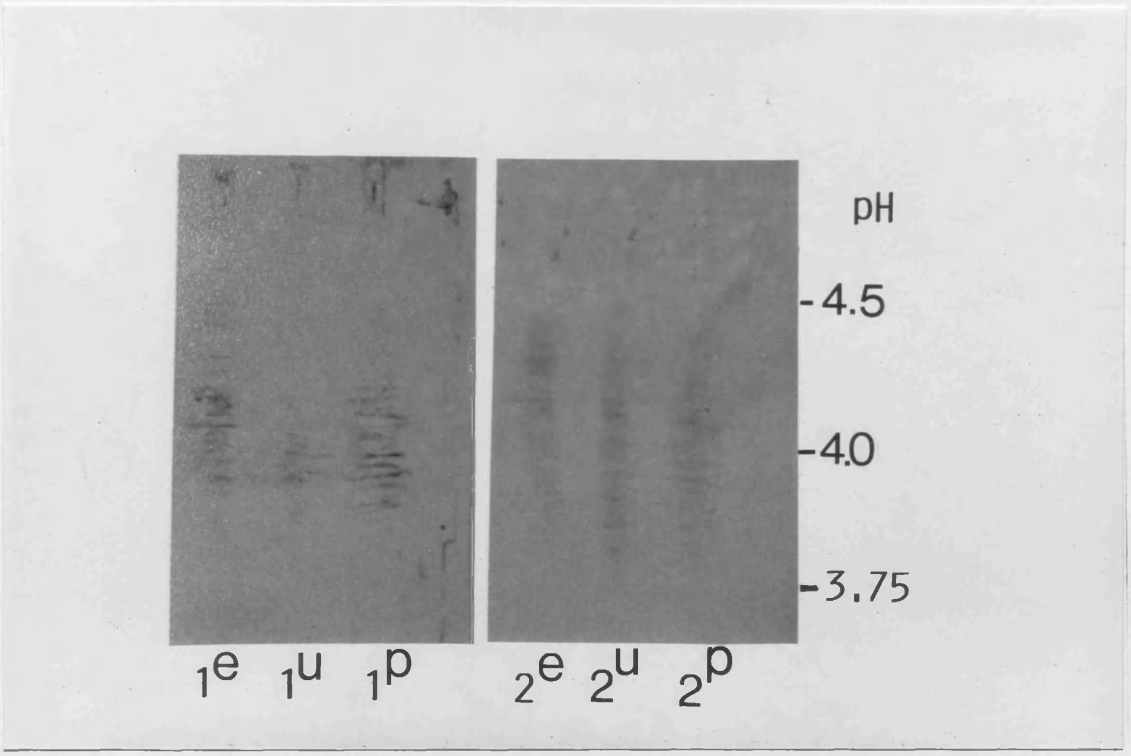
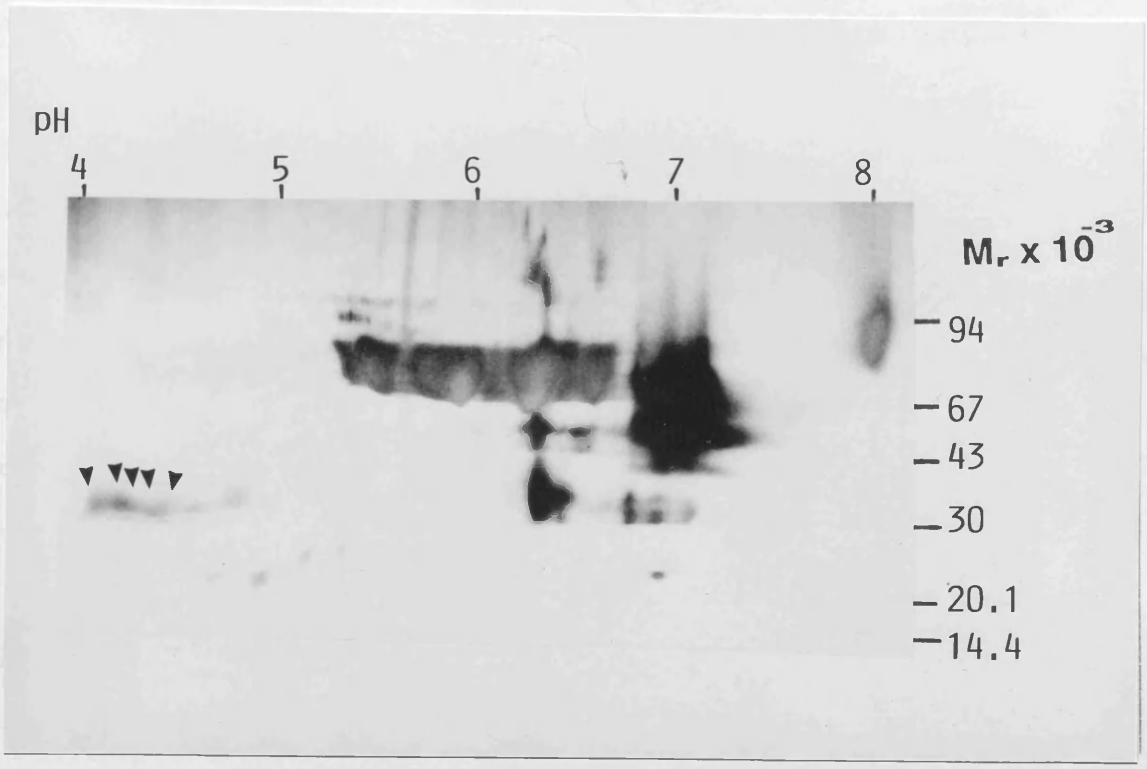


Fig. 3.45. 2-D PAGE, with pH 4-9 IPG in the first dimension, of control and Endo F-treated parotid salivary proteins from an RA patient

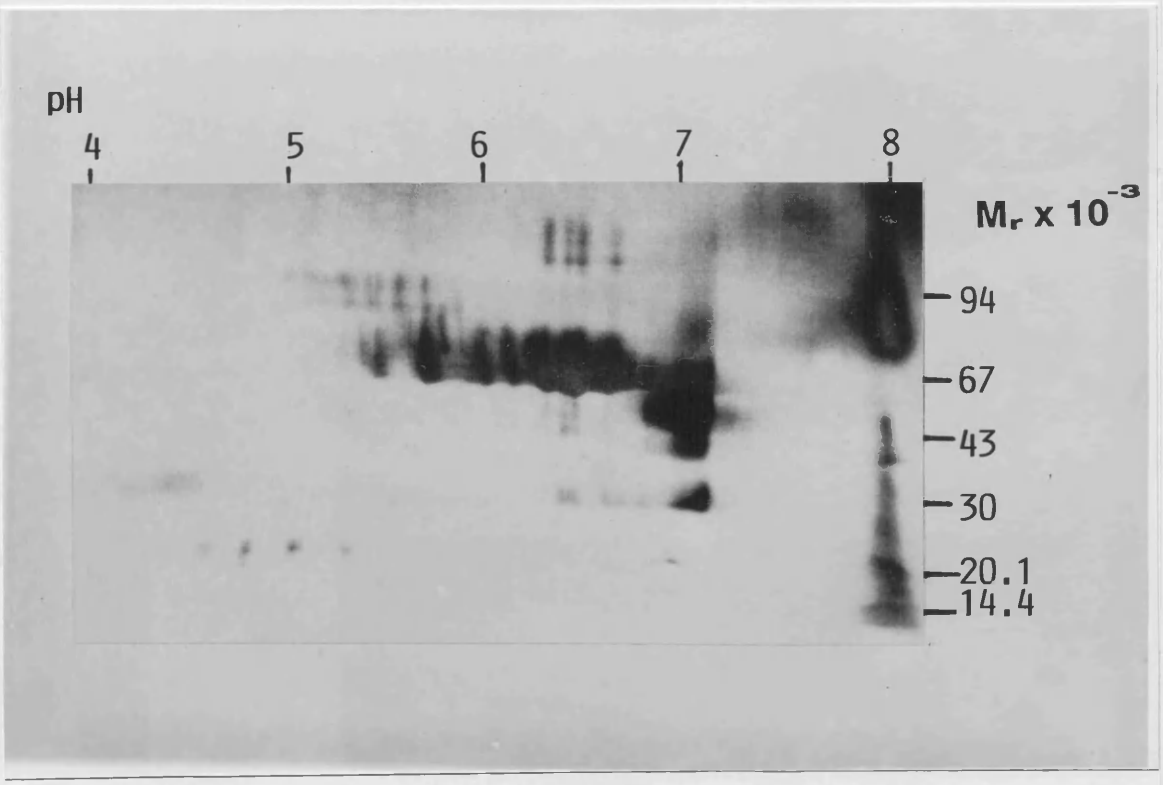
One hundredμg of protein was applied to each gel.
The gel in panel (a) was loaded with a control sample, while the gel in panel (b) was loaded with an Endo F-treated sample. The arrowheads in panel (a) indicate the anionic components associated with connective tissue disorders.
The control had been treated under the same buffer conditions in the absence of the enzyme.
Both gels were silver stained.

Figure 3.45

(a)



(b)



may be in fact be two groups of proteins which focus in the pH range under investigation, one of which is tissue kallikrein and another which is not.

By contrast, neuraminidase treatment had a profound effect on the pI's of the immunoreactive bands (Fig. 3.42) and the silver stained components (Fig. 3.39) probably because N-acetylneuraminic acid, being the terminal sugar residue in the oligosaccharide side-chain of a sialated glycoprotein, is more easily removed than the intact oligosaccharide side-chains.

3.5.6. Studies on Human Urinary Kallikrein

HUK was fractionated in a urea-containing pH 3.5-5.0 IPG which was then silver stained, and was found to display charge heterogeneity with approximately 12 pI forms which focused between pH 4.25-4.90 (Fig. 3.46). Parotid saliva samples from a normal healthy individual with relatively high levels of the anionic protein bands and from an SS patient were also fractionated in the same gel, and some of the bands in these samples focused at the same pI's as a number of the bands of HUK (Fig. 3.46). 2-D PAGE, with a pH 3.5-5.0 IPG in the first dimension, revealed that HUK has an M_r value of approximately 40,000 (Fig. 3.47(a)).

HUK is sensitive to neuraminidase treatment, after which there was reduced heterogeneity and its pI's being shifted to approximately 4.8-5.0 (Fig. 3.47(b)). This shows that the charge heterogeneity in HUK is also largely due to

Fig. 3.46. IEF in a pH 3.5-5.0 IPG of human urinary kallikrein and parotid salivary proteins

The gel was rehydrated with 8M urea in 20% (w/v) glycerol and 0.5% (w/v) pH 3.5-5 Ampholine.

Two μg of human urinary kallikrein was applied to the tracks labelled "1" and "2". Tracks "3" and "4" were each loaded with 50 μg of parotid salivary protein from a normal healthy individual and an RA patient, respectively. The normal subject selected had a higher level of anionic bands than other normal subjects.

The gel was silver stained.

Figure 3.46

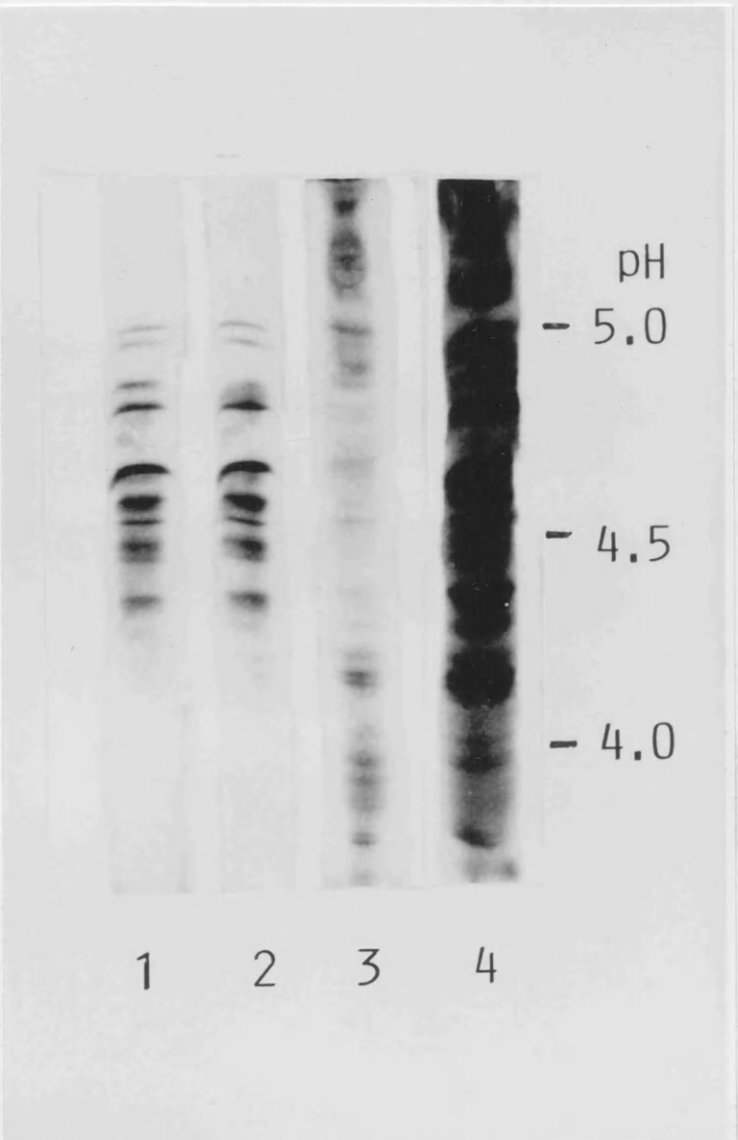


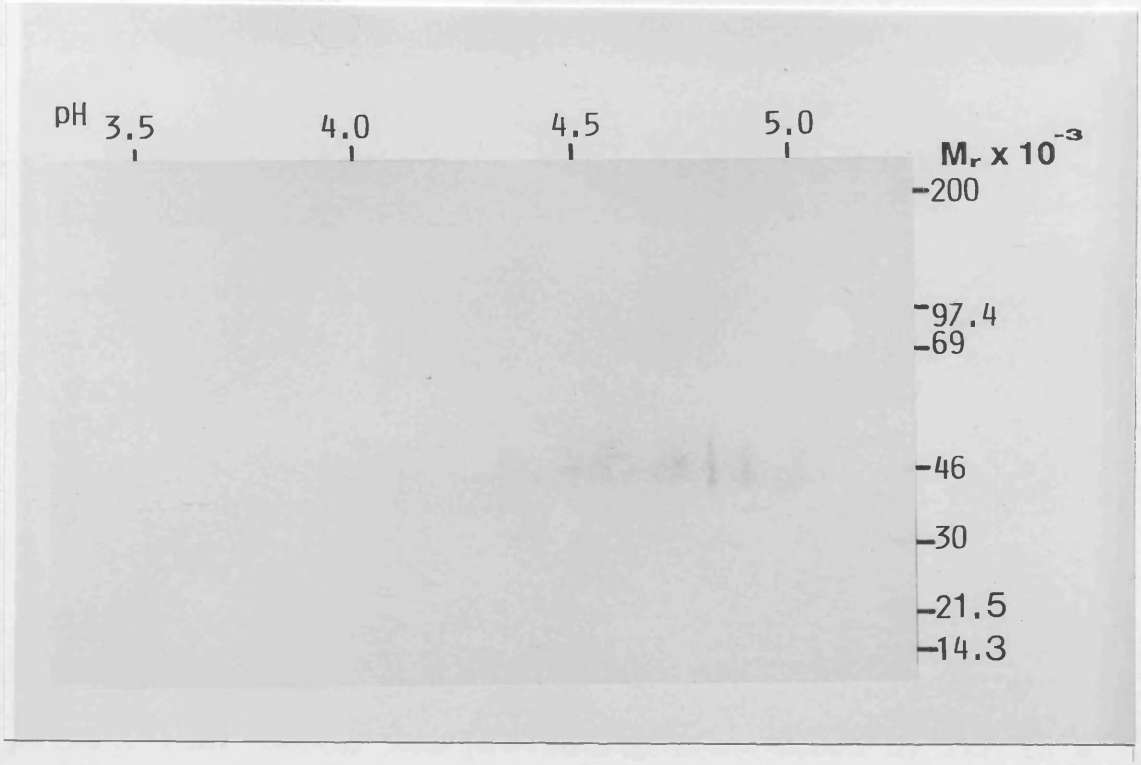
Fig. 3.47. 2-D PAGE, with pH 3.5-5.0 IPG in the first dimension, of control and neuraminidase-treated human urinary kallikrein followed by immunoblot for tissue kallikrein

Two μg of human urinary kallikrein (HUK) was applied to each gel. The gel in panel (a) was loaded with control HUK while the gel in panel (b) was loaded with neuraminidase-treated HUK. The control had been treated under the same buffer conditions in the absence of the enzyme.

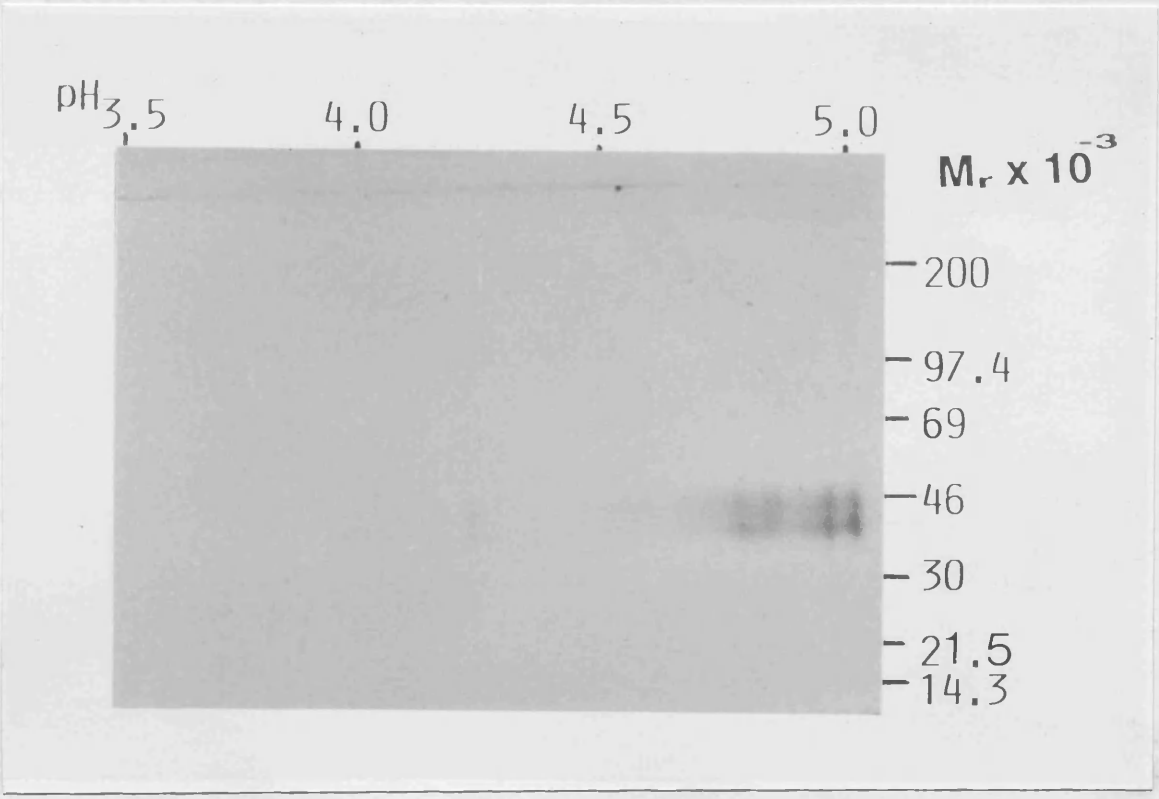
Rabbit antiserum to HUK was used as the primary antibody and anti-rabbit immunoglobulin-alkaline phosphatase conjugate as the detecting antibody.

Figure 3.47

(a)



(b)



differing contents of sialic acid in its oligosaccharide side-chains.

In an attempt made to deglycosylate HUK in its native state, with Endo F and PNGase F, IEF in a pH 3.5-5.0 IPG followed by immunoblotting for HUK showed that the endoglycosidases Endo F and PNGase F had no effect in altering the pI's of the immunoreactive bands (Fig. 3.48).

3.5.7. Separation of Parotid Salivary Proteins by FPLC

3.5.7.(i) Analysis of FPLC Fractions by SDS-PAGE and Immunoblotting

Parotid saliva samples from a normal healthy individual and an RA patient (the latter had previously been found by IEF in a pH 3.5-5.0 IPG to contain strongly-stained anionic bands) were fractionated by FPLC in a Pharmacia Mono-Q anion exchange column.

Fractionation by FPLC of parotid saliva from the normal healthy individual and the RA patient produced approximately 15 well-resolved peaks (Figs. 3.49(a) and (b)). Subsequent fractionations by FPLC of parotid saliva from 7 normal healthy subjects, 12 RA patients and 9 SS patients seemed to indicate that the differences in the peak patterns of the FPLC traces of the normal subjects and those of the RA or SS patients were due to individual-to-individual variation in these patterns, and no peaks that could be clearly identified as characteristic of RA or SS could be observed.

Fractions corresponding to each peak were collected,

Fig. 3.48. IEF in a pH 3.5-5.0 IPG of control, Endo F- and PNGase F-treated human urinary kallikrein followed by immunoblot for tissue kallikrein

The IPG was 1mm thick and cast on glass rather than on Gel-Bond film so as to facilitate easy removal prior to the electroblotting step. The gel was incorporated with 0.5% (w/v) pH 3.5-5 Ampholine and 4.46M urea.

Twoμg of human urinary kallikrein was applied to each track. The track labelled "e" was loaded with Endo F-treated sample, the track labelled "p" with PNGase F-treated sample, and track labelled "u" with control sample. The control had been treated under the same buffer conditions in the absence of either enzyme.

Rabbit antiserum to human urinary kallikrein was used as the primary antibody and anti-rabbit immunoglobulin-alkaline phosphatase-conjugate as the detecting antibody.

Figure 3.48

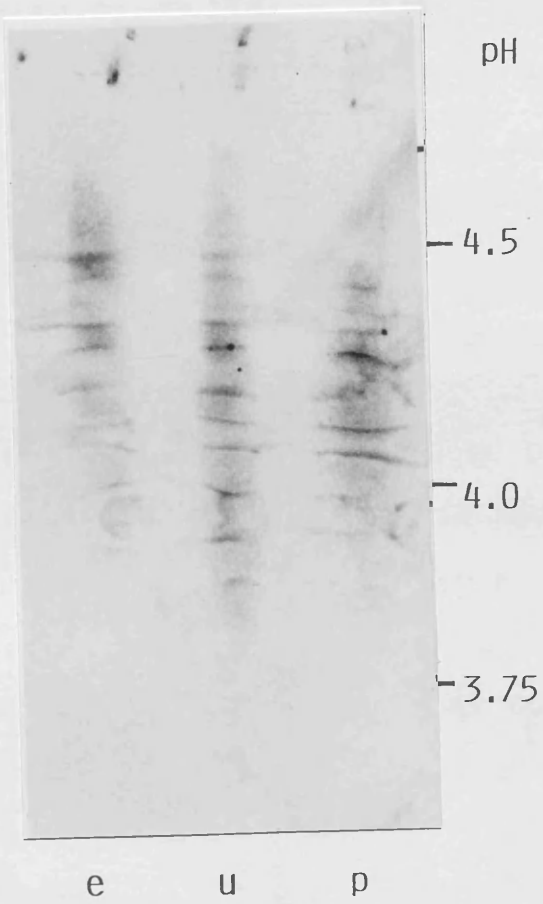


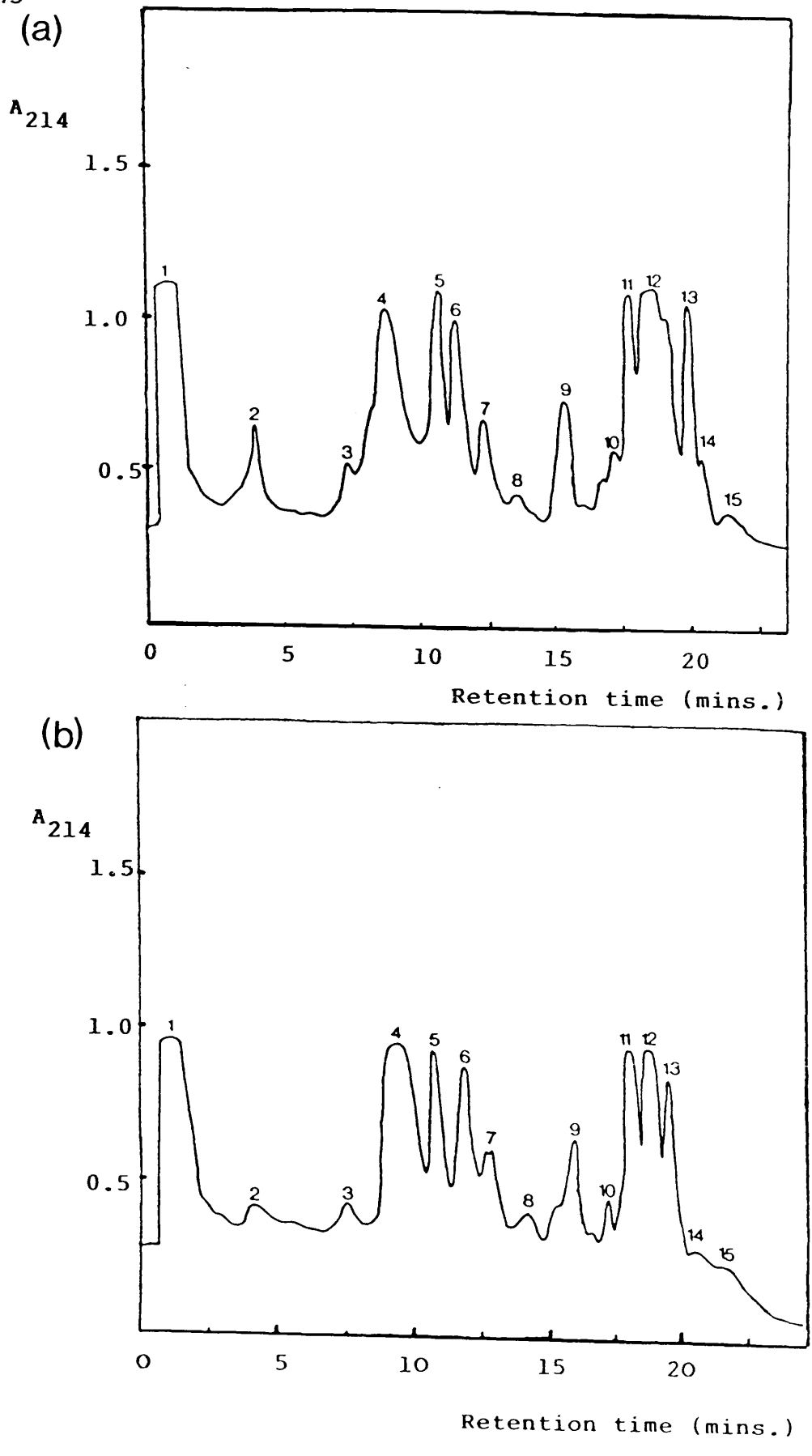
Fig. 3.49. Separation by FPLC, using an anion-exchange column (Mono-Q), of parotid salivary proteins from a normal healthy individual and an RA patient

Panel (a) shows the separation of proteins from a normal healthy individual.

Panel (b) shows the separation of proteins from an RA patient.

Column: Pharmacia Mono-Q HR 5/5, 5mm X 50mm; sample volume: 0.5ml; mobile phase: 20mM bis-tris propane, pH 7.0, as the initial buffer (A) and 1M sodium chloride in 20mM bis-tris propane, pH 7.0, as the final buffer (B); gradient: 0-30 %B from 3-14 minutes, 30-100 %B from 14-16 minutes; flow-rate: 1 ml/min; detection: UV (214nm), 2.0 AUFS.

Figure 3.49


(a)
A_{214}
1.5
1.0
0.5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0
5
10
15
20
Retention time (mins.)
(b)
A_{214}
1.5
1.0
0.5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0
5
10
15
20
Retention time (mins.)

and 50μl of each was analysed by SDS-PAGE followed by silver staining (Figs. 3.50(a) and (b)). Comparison of the gel in Fig. 3.50(a) with that in Fig. 3.50(b) showed that the gels loaded with FPLC fractions from the RA patient exhibited protein bands of M_r's between 27,000 and 30,000 in the tracks which had been loaded with FPLC fractions corresponding to peaks 4-9, while in the normal healthy control similar bands were not detectable. The bands in the later fractions had slightly higher M_r values than those in the earlier fractions, a finding that could be explained on the basis of the proteins being glycoproteins which are heterogeneous in their oligosaccharide moieties; those with larger oligosaccharide moieties being eluted in later fractions.

SDS-PAGE, followed by immunoblotting for tissue kallikrein using the alkaline phosphatase-conjugated anti-antibody method of Blake et al. (1984), of the FPLC fractions from an RA patient produced weakly stained immunoreactive bands corresponding to the protein bands of M_r's approximately 27,000. SDS-PAGE of these FPLC fractions was repeated, and another immunoblot for kallikrein was performed using the APAAP amplification method of Cordell et al. (1984). This procedure resulted in strongly stained bands of M_r approximately 27,000 in the tracks loaded with FPLC fractions corresponding to peaks 4-9 being visualised (Fig. 3.51). Goat antiserum to human serum albumin was shown, by double radial immunodiffusion, not to cross-react with HUK. It was

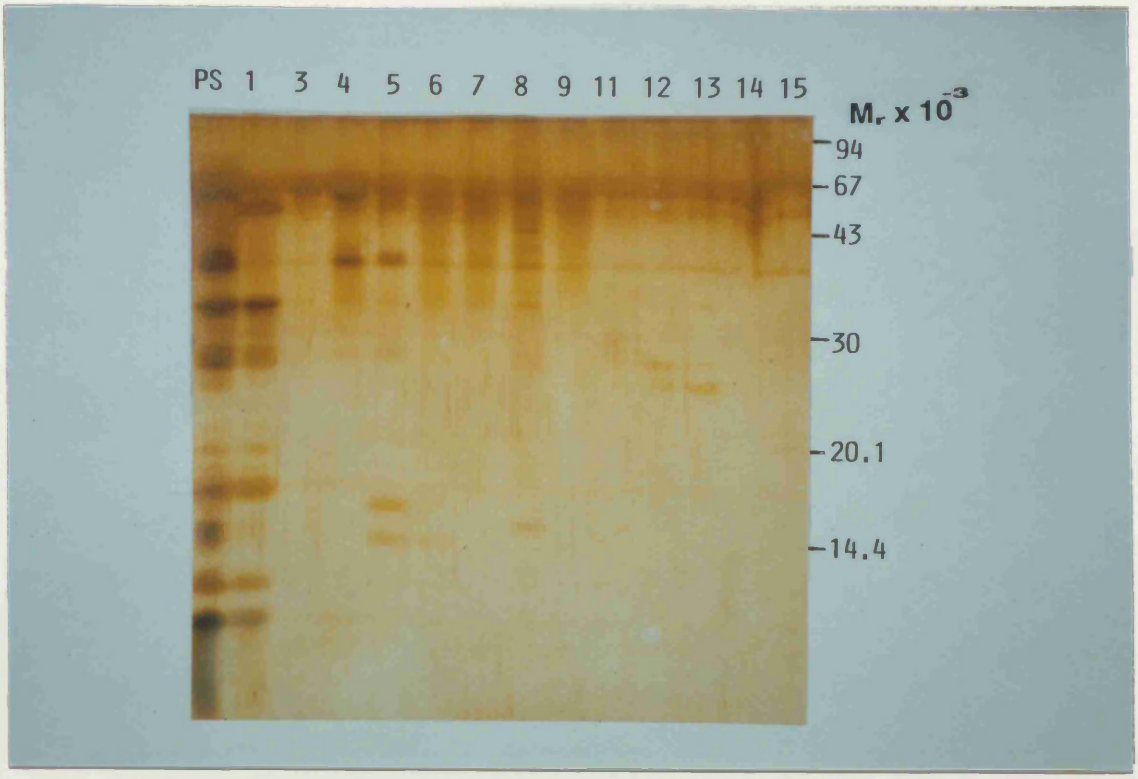
Fig. 3.50. Separation by FPLC, using an anion-exchange column (Mono-Q), of parotid salivary proteins from a normal healthy individual and an RA patient, followed by SDS-PAGE and silver stain

Panel (a) shows an SDS-PAGE gel of parotid salivary protein fractions, separated by FPLC, of a normal healthy individual. The track labelled "PS" was loaded with 40 μg of parotid salivary proteins, while the tracks labelled "1" to "15" were each loaded with 50 μl of each FPLC fraction corresponding to peaks 1-15 in Fig. 3.49(b), in the order in which they were eluted.

Panel (b) shows an SDS-PAGE gel of parotid salivary protein fractions, separated by FPLC, of an RA patient. The track labelled "PS" was loaded with 40 μg of parotid salivary proteins, while the tracks labelled "1" to "15" were each loaded with 50 μl of each FPLC fraction corresponding to peaks 1-15 in Fig. 3.49(b), in the order in which they were eluted. The concentration of both gels was 12.5 %T.

Figure 3.50

(a)



(b)

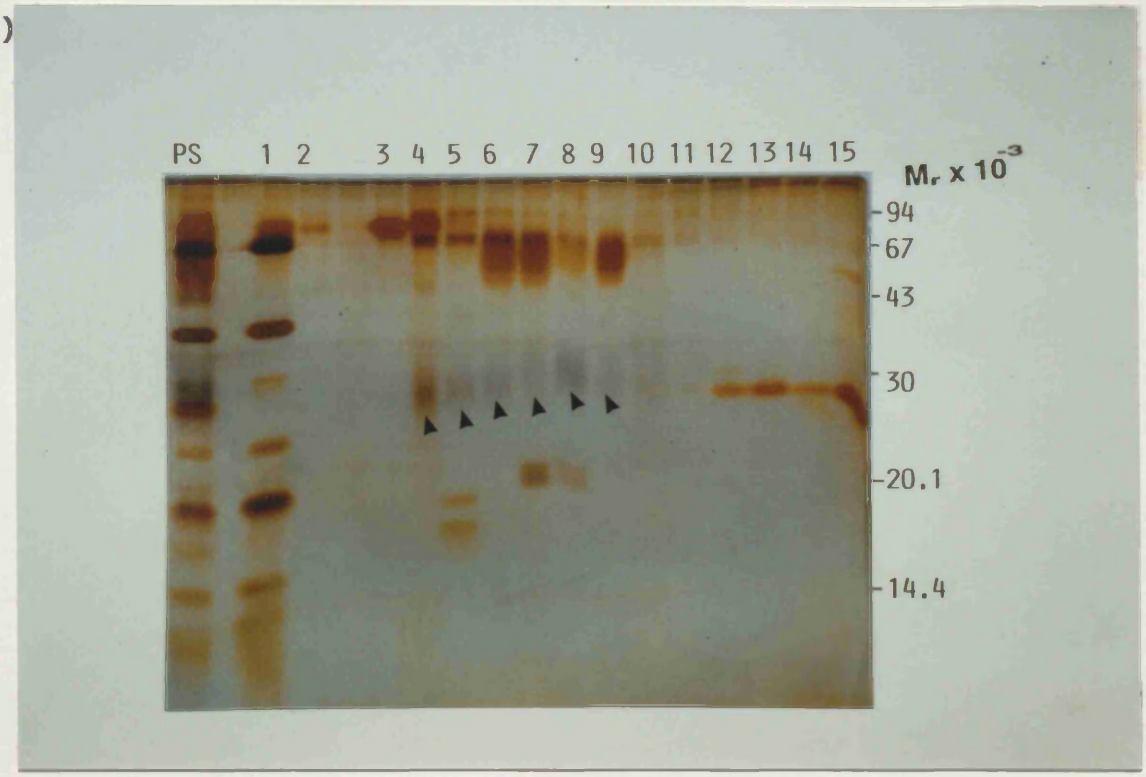


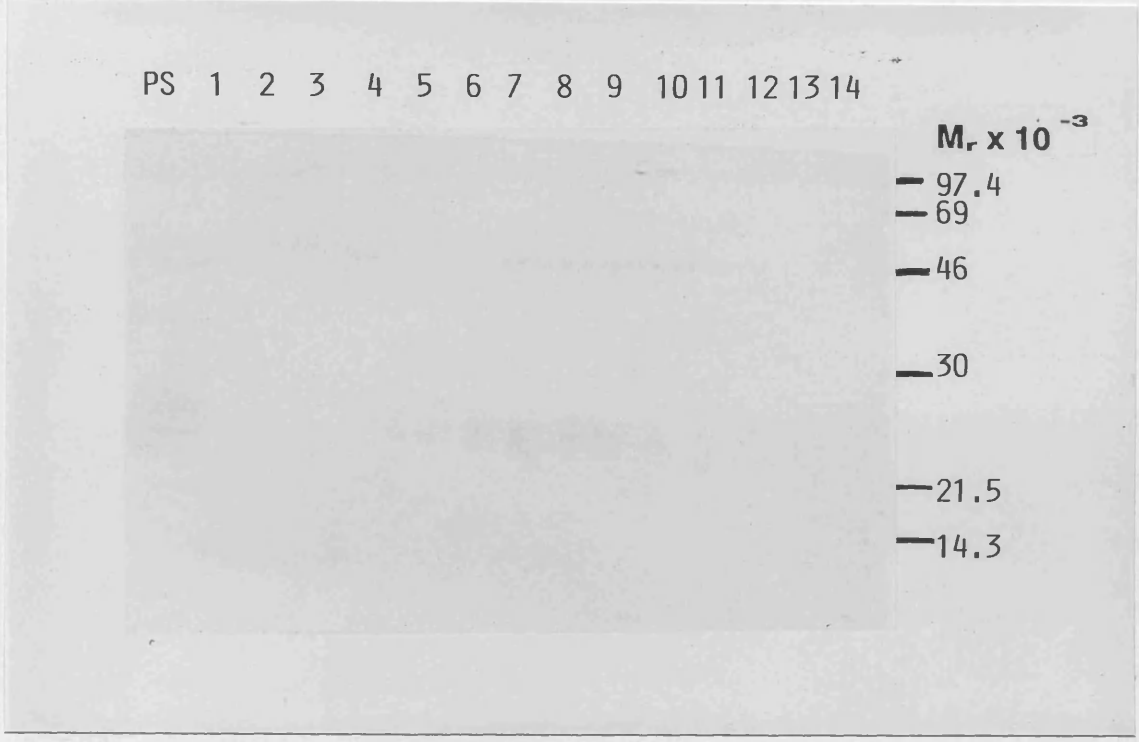
Fig. 3.51. Separation by FPLC, using an anion-exchage column (Mono-Q), of parotid salivary proteins from an RA patient followed by SDS-PAGE and immunoblot for kallikrein

The track labelled "PS" was loaded with 40μg of parotid salivary proteins. The tracks labelled "1" to "15" were each loaded with 50μl of each FPLC fraction corresponding to peaks 1-15 in Fig. 3.49(b), in the order in which they were eluted.

Goat antiserum to human urinary kallikrein was used as the primary antibody, followed by 3 stages of APAAP complex for detection.

The concentration of the gel was 12.5 %T.

Figure 3.51



the control experiment, the procedure of which is similar to that described in Section 3.5.4, which involved using goat antiserum to human serum albumin instead of goat antiserum to HUK, but retaining all the other steps involved in the APAAP amplification method. This did not result in any staining reaction.

The acidic PRP's, which have been shown by SDS-PAGE of purified standards to have M_r's of approximately 26,000 and 28,000 (Fig. 3.7(a)) and tended to co-electrophorese with the anionic proteins associated with connective tissue disorders, were more strongly bound to the anion-exchanger and were eluted in the fractions corresponding to peaks 12-15. These proteins were stained with a yellowish-brown colour by silver staining (Figs. 3.50(a) and (b)). SDS-PAGE, followed by staining with CBB R250, of fractions correponding to each FPLC peak showed pink-staining bands of M_r's of approximately 26,000 and 28,000 in the tracks which had been loaded with fractions corresponding to peaks 12-15 (Fig. 3.52).

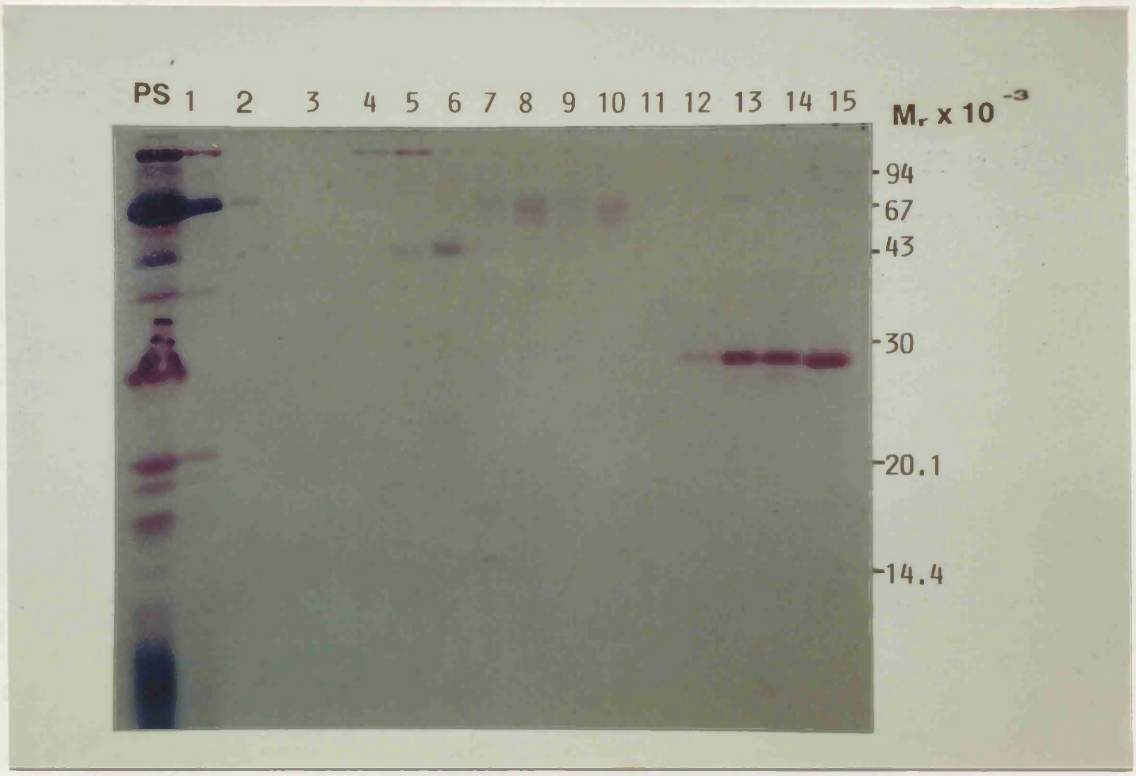
As the acidic PRP's and anionic proteins associated with connective tissue disorders were eluted in different fractions, FPLC provided a means of separating these two groups of proteins. As they have overlapping pI's and very close M_r's, they could not be separated from each other by preparative IEF or SDS-PAGE. However, by performing FPLC in an anion-exchange column, the acidic PRP's and the anionic proteins of interest were successfully separated with the

Fig. 3.52. Separation by FPLC, using an anion-exchage column (Mono-Q), of parotid salivary proteins from a normal healthy individual, followed by SDS-PAGE and Coomassie staining

The tracks labelled "PS" was loaded with 40 μg of parotid salivary protein. The tracks labelled "1" to "15" were each loaded with 50 μl of each FPLC fraction corresponding to peaks 1-15 in Fig. 3.49(b), in the order in which they were eluted

The concentration of the gel was 12.5 %T, and it was stained with CBB R250.

Figure 3.52



former being eluted in the later fractions. This provides the basis for the further characterisation of the anionic proteins associated with connective tissue disorders by SDS-PAGE followed by electroblotting onto PVDF membrane and the possibility of amino acid sequencing.

3.5.7.(ii) Electroblotting onto PVDF Membranes and Amino Acid Sequencing

As the FPLC fractions of parotid salivary proteins corresponding to peaks 4-9 have been shown by SDS-PAGE and immunoblotting to contain the anionic proteins associated with connective tissue disorders, these fractions were used for SDS-PAGE followed by electroblotting onto PVDF for the purpose of amino acid sequencing. After electroblotting, the PVDF membrane was stained with CBB R250. Following destaining of the membrane, the protein bands of interest (M_r 27,000) were removed for amino acid sequencing. This procedure is outlined in the flow-diagram in Fig. 3.53.

Staining with CBB R250 revealed faint bands of M_r 27,000 (Fig. 3.54), which were cut out and used for amino acid sequencing. However, the quantity of protein (6 pmol) on the membrane was too low for a sequence to be obtained.

3.5.7.(iii) Assay for Tissue Kallikrein Activity in Parotid Saliva

The kallikrein activity in parotid saliva samples from normal healthy individuals and RA and SS patients was

Fig. 3.53. Flow-diagram showing sequence of events from the separation of parotid salivary proteins from an RA patient to electroblotting onto PVDF membrane and amino acid sequencing

Path 1, which was followed before path 2, showed the presence of silver stained protein bands of M_r's between 27,000 and 30,000 in the fractions corresponding to peaks 4-9 in Fig. 3.49; and that bands of M_r approximately 27,000 in these fractions were immunoreactive with goat antiserum to tissue kallikrein. On the basis of this information, the procedures outlined in path 2 were carried out, in which the fractions corresponding to peaks 4-9 were pooled together, concentrated and subjected to SDS-PAGE. The SDS-PAGE gel was then electroblotted onto a PVDF membrane which was subsequently stained with CBB R250, after which protein bands of M_r approximately 27,000 were visualised. These were cut out and used for amino acid sequencing.

Figure 3.53

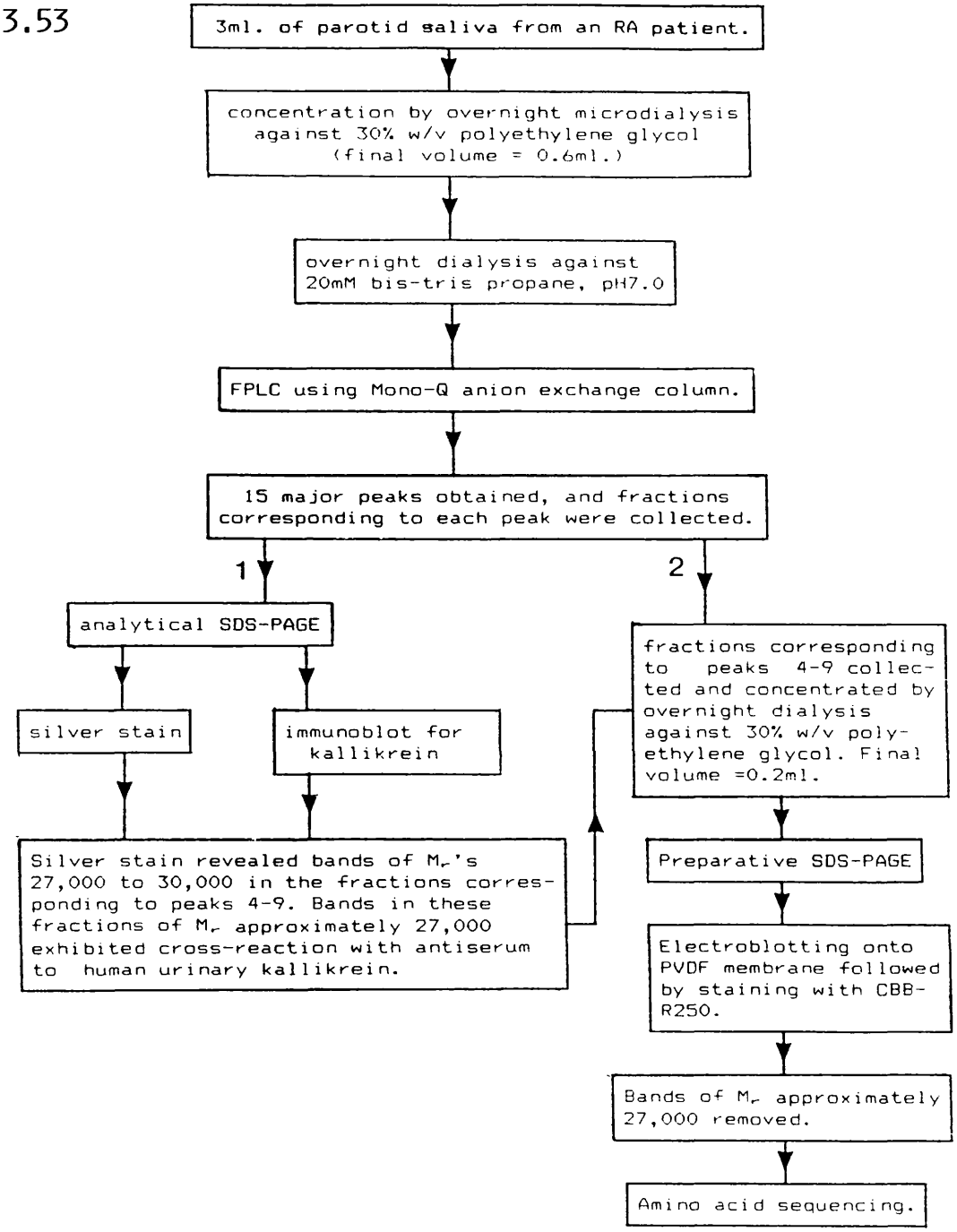
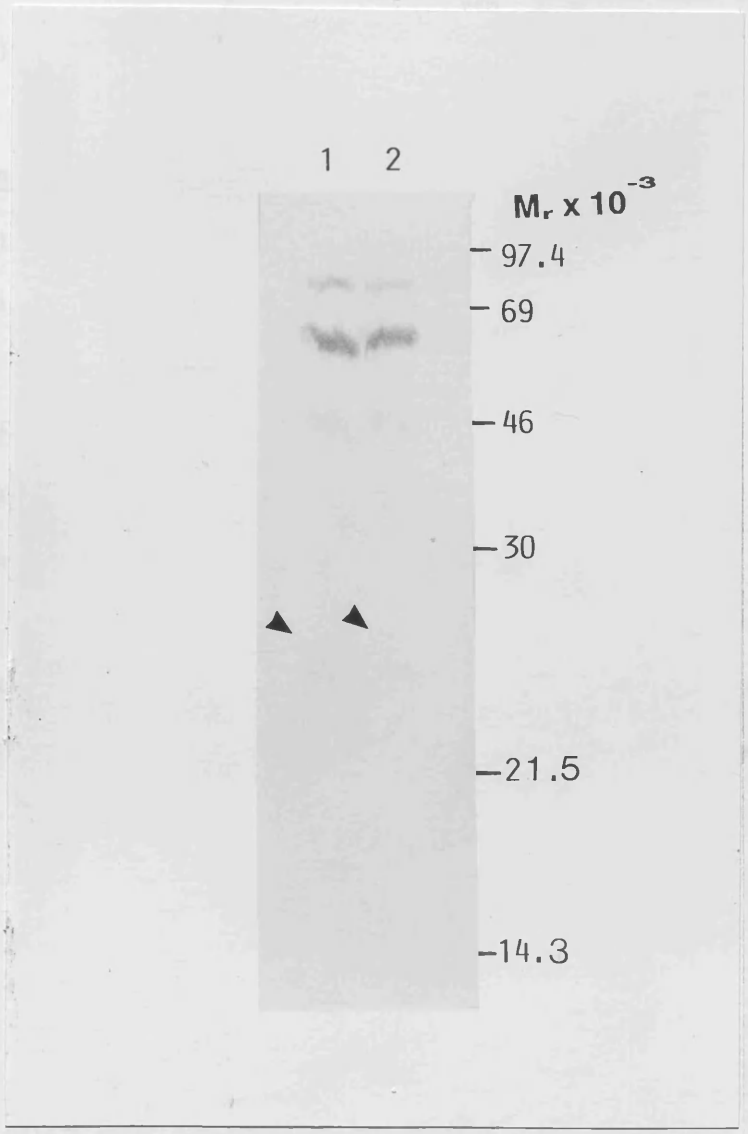

3ml. of parotid saliva from an RA patient.
concentration by overnight microdialysis against 30% w/v polyethylene glycol (final volume = 0.6ml.)
overnight dialysis against 20mM bis-tris propane, pH7.0
FPLC using Mono-Q anion exchange column.
15 major peaks obtained, and fractions corresponding to each peak were collected.
1
2
analytical SDS-PAGE
silver stain
immunoblot for kallikrein
Silver stain revealed bands of Mr's 27,000 to 30,000 in the fractions corresponding to peaks 4-9. Bands in these fractions of Mr approximately 27,000 exhibited cross-reaction with antiserum to human urinary kallikrein.
fractions corresponding to peaks 4-9 collected and concentrated by overnight dialysis against 30% w/v polyethylene glycol. Final volume =0.2ml.
Preparative SDS-PAGE
Electroblotting onto PVDF membrane followed by staining with CBB-R250.
Bands of Mr approximately 27,000 removed.
Amino acid sequencing.

Fig. 3.54. SDS-PAGE of the FPLC fractions from an RA patient which contained the anionic proteins associated with RA and SS, followed by electroblotting onto PVDF

The fractions which contained the anionic proteins of interest, i.e. the fractions corresponding to peaks 4-9 in Fig. 3.49, were pooled together and concentrated by overnight dialysis against 30% (w/v) polyethylene glycol, after which 50μl was applied to each track of the SDS-PAGE gel. The concentration of the gel was 12.5 %T.

The SDS-PAGE gel was electroblotted onto a PVDF membrane which was stained with CBB R250. After destaining, bands of M_r approximately 27,000 were obtained. They were cut out of the membrane for the purpose of amino acid sequencing.

Figure 3.54



measured using the chromogenic tripeptide substrate H-D-Val-Leu-Arg-NH-pNA. The absorbance values, measured at 405nm, obtained from 9 patients (4 RA and 5 SS) and 9 normal subjects were as follows:

Normal subjects

Mean= 2.27×10^{-3} U/ml

Standard deviation= 1.83×10^{-3} U/ml

RA/SS patients

Mean= 8.95×10^{-3} U/ml

Standard deviation= 5.48×10^{-3} U/ml

The Unit (U) of enzyme activity stated is the International Unit, which is defined as the amount of enzyme that causes the transfomation of 1 μmol/min of the substrate.

The kallikrein activity is higher in the RA and SS patients than in the normal subjects.

3.5.7.(iv) Latex Slide Test for Rheumatoid Factors

Parotid saliva samples from 13 SS patients, 10 RA patients and 10 normal healthy individuals were tested using the latex slide test for the presence of rheumatoid factors. Four of the SS samples (or 31%) and 3 of the RA samples (or 30%) exhibited agglutination of the human gamma-globulin-coated latex particles, which indicated a positive reaction. None of the samples from the normal healthy individuals resulted in agglutination. All 7 RA and SS patients whose

parotid saliva exhibited rheumatoid factor activity were subjected to IEF in pH 3.5-5.0 IPG's and found to contain elevated levels of the anionic bands of interest.

3.5.8. Discussion

The anionic salivary proteins associated with connective tissue disorders have been investigated by Fischer *et al*. (1968) using paper electrophoresis, Herzberg *et al*. (1973) using disc electrophoresis, Chisholm *et al*. (1973) using IEF in carrier ampholyte-containing polyacrylamide rod gels and Mairs and Beeley (1985) using IEF in carrier ampholyte-containing flat-bed polyacrylamide gels.

The IPG technique developed during the course of this study has allowed parotid saliva samples to be focused without prior treatment except for centrifugation, therefore making it possible to analyse salivary samples as small as 50μl. This makes the IPG technique especially ideal for the analysis of salivary proteins from RA and SS patients, as many of them, especially the latter, suffer from xerostomia, which makes the collection of large volumes of saliva extremely difficult or even impossible. The use of IPG's has also allowed the pH profile to be designed to suit the proteins of interest. Thus, IPG's of 2 acidic pH ranges, pH 2.8-4.5 and pH 3.5-5.0, were prepared to produce optimum resolution of the anionic salivary proteins. The addition of 8M urea to pH 3.5-5.0 IPG's optimised the resolution of the focused bands. In this study, IEF in IPG's was utilised in

order to further investigations into the nature of these anionic proteins. IPG's were used both in one- and two-dimensional systems.

The resolution of the protein bands obtained by IEF in IPG's was far superior to that obtained by IEF in carrier-ampholyte-based gels. In addition, because of the sensitivity of the pH gradients of carrier-ampholyte IEF gels towards electrolytes, the saliva samples have to be desalted before they were fractionated in such gels. This neccessitates relatively large volumes of saliva (preferably > 2ml). Using IEF in carrier ampholyte-containing flat-bed polyacrylamide gels, Mairs and Beeley (1985) found that the anionic proteins associated with connective tissue disorders tended to precipitate near their pI's, thus impairing the resolution of the focused bands. In this study, it was found that IEF in non-denaturing IPG's of pH range 3.5-5.0 produced poor resolution of these anionic proteins, but upon the addition of 8M urea to the gels, the resolution was greatly improved.

By performing IEF in urea-containing pH 3.5-5.0 hybrid carrier ampholyte-IPG's, the anionic salivary proteins associated with RA and SS have been found to display charge heterogeneity, with pI's 3.65-4.75.

Using 2-D PAGE with IEF in pH 3.5-5.0 hybrid carrier ampholyte-IPG's in the first dimension and SDS-PAGE in linear polyacrylamide gradient gels in the second, followed by silver-staining, the M_r of the anionic proteins as-

sociated with connective tissue disorders has been established as approximately 32,000. In the 2-D PAGE gels which were loaded with parotid saliva from normal healthy individuals, protein spots of a similar M_r were either not detectable or appeared as only faintly-staining components of pI's 3.65-4.25. Thus, on the basis of the evidence from silver staining of the 2-D PAGE gels alone, these proteins appeared to be less heterogeneous with respect to pI's in normal healthy individuals compared to the RA and SS patients.

In the majority of normal healthy individuals, these components were not evident in silver stained one-dimensional pH 3.5-5.0 IPG's. That they were detectable in 2-D PAGE, but not in IEF gels is probably because of the greater sensitivity exhibited by SDS-denatured proteins towards silver staining(Heukeshoven and Dernick, 1985).

IEF of human salivary proteins in IPG's of extremely acidic pH range (i.e. pH 2.8-4.5) produced no protein bands focusing below pH 4.1, and no differences could be detected between the IEF band patterns of RA or SS patients and that of normal healthy individuals. Therefore, there is little information to be gained by studying this pH range. However, in their study, Greaves et al. (1989) chose to concentrate on the protein bands focusing below pH 4 because the acidic PRP's tended to dominate the IEF pattern above this pH value. Although pH 2.8-4.5 IPG's did not succeed in fractionating the anionic proteins of interest, pH 3.5-5.0

IPG's, however, provided optimum resolution of these proteins.

Using 2-D PAGE, acidic PRP's of M_r's 26,000 and 28,000 have been located in the pH 3.5-5.0 range. Although the acidic PRP's did not display charge microheterogeneity, they exhibited considerable individual-to-individual variation, which has been attributed to genetic polymorphism by Azen and Oppenheim (1973) and Azen (1978). The overlap in the pI's of the PRP's and that of the anionic salivary proteins associated with RA and SS, together with their close M_r values have played a large part in hindering investigations into the nature of the latter group of proteins. However, 2-D PAGE has successfully resolved these two groups of proteins, thus providing a valuable method for the further study of the anionic salivary proteins associated with connective tissue disorders.

Although the anionic salivary proteins associated with RA and SS have been investigated by Fischer et al. (1968), Herzberg et al. (1973), Chisholm et al. (1973) and Mairs and Beeley (1985), their identity remained uncertain until Greaves et al. (1989) reported that a number of the protein bands focusing over the pH range 3-4 cross-reacted with rabbit antiserum to HUK. That the pI's of these bands were shifted by neuraminidase treatment led them to postulate that the salivary kallikrein in connective tissue disorder patients could be unusually glycosylated, i.e. in having higher contents of sialic acid, than the salivary kallikrein

in normal individuals. Although immunoblotting was performed on isoelectrically focused parotid salivary proteins from connective tissue disorder patients and normal subjects with a high concentration of anionic proteins, no such experiments were performed using samples from normal subjects with low concentrations of the anionic proteins. In addition, the resolution of the IEF gels appeared to be poor. Thus, the occurrence of novel or unusual glycosylation of salivary kallikrein in connective tissue disorder patients remained questionable. Another inadequacy of the study of Greaves et al. (1989) was the omission of the desalting procedure although carrier ampholyte-based IEF, the pH profile of which is sensitive to electrolytes, was used. The salivary samples were freeze-dried and reconstituted to a protein content of 6mg/ml, which entailed a four- to five-fold concentration, and applied to the IEF gels without prior dialysis. The high levels of electrolytes in parotid saliva will thus cause distortion of the pH gradient and impair the resolution of the protein bands. Finally, no attempts were made to improve the resolution of the pH range used, or to further characterise the anionic proteins, for example, by establishing their M_r's.

Immunoblotting of the pH 3.5-5.0 IPG's and 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension has demonstrated that the anionic salivary proteins associated with RA and SS focused within the same pH range as components which were immunoreactive with both commercial goat

antiserum to HUK and laboratory-produced rabbit antiserum to HUK. In the immunoblots of the IEF gels, the rabbit antiserum produced a strong staining reaction, and the goat antiserum a weak reaction. However, in the immunoblots of 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension, both antisera produced weak staining reactions when the alkaline phophatase-conjugated anti-antibody method of Blake et al. (1984) was used. The alkaline phosphatase signal was, however, successfully amplified using APAAP complexes (Cordell et al., 1984), and the use of 3 stages of APAAP complexes resulted in an intensification of the signal, both in the immunoblots of pH 3.5-5.0 IPG's and 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension. The pI's and M_r of the immunoreactive bands correlated closely with those of the RA- and SS-associated anionic salivary proteins which were visualised in 2-D PAGE gels after silver staining.

When parotid saliva from RA patients and normal healthy individuals was fractionated in pH 3.5-5.0 IPG's which were immunoblotted for tissue kallikrein using the alkaline phophatase-conjugated anti-antibody method of Blake et al. (1984), the pI's of the immunoreactive bands were found to be 3.65-4.25. In RA patients, they were more intensely stained than in normal individuals. However, amplification of the alkaline phosphatase signal using APAAP complexes (Cordell et al., 1984) revealed immunoreactive bands of a broader pI range, i.e. 3.65-4.75, both in the RA patients and in the normal individuals. In both the RA patients and

normal individuals, the bands of pI's 4.25-4.75 are most probably the minor isozymes of salivary kallikrein, and therefore the alkaline phosphatase-conjugated anti-antibody method was not senstive enough to detect them. They were, however, successfully visualised using APAAP complexes.

Silver staining of 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension revealed protein spots of pI's 3.65-4.75 and M_r approximately 32,000 in the RA and SS patients.The silver stained components of similar M_r, however, were less heterogeneous in the normal individuals (i.e. pI's 3.65-4.25). Silver staining failed to reveal the bands of pI's 4.25-4.75 in the normal healthy individuals although components in this pH range were detected by immunoblotting for kallikrein followed by amplification of the alkaline signal using APAAP complexes. This may be explained on the basis of the silver stain being less sensitive than APAAP complexes for protein detection. However, in the RA and SS patients, the level of salivary kallikrein is elevated and therefore the isozymes of pI's 4.25-4.75 that could be detected by silver staining might correspond to this enzyme. The evidence obtained from the silver stained 2-D PAGE gels, with pH 3.5-5.0 IPG's in the first dimension, could also suggest that the parotid saliva of RA and SS patients contains a larger proportion of less sialated kallikrein than that of normal subjects if that is in fact its identity.

Preparative IEF is unsuitable for the purification of

the anionic proteins of interest because of the presence of the acidic PRP's which focus over the same pH range. In addition, due to the closeness of the M_r values (approximately 26,000 and 28,000 for the acidic PRP's and 32,000 for the anionic proteins of interest), preparative SDS-PAGE would also be unsuitable for separating these two groups of proteins. However, FPLC using an anion-exchange (Mono-Q) column successfully separated these two groups of proteins as the acidic PRP's were more strongly bound to the anion-exchanger, and eluted in later fractions, than the anionic proteins associated with connective tissue disorders. This could provide a valuable step for the further purification of the anionic proteins associated with RA and SS. During the course of this study, attempts were made to subject the FPLC fractions containing these anionic proteins to further purification procedures, including size-exclusion HPLC (Welling and Welling-Wester, 1989) and lectin-affinity chromatography, including the use of Wheat Germ agglutinin and Ricinus Communis agglutinin (Gallagher, 1989). These attempts were unsuccessful, mainly because of insufficient amounts of saliva samples.

When the FPLC fractions of an RA patient which contained the anionic proteins of interest were analysed by SDS-PAGE in a 12.5 %T gel followed by immunobloting for kallikrein, the immunoreactive bands exhibited an M_r of 27,000. This value agrees very well with the M_r of purified salivary kallikrein, also determined by SDS-PAGE, estab-

lished by Hare and Verpoorte (1982). However, in the linear polyacrylamide gradient gels which were used in the second dimension of 2-D PAGE, the components which were immunoreactive with antiserum to HUK displayed an M_r of 32,000. This discrepancy in M_r values is possibly due to the anomalous behaviour of kallikrein under the conditions for SDS-PAGE. Being a glycoprotein, it binds SDS only at the protein part of the molecule (Hames, 1981). Although there is some evidence that a number of glycoproteins do not behave anomalously in gradient SDS-PAGE gels, because in such gels molecular sieving would predominate over the anomalous charge effect caused by the reduction in SDS-binding, sialic acid-containing glycoproteins may still behave quite differently under such conditions (Hames, 1981). Some of the carbohydrate moieties of HUK are sialated (Iketika et al., 1983b) and the sensitivity of the pI's of salivary kallikrein towards neuraminidase treatment strongly suggests that it is a highly sialated glycoprotein. Thus the determination of the M_r's of many glycoproteins by SDS-PAGE is very much an approximation. Fujimoto et al. (1973) have reported that the M_r of salivary kallikrein, determined by gel filtration, was 28,000. However, when ultrafiltration was used, a value of 23,500 was established. These values are probably closer to the actual M_r of salivary kallikrein than the value obtained by SDS-PAGE.

On the basis of the information obtained by immunoblotting, there is no evidence to suggest the occurrence of un-

usual glycosylation of kallikrein in the parotid saliva of RA and SS patients. Instead, in the RA and SS patients, there appears to be an elevation in the levels of all the different pI forms of salivary kallikrein. The chromogenic assay for tissue kallikrein, used in this study, has shown that the enzyme activity was higher in RA and SS patients than in the normal subject. A similar finding in SS patients has also been reported by Friberg et al. (1988).

Urinary kallikrein and the anionic proteins associated with connective tissue disorders were found to be sensitive to neuraminidase treatment, as was salivary kallikrein as demonstrated by immunoblotting, thus showing that the charge microheterogeneity in both is largely due to the differing contents of sialic acid in their carbohydrate moieties. Incubation of parotid saliva samples from an RA patient with Endo F or PNGase F followed by IEF in a pH 3.5-5.0 and silver staining revealed that the intensity of the anionic bands of interest was very substantially reduced by Endo F and PNGase F treatment. The process of deglycosylation, however, did not appear to be complete as some anionic bands, although less intensely stained, could still be visualised in the IPG tracks which had been loaded with the Endo F- and PNGase F-treated samples. However, components detectable using an immunoblot for tissue kallikrein were still present in this pH region, and their intensity did not appear to be changed.

Immunoblotting, using antiserum to HUK, of IPG's of pH

range pH 3.5-5.0 which had been loaded with HUK also showed no change in the band pattern after Endo F or PNGase F treatment. It is possible that Endo F and PNGase F treatment may have resulted in a shift of some of the immunoreactive bands to pI values above that of the pH range of the IPG used. However, immunoblotting of an IPG of broader pH range (i.e. pH 4-9) for kallikrein was unsuccessful; only non-specific binding of the antiserum to HUK, to some of the isozymes of α-amylase was observed.

Endoglycosidases such as Endo F and PNGase F may not be effective in the removal of large quantities of carbohydrates from glycoproteins as the intact molecules may not be accessible to the enzyme (Dunbar, 1987). Endo F will slowly hydrolyse high mannose oligosaccharides and complex biantennary oligosaccharides, but large amounts of the enzyme are required for complete deglycosylation (Tarentino et al., 1985). PNGase F effects the deglycosylation of some glycoproteins with complex or multiantennary oligosaccharides even in their native state. Glycoproteins with high mannose or hybrid carbohydrates require prior denaturation (e.g. by treatment in SDS) to make them susceptible to deglycosylation, possibly due to the limited accessibility of these types of oligosaccharides in the native glycoproteins (Mussar et al., 1989). In contrast, the pI's of the silver stained anionic bands and the bands which were immunoreactive to antiserum to kallikrein were readily altered by neuraminidase treatment, almost certainly because the ter-

minal sialic acid residues are more accessible to the enzyme than whole oligosaccharide moieties. The amounts of parotid saliva from RA and SS patients and commercial HUK available, however, were insufficient for denaturation in SDS to be carried out prior to the enzymic digestions.

Evidence that the anionic salivary proteins associated with connective tissue disorders may be tissue kallikrein includes the close correlation between the pI's and M_r's of the silver stained and immunoblotted components in 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension, and the results from neuraminidase degradation which showed that both HUK and the components in saliva which cross-reacted with antiserum to HUK are sialated.

Enzymic degradations in Endo F or PNGase F have shown that the anionic proteins associated with RA and SS are susceptible to degradation by these enzymes but HUK and salivary kallikrein do not appear to be as susceptible to such treatment. This result could be interpreted in two ways; the first is that the deglycosylation effected by these enzymes was not complete, as is often the case when attempting to deglycosylate N-linked oligosaccharides from native glycoproteins (Dunbar, 1987; Tarentino et al., 1985; Mussar et al., 1989). In addition, neither Endo F nor PNGase F will have any effect in the removal of O-linked oligosaccharides from glycoproteins. The structures of carbohydrate side-chains of salivary kallikrein remain to be elucidated, but Kellermann et al. (1988) have identified 6 carbohydrate

side chains on the HUK molecule; 3 are N-glycosidically linked and the remaining 3 are O-glycosidically linked. The second possible interpretation is that salivary kallikrein and the anionic proteins associated with connective tissue disorders are two separate groups of proteins which focus close to one another in IPG's and are co-electrophoresed in SDS-PAGE gels. This may explain the slight discrepancy between the M_r's of the silver stained (M_r's between 27,000 and 30,000) and immunoblotted components (M_r approximately 27,000) in the SDS-PAGE gels which had been loaded with the FPLC fractions of parotid saliva from an RA patient.

Although the IPG's of pH range 3.5-5.0 provided optimum resolution of the anionic proteins associated with connective tissue disorders, it was not suitable for comparing the IEF band patterns of native and glycosidase-treated HUK, salivary kallikrein or the anionic proteins associated with connective tissue disorders because after deglycosylation the pI's of most of the products appeared to have been shifted to values close to, or even beyond, the upper limit of the pH range. The formulae provided for IPG's of broader pH ranges, e.g. pH 4-7 or pH 4-9, do not, however cover the pH region of interest. When they finally become available, Immobilines of pK's 0.8, 3.1 and 10.3 will allow the preparation of IPG's with ranges spanning the pH extremes (Altland, 1990), thus allowing the preparation of IPG's of, for example, pH 2.5-10.5. An IPG of such a range would make it possible to compare the IEF band patterns of native and

enzymically-digested HUK, salivary kallikrein and the anionic proteins associated with connective tissue disorders on the same gel.

The effect of drugs on the levels of the anionic proteins is difficult to assess as a wide variety of drugs is used in the treatment of RA and SS. Of the 13 primary SS patients who had high levels of the anionic bands, 9 were receiving topical treatment for dry eyes and mouth (such as tear and saliva substitutes) and drugs used in the treatment of xerostomia such as diuretics, antihistamines and antidepressives (Petersen, 1986). The majority of patients with xerostomia were also receiving these drugs, but they did not exhibit elevated levels of the anionic proteins. It is therefore unlikely that the presence of these proteins could have been induced by drugs. The remaining 4 of the primary SS patients who had high levels of the anionic bands were treated with the seed oil Efamol, which was obtained from a cultivated strain of evening primrose (Manthorpe and Prause, 1986). The mode of action of Efamol is unknown, although it may have a weak immunomodulatory effect on prostaglandin biosynthesis. Its effect on the composition of saliva has not been investigated.

Drugs which are used in the treatment of SS and which are known to have an effect on the composition of saliva include bromhexine, which reduces the levels of sodium ions, sIgA and IgG (Nahir et al., 1979) and corticosteroids (prescribed in only very severe cases) which reduce the

level of kallikrein (Friberg et al., 1988). However, none of the SS patients involved in this study were receiving either of these drugs.

The 17 secondary SS patients and 25 RA patients who had high levels of the anionic bands were receiving treatment with disease modifying anti-rheumatic drugs (e.g. salazopyrine, oral gold and penicillamine), immunosuppressive drugs (e.g. flurbiprofen, ibuprofen, sodium salicylate, cyclophosamide and methotrexate and steroids (e.g. prednisone and prenisolone) (Olsen et al., 1988; Arnold et al., 1988; Abramson and Weissman, 1989; Cush et al., 1990). Of these, only steroids have been shown to have an effect on the composition of saliva. An example is corticosteroids which reduces the level of salivary kallikrein (Friberg et al., 1988). Both testosterone and the sodium-retaining steroid fluodrocortisone, which are not used in the treatment of RA or SS, increase the level of salivary kallikrein (Van Leeuwen et al., 1984; Horwitz et al., 1982).

Two of the 5 patients with sialadenitis, but without SS, whose parotid saliva samples were investigated in this study were receiving the immunosupressive drug flurbiprofen, another 2 were receiving steroid treatment and the remaining one not receiving any drugs. None of these patients, however, was found to exhibit high levels of the anionic proteins in their parotid saliva.

Thus, it is extremely unlikely that the levels of the anionic proteins associated with RA and SS could have been

elevated as a result of drug treatment. Some of the evidence in this study indicates that these proteins may be kallikrein. The levels of salivary kallikrein has been shown to be reduced by corticosteroid treatment (Friberg et al., 1988). Anti-inflammatory drugs such as corticosteroids act by inhibiting the production of prostaglandins (Friberg et al., 1988), and prostaglandin-dependent mechanisms are involved in controlling the release of kallikrein (Vio et al., 1983). As the mode of action of disease-modifying anti-rheumatic drugs also involves the inhibition of prostaglandin biosynthesis (Cush et al., 1990) it is highly possible that these drugs may have a similar effect in lowering the levels of salivary kallikrein. No studies, however, have been made on the effect of disease-modifying anti-rheumatic drugs on salivary composition. The levels of salivary kallikrein in the SS patients, even after receiving corticosteroids, were higher than in the normal subjects (Friberg et al., 1988).

All the RA and SS patients whose parotid saliva was rheumatoid factor-positive were found, by IEF in pH 3.5-5.0 IPG's, to have intensely-stained anionic bands. As the role of rheumatoid factors in the immunopathogenesis of RA and SS still remains obscure (Müller et al., 1989), the significance of this is unknown. Rheumatoid factor has been demonstrated to be present in the saliva of SS patients, but has not been connected to any measurement of the severity of the disease (Atkinson et al., 1989**a**). However, the levels of rheumatoid factor may reflect the activation of

B-lymphocytes in the diseased exocrine glands (Müller et al., 1989).

4. General Discussion and Conclusions

Human saliva contains a number of proteins, some of which are of value for diagnostic purposes. At present the application of sialochemistry, in particular, the fractionation of human salivary proteins by electrophoretic methods, remains limited. One reason for this is the lack of familiarity of clinicians towards such techniques. Another is that many of the electrophoretic techniques currently used for fractionating human salivary proteins require considerable preparation of the samples prior to fractionation. Preparation of saliva samples include the use of techniques such as centrifugation, lyophilisation, dialysis and denaturation (e.g. by treatment in SDS or urea). Such procedures require relatively large volumes of saliva, which may be difficult to obtain from patients. In addition, they may result in the loss of some proteins, e.g. proteins of low M_r may be lost by passage through dialysis membranes.

In this study, some of the latest electrophoretic techniques which have resulted in high resolution of the separated components, including SDS-PAGE, IEF in IPG's and 2-D PAGE using IPG's in the first dimension, have been utilised in the study of human salivary proteins. They were used in conjuction with protein detection methods of high sensitivity such as protein blotting and silver staining. These methods produced high resolution of the protein components of human saliva. The IPG technique has allowed salivary samples to be fractionated with little prior

preparation except for centrifugation, thus allowing relatively large numbers of samples to be fractionated. 2-D PAGE may benefit from this if IPG's are used in the first dimension. All these developments have advanced the techniques for the analysis of human salivary proteins.

Although one-dimensional separation techniques such as SDS-PAGE or IEF may not successfully separate proteins of very close M_r or pI values. 2-D PAGE overcomes this problem. These techniques, together with immuno- and lectin-affinity blotting, were used to characterise the proteins in human parotid saliva. Most of the major components in SDS-PAGE and 2-D PAGE gels have been identified in this study.

IEF in hybrid carrier ampholyte-IPG polyacrylamide gels has made it possible to analyse the proteins in human saliva without prior desalting of the samples, thus allowing the proteins in small volumes of saliva to be analysed. IPG's were also used for the first-dimensional fractionation of salivary proteins in 2-D PAGE. In order to visualise or characterise the components in IEF and 2-D gels, sensitive protein detection techniques such as silver staining and immunoblotting were used. Such detection procedures often did not require concentration of the salivary samples prior to electrophoretic separation, and as such are useful for the analysis of salivary proteins from patients with disorders which affect the functions of the salivary glands. After these techniques had been used in a general study of human salivary proteins, they were applied specifically to the

investigation of the nature of the anionic salivary proteins associated with connective tissue disorders such as RA and SS.

Although the literature on sialochemistry in SS, and to a lesser extent in RA, is vast and still growing, sialochemistry has yet to achieve a significant role in the diagnosis and monitoring of patient progress in these disorders (Thorn et al., 1989). Reasons for this include the unfamiliarity on the part of clinicians towards the techniques used in the chemical analysis of saliva, the lack of specificity or sensitivity of certain tests, and the lack of studies which have correlated the sialochemical data with the severity of the disorder. One of the few studies which have correlated sialochemical with clinical data is that of Michalski et al. (1975) who found a direct relationship between the concentration of salivary β_2-microglobulin and the degree of local lymphocytic infiltration of the labial salivary glands in SS patients.

In view of its non-invasiveness, the possibility of using sialochemical data in the diagnosis and monitoring of the disease process in RA and SS remains an attractive one, especially when used in conjunction with one or more of the other clinical tests. Unlike biopsy and scintigraphy which are very painful to the patient, saliva samples are in most cases easily collected. However, a recurrent problem encountered when collecting saliva from SS patients is that because a large proportion of them suffer from xerostomia, it

is difficult to collect large volumes of saliva (such as > 2ml). However, the electrophoretic and protein detection methods developed in the course of this study allowed the proteins in very small volumes (often < 50μl) of saliva to be analysed.

The results obtained by using IEF in IPG's and 2-D PAGE with IPG's in the first dimension followed by immunoblotting, indicate that there is some evidence that the identity of the anionic salivary proteins associated with RA and SS is tissue kallikrein. Future research could be carried out in order to elucidate the amino acid sequence of these anionic proteins and compare them with the sequence of urinary kallikrein and other sequences on databases. If sufficient volumes of saliva samples are available, salivary kallikrein might also be isolated, for example, following the method of Fujimoto et al. (1973), its amino acid sequence obtained, and a more detailed comparison made. Alternatively, adsorption of parotid saliva with antiserum to HUK, followed by IEF in an IPG of pH range 3.5-5.0 followed by immunoblotting to confirm that kallikrein removal is complete, and silver staining of the gel to ascertain whether or not this results in a decrease in the levels of the anionic salivary proteins associated with RA and SS could resolve this problem.

The diagnostic significance of kallikrein in SS is still uncertain. The function of tissue kallikrein, especially in saliva and the salivary glands, has not yet been

elucidated. However, as tissue kallikrein has been localised mainly in the striated duct cells of the salivary glands, the levels of this enzyme in saliva may serve as a marker of striated cell function in SS, and may indicate the extent of damage to these cells (Thorn et al., 1989). However, using a chromogenic substrate to measure the kallikrein activity in the whole saliva of SS patients, Friberg et al. (1988) failed to find any relationship between the extent of inflammation in the labial glands and kallikrein activity.

As immunoblotting of 2-D PAGE gels with pH 3.5-5.0 IPG's in the first dimension followed by amplification of the alkaline phosphatase signal using APAAP complexes has shown that the pI's of the components which were immunoreactive towards antisera to tissue kallikrein were the same both in the RA and SS patients and in normal subjects, it is unlikely that kallikrein in the saliva of these patients is more extensively sialated as postulated by Greaves et al. (1989). However, in view of the number of reports of unusual glycosylation in some plasma proteins in the course of the inflammatory process, the carbohydrate moieties of salivary kallikrein in RA and SS deserve further investigation. The plasma proteins, the levels of which are increased, usually two- to a hundred-fold, during inflammation are often referred to as "acute-phase proteins" (Putnam, 1982). They are unrelated in their amino acid sequences, have different biological functions, and play an important role in the innate immunity to infection. Many acute-phase proteins have

high carbohydrate contents.

An example is α_1-acid glycoprotein, which is synthesized by liver parenchyma cells (Nicollet et al, 1982). Like many other acute-phase proteins, α_1-acid glycoprotein has a carbohydrate content of 45% of the total weight of the protein (Arnaud and Gianazza, 1982). A large degree of its charge microheterogeneity is due to uneven sialation. Some degree of heterogeneity is present after desialation, and the asialo-variants could represent different gene products.

Alterations in the microheterogeneity of α_1-acid glycoprotein have been noted in the course of disease processes. For example, Mackiewicz et al. (1986) found an increase in Concavanalin A non-reactive variants in RA patients.

Another example of an acute-phase protein is α_1-antichymotrypsinogen, which is a serum protein (M_r approximately 68,000) with a carbohydrate content of 26%. In advanced cancer and RA its level remains high for many months (Bowen et al., 1982). Its carbohydrate portion is microheterogeneous, and in the event of acute diseases and there is an increase in Concavanalin A-binding variants.

Thus in view of these examples, the carbohydrate moieties of the anionic salivary proteins associated with RA and SS merit further studies. Methods that could be used for this purpose include lectin-affinity electrophoresis (Faye and Salier, 1989) and HPLC (Hounsell, 1986). Therefore future work could consist of ascertaining whether or not the

identity of the anionic salivary proteins associated with connective tissue disorders is kallikrein, and whether salivary kallikrein in RA and SS patients is abnormally glycosylated as compared with normal healthy individuals.

IEF of parotid saliva from RA and SS patients in IPG's of pH range 3.5-5.0 followed by silver stain to detect the presence of the anionic proteins of interest has shown good correlation between elevated levels of these bands and the diagnosis of the disorders; therefore, although further investigations are needed in order to establish the identity of the anionic proteins, the use of IEF to analyse saliva samples from connective tissue disorder patients is still potentially useful for diagnostic purposes.

In this study, using SDS-PAGE in gradient gels (the second dimension of 2-D PAGE), it was established that the M_r of urinary kallikrein is approximately 40,000 while that of the component in saliva which cross-reacted with antiserum to tissue kallkrein is approximately 32,000. Kallikreins from different tissues are closely related or even identical in their polypeptides (Kellermann et al., 1988). Hare and Verpoorte (1982) have established, using homogeneous SDS-PAGE gels, that the M_r of human salivary kallikrein is 27,000. Thus on the basis of M_r values it is almost certain that salivary kallikrein is less glycosylated than its urinary counterpart.

The main difficulty encountered when attempting to purify salivary kallikrein is its low levels in human

saliva. For example, Fujimoto et al. (1973) succeeded in isolating only 1.5mg of the enzyme from 4 litres of mixed human saliva. Although the level of kallikrein is elevated in the saliva of RA and SS patients, their low salivary flow-rates, especially in the SS patients, make the collection of reasonably large volumes very difficult or even impossible.

Urinary kallikrein occurs in both inactive and active forms. The inactive form has an additional 7 amino acid residues at the amino terminus of active kallikrein (Takahashi et al., 1986). Using a radioimmunoassay (RIA) technique, Oza et al. (1981) discovered that between half to two-thirds of the kallikrein in urine occurs as the inactive form. The proportion of active to inactive forms of salivary kallikrein has not yet been ascertained. As the salivary kallikrein activity, measured by chromogenic assays, in SS patients is known to be elevated (Friberg et al., 1988) there is obviously an increase in the active form of kallikrein in SS patients. However, if there is also an increase in the level of the inactive form of salivary kallikrein, chromogenic assay methods cannot give any indication of this. Therefore it is possible that IEF in pH 3.5-5.0 IPG's followed by immunoblotting for kallikrein could provide a more sensitive indication of the of the levels of salivary kallikrein, both active and inactive. An alternative method is the use of RIA to measure the kallikrein levels in human saliva.

During the course of this study, attempts were made to visualise the active form of both urinary and salivary kallikrein following IEF in non-denaturing pH 3.5-5.0 IPG's by overlaying the focused gels with cellulose acetate membranes which had been impregnated with the fluorogenic substrate for kallikrein, D-Val-Leu-Arg-AFC (AFC = 7-amino-4-trifluoromethylcoumarin) (Smith, 1984). These attempts were unsuccessful, probably due to the low amounts of kallikrein present in parotid saliva, to the lack of sensitivity of the method used, or to the presence of a substantial amount of the enzyme in its inactive form.

Much of the protein analyses which have been carried out on human body fluids, including cerebrospinal fluid, urine, serum and saliva are still purely descriptive, and the functions and origins of many proteins have yet to established (Büeler et al., 1989). Monitoring the levels of, or the analysis of changes in glycosylation, of some of these proteins, such as the acute phase proteins in serum, is at present of significance in the diagnosis of a number of diseases. This study has concentrated in developing and refining electrophoretic techniques for the analysis of human salivary proteins, and has investigated and partially characterised the anionic salivary proteins associated with connective tissue disorders as an example of how electrophoretic techniques may be of value in the diagnosis of these disorders.

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