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Review

Clinical applications of electrophoresis of human salivary proteins

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ABSTRACT

Human salivary proteins have been studied by electrophoresis in denaturing and non-denaturing polyacrylamide gel electrophoresis (PAGE) as well as by isoelectric focusing (IEF) and two-dimensional procedures, and the clinical applications of this have been reviewed. Whilst non-denaturing PAGE is useful in studying polymorphisms, sodium dodecylsulphate PAGE appears to be otherwise preferable. Immobilized pH gradients containing carrier ampholytes (CAs) give better resolution than CA-based IEF and overcome the problems of cathode drift and loss of basic material. Proline-rich proteins stain poorly with conventional procedures and special techniques are necessary. In clinical studies, findings must be viewed over and above the large number of polymorphisms which occur normally. Studies relating salivary protein and peptide profiles to dental caries susceptibility are encouraging. Specific protein abnormalities have been associated with connective tissue disorders and could form the basis of new non-invasive diagnostic procedures. Protein differences associated with cystic fibrosis and diabetes mellitus, however, merit reinvestigation with the new procedures now available. Detection of HIV antigens in saliva is a new area of research. In the light of new techniques available and new information which has arisen from DNA studies, future prospects for the clinical applications of electrophoresis of saliva look good.

CONTENTS

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LIST OF ABBREVIATIONS

CA	Carrier ampholyte
2D	Two-dimensional
EGF	Epidermal growth factor
GACELISA	IgG antibody capture enzyme-linked immunosorbent assay
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
NGF	Nerve growth factor
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecylsulphate

1. INTRODUCTION

Human saliva is secreted by three pairs of major glands (parotid, submandibular and sublingual) and numerous minor ones (labial, buccal, palatine and glossopalatine). Whilst the parotid glands are the major contributors to stimulated saliva, in the resting state much of the fluid present in the mouth is the viscous submandibular/sublingual secretion, with the minor glands keeping the tissues moist. Mixed or whole saliva (oral fluid) consists of the secretions of the major and minor glands, together with gingival exudate (crevicular fluid) micro-organisms and cell debris.

The major proteins in human saliva are summarised in Table 1; in duct saliva these consist of ~25% α-amylase and ~65% proline-rich proteins, with the remaining ones comprising ~10%. The protein concentration normally ranges from ~0.5 to 3.0 mg/ml and the solution is hypotonic as compared to serum, with the ionic composition depending on the degree of stimulation. Most of the proteins are synthesised within the salivary gland and are salivary gland-specific (*e.g.* α-amylase, proline-rich proteins, histatins), but some, such as albumin, are derived from serum [1,2].

Saliva is unusual in containing many proteins and polypeptides which are salivary-specific and rich in certain amino acids. The major group of these is a unique multigene family of proline-rich phosphoproteins of unusual amino acid composition and because of which they stain poorly with conventional electro-

TABLE 1

HUMAN SALIVARY PROTEINS OF GLANDULAR ORIGIN

Proline-rich proteins
Enzymes (α-amylase, kallikrein, lysozyme, lacteroperoxidase, etc.)
Lactoferrin
Histatins
Cystatins
Statherin
Immunoglobulins (IgA, IgG, IgM, etc.)
Secretory component
Albumin
Blood group substances
Mucins
Sulphated glycoproteins
Transcalciferin
Polypeptide hormones (EGF, NGF, etc.)
etc.

phoretic staining procedures. They are divided into three groups, acidic, basic and glycosylated, and their genes have been cloned [3]. They are a highly complex polymorphic set of proteins and include six acidic ones [4], nine non-glycosylated basic ones [5,6] and a major basic glycosylated one [7]. Their functions are still uncertain but are thought to include calcium binding, inhibition of hydroxyapatite precipitation, binding of dietary tannins and binding of micro-organisms to enamel surfaces with which they are coated.

Another group includes twelve related histidine-rich polypeptides known as "histatins" [8,9] which have antifungal properties [10] and may constitute a non-immune defence system. A related polypeptide derived from a common ancestral sequence is statherin [11], a tyrosine-rich acidic peptide which also inhibits calcium phosphate precipitation from saliva and may be important in limiting dental caries [12]. A further family of unusual proteins is the cystatins which are thought to have an antiviral function [13].

Other antimicrobial proteins in saliva include the immunoglobulin sIgA, lysozyme, lactoperoxidase and lactoferrin; α-amylase may also have an antibacterial role [14], and some mucins cause bacterial aggregation [7].

Salivary secretion is under the control of the autonomic nervous system and flow can be stimulated by sialogogues such as citric acid or by mechanical action such as chewing. Parasympathetic impulses evoke most of the fluid secreted and cause varying degrees of exocytosis whilst the sympathetic system modulates salivary composition but causes little mobilisation of fluid [15].

The main function of saliva is the maintenance of the health of the oral tissues, with xerostomia giving rise to rampant dental caries, increased infections, glossitis, epithelial dryness and other soft tissue disorders. But some components have

roles elsewhere in the body, for example epidermal growth factor (EGF) may be important in maintaining the integrity of the gastric mucosa, and proline-rich proteins may have a protective effect against the anti-digestive and potentially carcinogenic affect of dietary tannins [16]. Whilst a range of pathological disorders have oral manifestations [17], only few are currently known to modulate salivary protein composition. These include connective tissue disorders, cystic fibrosis and diabetes mellitus. In addition, antibodies to disease-specific antigens (*e.g.* HIV) may also be present. However, progress has been restricted until very recently by the limited methodology available for electrophoretic analysis of saliva. Introduction of the use of immobilised pH gradients (IPGs) for isoelectric focusing (IEF), immunoblotting and modifed staining procedures have resulted in substantial improvements in the resolution of salivary proteins which should lead to considerable developments in the future.

2. ELECTROPHORETIC ANALYSIS OF SALIVA

Whilst attempts have been made to study the proteins in saliva by electrophoretic procedures for many years, only fairly recently has useful information begun to be obtained. This is partially attributable to the low protein and high salt content of the saliva with the consequent need for sample preparation by dialysis and lyophilisation, and partially to the fact that the major group of proteins present, the proline-rich proteins, was difficult to detect by the staining procedures used and was therefore largely unnoticed. Recent developments such as new and modified staining procedures, together with techniques giving improved resolution, have opened up a new era in salivary protein electrophoresis.

2.1. Electrophoresis in denaturing and non-denaturing gels

The introduction of disc electrophoresis in non-denaturing conditions in polyacrylamide gels enabled considerable strides to be made in the study of salivary proteins and up to twenty or more components were resolved. In order to obtain optimum information about the full range of proteins present, however, both anionic and cationic gels were necessary [18–21]; staining was with Coomassie Blue. However, whilst the technique offers the advantage of separating species of similar molecular mass but differing charge, no procedure provides information about the complete spectrum of proteins present, and the pHs used frequently result in some loss of material by denaturation. Accordingly, with the exception of studies on polymorphic species (Section 3.1.), the procedure has largely been superceded by electrophoresis in denaturing conditions, in particular sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) [22].

SDS-PAGE has been widely used in the study of salivary proteins in the last decade. The technique has been used to study whole saliva [23–26] but rather variable results were obtained, presumably on account of the heterogeneous na-

ture of the fluid studied, the variable contributions of different gland secretions and the effects of bacteria. SDS-PAGE of parotid saliva has revealed more consistent results and has indicated individual-to-individual variations [27,28] as well as showing differences between parotid and palatine secretions [29,30]. When stained with Coomassie Blue R-250, whilst α-amylase, histatins, etc. stain blue, in the case of proline-rich proteins a metachromatic effect occurs and pink–violet bands are formed. This was first reported in 1978 [31] and the proteins involved were subsequently shown to be the proline-rich ones [32,33]. The effect was found to be specific for Coomassie Blue R-250, with proline-rich proteins staining poorly, if at all, with G-250; for metachromasia to occur, the destaining solution must be free from organic solvents [29]. A similar effect is observed with collagens [34]. Accordingly, proline-rich proteins can now easily be distinguished from other proteins on SDS-PAGE gels.

Silver staining procedures have led to substantial improvements in the sensitivity of detection of proteins [35] and glycoproteins are stained a characteristic yellow by this technique [36]. Proline-rich proteins, however, especially the acidic ones, are difficult to stain by this procedure [37] probably because they are poor in the basic and sulphur-containing amino acids thought to be responsible for the binding of silver ions [38,39]. However, the problem can be overcome by use of a modification of a more recent procedure [40] which results in the proteins which

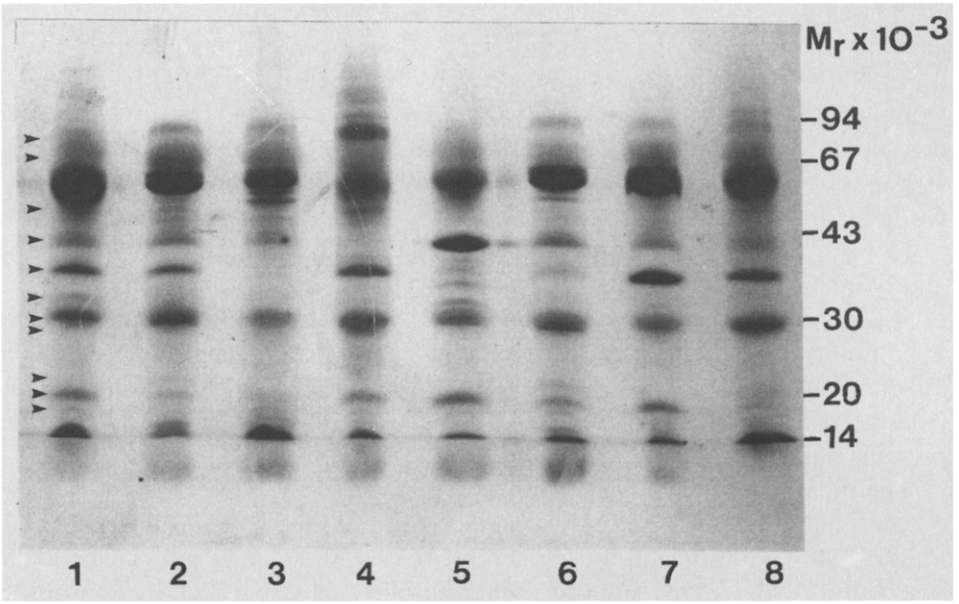

Fig. 1. SDS-PAGE of human parotid saliva from eight different individuals (1–8) on a 12.5% gel. The protein load was 75 μg and staining was with Coomassie Blue R-250. The bands indicated by arrows stained pink–violet and are proline-rich proteins; all other components stained blue.

stain pink–violet with Coomassie Blue R-250 staining yellow-brown while those which stained blue being stained brown–black [41]. Interestingly contaminant bands reported on silver-stained SDS-PAGE gels of a variety of samples have been attributed to contamination by components of skin, serum and saliva [42].

Optimum results for SDS-PAGE of parotid saliva are obtained using 12.5% gels followed by Coomassie Blue R-250 staining (Fig. 1). Improved resolution of the lower-molecular-mass components can be achieved by means of a 5–20% linear gradient gel [43]. Two major blue staining components (corresponding to α-amylase), ten or more pink–violet staining proline-rich proteins and some low-molecular-mass and minor blue staining components are evident; most of these components have been characterised [41]. Additional minor components are revealed on silver staining. There is substantial individual-to-individual variation but the patterns produced are characteristic for each individual and appear to be independent of time of collection, flow-rate, etc. However, it should be noted that because of the unusual amino acid composition of many salivary proteins, their migration rates in SDS-PAGE are atypical and the procedure cannot be used to determine their molecular masses.

2.2. Isoelectric focusing

Early attempts at IEF of salivary proteins in carrier ampholytes (CAs) involved the use of polyacrylamide gel rods and Lissamine green staining [44]; up to 20–30 well defined bands could be observed with parotid saliva and the technique gave better resolution than hitherto achieved with other electrophoretic procedures. Subsequently the technique was improved by the use of flat-bed gels and Coomassie Blue G-250 staining [45] and a procedure was developed which gave considerably greater resolution and sensitivity than previously achieved [46]; 30–40 bands were observed with whole saliva but the patterns produced from different individuals were very similar.

However, because of the low protein and high salt content of saliva, desalting and lyophilisation were necessary prior to analysis. This is time-consuming and necessitates large sample volumes ($\sim$1–2 ml) which are frequently not available in pathological disorders associated with impaired salivary gland function. These difficulties were partially overcome by the use of silver staining for whole [47] and palatine saliva [48], with immunoblotting being used for the further study of the proteins separated [49].

The most recent development, however, involves the separation of salivary proteins in hybrid CA-IPG polyacrylamide gels followed by either silver or Coomassie Blue G-250 (which was preferable to R-250) staining [50]. (A colloidal suspention of the G-250 stain in trichloroacetic acid is particularly convenient because destaining can usually be achieved in an hour [51].) Use of IPGs overcomes the problems of cathode drift and distortion by electrolytes which are associated with CA-IEF. They also enable gradients to be tailor-made to facil-

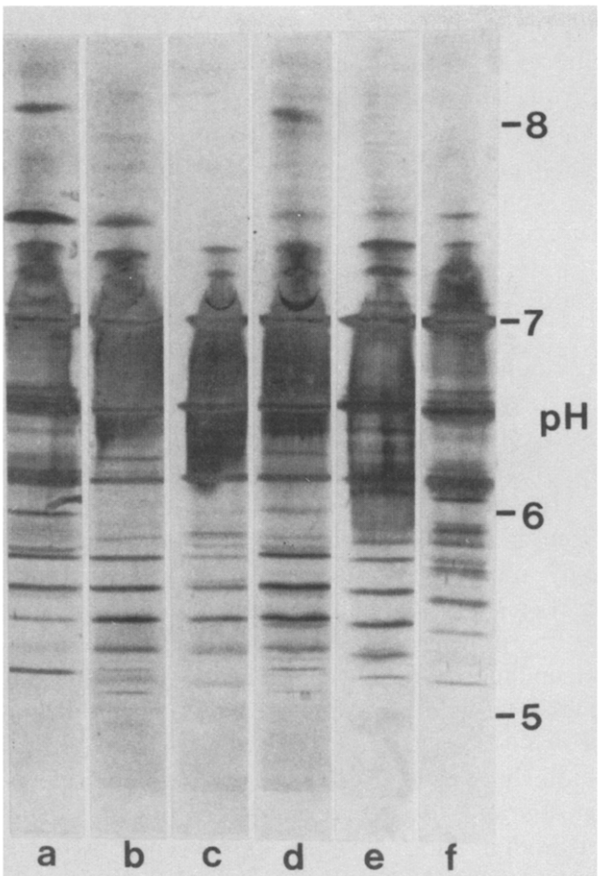

Fig. 2. IEF of parotid saliva from six different individuals (a–f) on a pH 4–9 IPG. The protein load was 25 μg per sample and the gel was silver-stained [50].

itate optimum resolution of the proteins of interest. Using this system, it is also possible to study proteins of p$I > 8$, including the basic proline-rich proteins; with CA gradients this is not possible because they are lost from the end of the gel. However, use of IPGs sometimes gives rise to streaking and sample precipitation at the application site, but addition of carrier ampholytes to the IPGs to form a hybrid CA-IPG system [52,53] overcomes this difficulty. Formation of droplets of fluid on the gel surface can be prevented by inclusion of 20% glycerol in the rehydration solution [54] and band resolution improved still further by the addition of 8 *M* urea. Fig. 2 shows the results obtained on analysis of parotid saliva from six different individuals in the CA-IPG system. Resolution was considerably better than with CA systems alone and showed few major but several minor individual-to-individual variations. Whilst these differences were somewhat

greater than with CA-IEF [46], the substantial individual-to-individual differences in proline-rich protein patterns as revealed by SDS-PAGE (Fig. 1) were not evident. This probably results from failure of the proline-rich proteins to stain with either the silver or Coomassie Blue (G-250 or R-250) procedures in the presence of CAs and/or IPGs, because two-dimensional (2D) analysis with IPGs in the first dimension (Section 2.3) confirms that proline-rich proteins of pI <4 to pI >8 are well separated in the CA-IPG system.

2.3. Two-dimensional electrophoretic analysis

2D Electrophoresis of human saliva has been reported by several authors. Whole saliva was first studied by a system involving CA-IEF in the first dimension and 10–20% gradient SDS-PAGE in the second. Staining was with Coomassie Blue R-250. Only α-amylase, immunoglobulins, albumin and a few minor components were detected [55]. However, if an organic solvent had been omitted from the destain procedure (Section 2.1) then proline-rich proteins might also have been observed. Subsequently, silver staining has been used for the detection of proteins in whole saliva after 2D analysis in either a modified O'Farrell system [56] which involved IEF in the first dimension and either SDS-PAGE or non-denaturing PAGE in the second, and more than 250 components were found [57]. However, because of the complexity of whole saliva, these would have predominantly been either bacterial proteins or bacterial degradation products of duct saliva, and the stain used was unlikely to have revealed the proline-rich proteins.

Using IEF in the first dimension and non-denaturing PAGE in the second, followed by silver staining and immunoblotting, maps have been constructed showing the location of proteins in submandibular/sublingual [58] and parotid [59] salivas on 2D electrophoretic analysis. The main components observed were α-amylase, albumin and immunoglobulins. Proline-rich proteins were not stained by this procedure either and the pI values reported for the immunoglobulins are higher than those observed elswhere [60]. In addition to the staining limitations of all of these methods, any 2D system based on CA-IEF in the first dimension will lead to loss of basic components including proline-rich proteins.

A 2D electrophoretic system for the analysis of salivary proteins involving IPGs in the first dimension and 3–15% linear gradient SDS-PAGE in the second followed by silver staining (Section 2.1) has now been developed. By means of a combination of staining for proline-rich proteins and immunoblotting, all of the major antigens in parotid saliva have been identified [61]. Excellent resolution was obtained and typical results are shown in Fig. 3. Proline-rich proteins of pI ~ 4 to >8 (*i.e.* both basic and acidic) ar well resolved, together with α-amylase and the other major proteins present. Because of genetic polymorphisms, the patterns produced vary from individual to individual but usually some 70–80 or more components are observed.

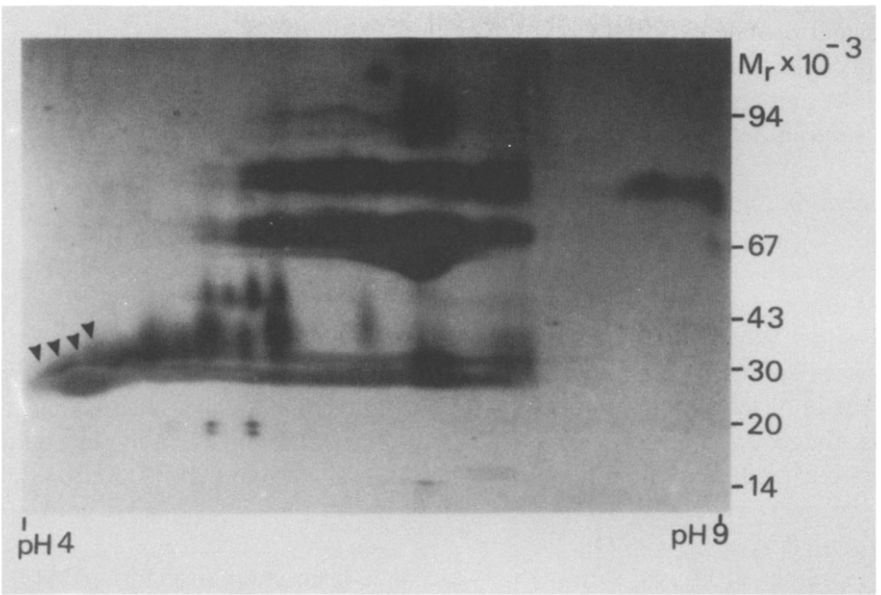

Fig. 3. 2D analysis of parotid saliva from a rheumatoid arthritis patient using a pH 4–9 IPG in the first dimension and a 3–15% linear gradient SDS-PAGE in the second. The protein load was 100 μg and the gel was silver-stained. The components indicated by arrows stain black and are characteristic of rheumatoid arthritis and secondary Sjögren's syndrome.

2.4. Other methods of electrophoretic analysis

Whilst analytical electrophoresis of saliva in small-bore tubes seems attractive as an alternative procedure for salivary analysis in the clinical laboratory because of the small sample volumes required and the rapid separation produced, the complexity of the patterns produced on isotachophoresis and the problems of identification of the components present have limited further development [62]. However, with the recent upsurge of interest in capillary electrophoretic procedures and the development of instrumentation [63], this technology may offer considerable potential for salivary analysis in the future.

3. CLINICAL APPLICATIONS

Electrophoretic analysis of saliva as an aid to clinical diagnosis is attractive as it offers a simple rapid non-invasive method for the short- and long-term monitoring of pathological disorders and drug therapy, as well as being a research tool. Unfortunately, until recently, progress has been limited by the methodology available. Furthermore, the fluid collected must be clearly defined, and because of circadian variations in salivary composition and flow-rate, the time of collection must be specified, as well as the type of stimulation used.

A further problem arises on account of the complexity of the polymorphisms which occur in salivary proteins and the problem of interpreting clinical variations over and above these. Nevertheless, provided one is aware of these limitations, useful information can be still be obtained.

3.1. Polymorphisms

Although salivary polymorphisms may superficially appear to restrict the clinical usefulness of electrophoresis of saliva, they have also been shown to be of clinical value. Whilst a detailed account of these polymorphisms has been published elsewhere [64], a general understanding of their nature is an essential base to current knowledge and future research into the clinical relevance of salivary analysis. Indeed, as well as being of diagnostic use, different phenotypes could well be associated with differences in susceptibility to oral and systemic disease.

3.1.1. α-Amylase

The isozymes of human salivary amylase have been characterised by PAGE [65] and IEF [66–68]. Whilst the phenotypic variants and their frequencies depend on the populations studied, analysis of familial data is indicative of the inheritance of autosomal co-dominant alleles. Because the products of the salivary amylase (Amy1) gene (S-type) are distinct from those of the pancreatic (Amy2) gene (P-type), electrophoretic separation of amylase isozymes in serum is used to assess salivary and pancreatic function in disorders affecting these glands such as mumps [69], pancreatitis [70–72], cystic fibrosis [73,74], diabetes mellitus [75–77] and hyperamylasaemia [78,79]; an automated routine procedure has now been developed [80]. Whilst differential assay of serum amylase activity involving inhibition of salivary amylase either by an inhibitor of wheat origin [81] or monoclonal antibody [79] gives similar results to electrophoresis and densitometric scanning in some conditions, it is less efficient in the investigation of cystic fibrosis [74] and fails to detect macroamylasaemia; accordingly, electrophoretic procedures provide the best method of analysis of pancreatic and salivary amylase isozymes [71,72,79].

3.1.2. Proline-rich proteins

PAGE and IEF have revealed the complexity of the products of the salivary gland genes coding for proline-rich proteins. Whilst early studies on these polymorphisms suggested that some thirteen linked loci coded for them, recent studies at DNA level have shown that there are only six, with two PRH genes coding for the acidic proline-rich proteins and four PRB genes for the basic and glycolysated ones. These are arranged as a cluster on chromosome 12 at band p13.2 in the order 5′-PRB2-PRB1-PRB4-PRH2-PRB3-PRH1-3′ spanning a distance of ~700 kbp (kilo base pairs). The three acidic polymorphisms Db, Pa and PIF are coded for by three alleles at the locus PRH1, and the two acidic ones PRH1/PRH2 and

Pr at the second locus, PRH2. The basic polymorphisms PmF, PmS and Pe are encoded by PRB1, Ps by PRB2 and Po by PRB4, whilst the major glycosylated proline-rich protein G1 is assigned to PRB3 and CON1/CON2 to PRB4 [82–86]. Differential RNA splicing and post-translational modification generate multiple products from a single gene. The complexity of the situation continues to increase with new polymorphisms continuing to be reported; these include acidic products As, At and Au of PRH1 [87,88], basic variants Pc and Pmo1/2 and new variants of Ps [89–91]. In addition, the occurrence and frequency of various polymorphisms may depend on the ethnic origin of the population studied. Accordingly, research into the clinical use of electrophoretic analysis of proline-rich proteins must be assessed in the light of the genetic situation which pertains. Despite this, their electrophoretic analysis has been reported as a procedure for paternity testing [92].

3.1.3. Histatins and cystatins

Histatins represent yet another complex family of salivary-specific proteins, the genes for which are located on chromosome 4 at band q13; three histatin mRNAs are expressed [8,9,93]. Cystatins too appear to be a genetically related complex family of proteins [94].

3.2. Dental caries

Dental caries is perhaps the most widespread disease in the human population, and although dietary fermentable carbohydrate is the major causative factor, there is considerable individual-to-individual variation in susceptibility to the disorder. Salivary protein polymorphisms could be responsible for these differences. Any procedure which would identify high-risk individuals would be of considerable clinical importance.

Initial studies using PAGE indicated that there were higher levels of anodal proteins, predominantly amylase, and lower levels of cathodal proteins in caries-susceptible individuals as compared with caries-resistant ones [20]. However, the experiments involved pooled saliva samples from the two groups, and differences arising from polymorphisms were not taken into account. A subsequent comparison of the acidic proline-rich protein polymorphisms Pr, Pa and Db from caries-resistant and caries-susceptible adults showed no significant differences between the two groups although a familial nature of caries resistance was indicated [23,95]. But in a more detailed study involving a larger population of children, a positive correlation between Pa and Pr phenotypes and caries was observed [96]. Comparison of a group of anionic proteins (p*I* 4.70–5.05, MW 14 000–17 000) from whole saliva of caries-free and caries-active individuals showed that whilst the proteins from the two groups were electrophoretically and immunologically similar, they differed in their biological activities towards oral streptococci [97]. Some of these proteins may correspond to the low-molecular mass peptides pres-

ent in whole saliva which are derived by bacterial cleavage of acidic proline-rich proteins and which retain their biological activities [98]. Although attention has been directed towards the role of acidic proline-rich proteins in the adhesion oral micro-organisms to apatite surfaces [99,100], electrophoretic analysis followed by immunoblotting indicated that the main proteins in *in vivo* pellicle are in fact α-amylase, cystatins, mucin and sIgA [101]. Clearly, electrophoretic studies of salivary proteins and their relationship to caries susceptibility is becoming a very active and hopefully fruitful area of research.

3.3. Connective tissue disorders

Autoimmune connective tissue disorders affect salivary gland function in addition to their more generally recognised features. Sjögren's syndrome is characterised by the triad of symptoms keratoconjunctivitis sicca, xerostomia and a connective tissue order, usually rheumatoid arthritis (a glycosylation disorder [102]) but which may also be systemic lupus erythematosus, osteoarthritis, progressive sclerosis or mixed connective tissue disease. The classic lesion of the disorder is lymphocyte infiltration of the salivary, lachrymal and other exocrine glands. Analysis of saliva from rheumatoid arthritis and Sjögren's syndrome patients has revealed the presence of additional acidic proteins by electrophoresis [103–105] and IEF [106]. Using CA-IEF in polyacrylamide gel slabs, parotid saliva from 33 patients with rheumatoid arthritis but without clinical evidence of salivary gland

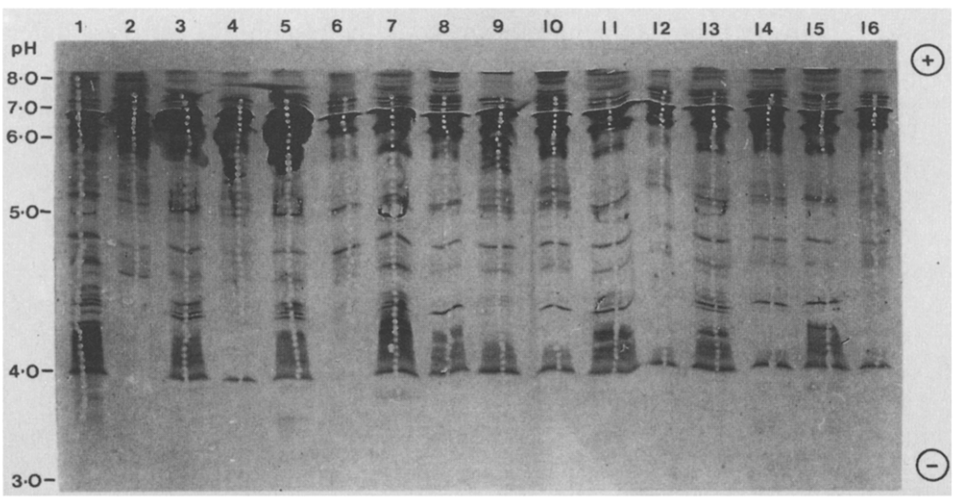

Fig. 4. IEF of parotid saliva in CAs in a pH 3–8 gradient. Odd-numbered samples were from rheumatoid arthritis patients and the even from normal healthy controls; subjects 8 and 10, however, had a familial history of rheumatoid arthritis. The sample loads were ~0.5 mg of protein and the gels were stained with Coomassie Blue G-250. (The gel shows where discs were excised for immunodiffusion studies [107].)

involvement was shown to contain multiple abnormal proteins of p*I* 3.95–4.25 (Fig. 4) whereas only 2 out of 16 normal individuals exhibited a similar feature [107]. In addition saliva from rheumatoid arthritis patients has been shown to contain rheumatoid factors of all the major immunoglobulin classes but of restricted heterogeneity and unusually low p*I* [107,108]; a similar feature has been reported for rheumatoid factor from serum [109]. Subsequently it has been claimed that the abnormal proteins revealed on IEF may correspond to sialated tissue kallikreins [110]. However, the experiments involved immunoblotting of poorly resolved proteins of p*I* <4.0 with antikallikrein antisera on a one-dimensional IEF gel; as kallikrein has a p*I* range of 3.75–4.25, it was not possible to determine whether the proteins involved were in fact kallikrein or other components of similar p*I*. Extensive studies on SDS-PAGE of parotid saliva from connective tissue disorder patients have, however, revealed no abnormalities other than perhaps an increased intensity of lactoferrin (the levels of which are elevated in Sjögren's syndrome [111]) nor was there any association with any proline-rich protein band frequencies [112]. A probable explanation is that the abnormal proteins of interest co-migrate with major proteins and are therefore masked. The suggestion that salivary protein differences are evident on SDS-PAGE [113] may arise from the problem of differentiating these from polymorphisms.

On IEF in CA systems, the proteins associated with connective tissue disorders tend to precipitate out at their p*I* values thereby making further study impossible. Using IEF in IPGs, however (Section 2.3), substantially improved resolution can be obtained and this is optimised by use of a pH 3.5–5.0 gradient (Fig. 5) [50]. By means of a 2D system involving IPGs in the first dimension and SDS-PAGE in the second, the proteins can be separated from acidic proline-rich proteins and appear as a heterogeneous group of molecular mass $\sim 32\,000$ (Fig. 3). Whilst being present in all individuals, their levels in healthy controls are normally very low but are occasionally slightly elevated. In rheumatoid arthritis and secondary Sjögren's syndrome, however, their level is substantially raised in most subjects [61]. This new procedure appears to offer the possibility of becoming an additional technique which could aid substantially in the diagnosis of oral and connective tissue disorders.

3.4. Cystic fibrosis

Cystic fibrosis, the most common lethal genetic defect among caucasians, is an epithelial exocrinopathy characterised by alterations in ion transport and mucous secretion. It is an autosomal recessive disease and the gene involved is located on the long arm of chromosome 7 between bands q21 and q22. Although the gene responsible has now been isolated [114–116], the physiological functions of the proteins encoded have yet to be established. The organs affected include the lungs, sweat glands, pancreas and salivary glands. Histologically, precipitated secretions are evident in the submandibular, sublingual, buccal and labial glands,

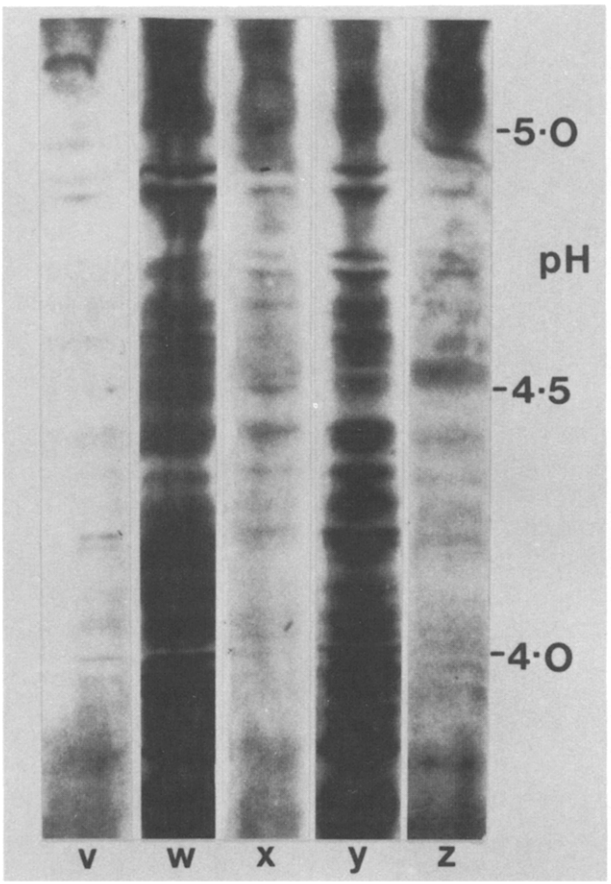

Fig. 5. IEF of human parotid saliva using a pH 3.5–5.0 IPG rehydrated with 8 *M* urea in 20% glycerol and 0.5% pH 3.5–5.0 Ampholine. Tracks v, x and z were loaded with 100 μg salivary proteins from healthy individuals, while track w was from a Sjögren's syndrome patient and track y a rheumatoid arthritis patient. The gel was silver-stained [50].

but the parotid (a pure serous gland) is histologically normal [117–119]. However, at a functional level, all the salivary glands may well be involved [120].

Although impaired pancreatic function in cystic fibrosis leads to reduced amylase production, salivary production may be elevated [121] and serum levels may be also be increased [73,74]; some of the isozymes have increased electrophoretic mobility [122].

The proteins in parotid saliva from cystic fibrosis patients were first studied by PAGE and additional components were reported [123]. A more detailed study involving IEF and 2D analysis with SDS-PAGE in the second dimension showed that the most prominent differences were the presence of two components of p*I*

8.0 and 5.8 of molecular mass ~ 10 000 in cystic fibrosis subjects as compared with controls. Further quantitative differences were apparent on silver staining; these included components of p*I* 5.8–8.0 and molecular mass 7600–10 000 [124]. However, the number of individuals studied was not stated, nor were the problems arising from polymorphisms considered. More recently, a salivary acid glycoprotein which binds cobalamin (R binder) has been shown to have an increased p*I* (from 3.78 to 4.3) and reduced heterogeneity in cystic fibrosis [125] but kallikrein, which may be involved in the control of sodium secretion, was electrophoretically identical to controls [126]. No structural differences were found between the neutral and sialic acid-containing oligosaccharide units of low-molecular-mass submandibular/sublingual mucin [127]. It would therefore appear that with the improved electrophoretic techniques now available and in the light of rapid advances in the last two years in the understanding of the genetic lesion involved, further electrophoretic studies on cystic fibrosis saliva are now merited.

3.5. Diabetes mellitus

Oral manifestations of diabetes mellitus include increased prevalence and severity of periodontal disease and variations in salivary flow-rate and composition [120], the nature of which may depend on the extent and state of autonomic neuropathy associated with the disorder [128,129]. The antimicrobial defence capacity of saliva, however, does not appear to be impaired [130].

Electrophoretic analysis of serum amylase isozymes has shown that, in many cases, the hyperamylasaemia associated with diabetic ketoacidosis is salivary in origin [75,76] and pancreatic function is decreased [131]; this technique could also be of value when investigating diabetic serum macroamylase [77]. PAGE of parotid saliva indicated the presence of increased levels of proteins with the same mobility as γ-globulins in diabetics as compared with controls [132] and increased immunoglobulin levels have been reported subsequently [130]. However, the technique involved polyacrylamide gel rods and Buffalo Black staining. Accordingly it lacked sensitivity and again the problems of polymorphisms were not considered. Thus, with diabetes mellitus too, electrophoretic analysis of saliva using techniques recently developed is much needed.

3.6. Human immunodeficiency virus

Saliva has recently gained attention as a fluid for detection of HIV antibodies. The saliva of HIV-positive individuals has been shown to only rarely contain infectious HIV particles, but always contains immunoglobulins directed against viral proteins. The presence of IgG antibody to HIV in saliva has been proven to be an accurate and convenient method of HIV antibody testing using a GACELISA system, as this method is based on the proportion of IgG antibody in saliva rather than actual concentrations. Using this system, IgG antibody has

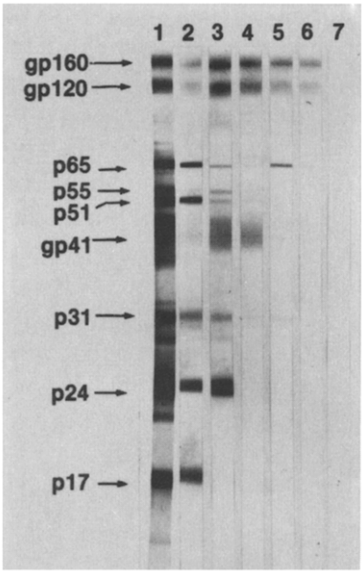

Fig. 6. Western blot patterns of antibodies to HIV antigens. Lane 1 is a positive serum control, lanes 2–6 are saliva samples from different individuals and lane 7 is a non-reactive saliva sample control [136].

been detected in all components of saliva, including the secretions of major glands, minor glands and crevicular fluid [133]. Using Western blots, antibodies to most HIV antigens probed for were detected in whole and parotid saliva from a high percentage of HIV-seropositive individuals studied (Fig. 6) [134–136]. Collection of whole saliva by the "Salivette" system has proven to be a most convenient method of anonymous testing for epidemiological purposes [137].

3.7. Other clinical applications

At present, other clinical applications of the electrophoresis of saliva are limited. An area where information would be very valuable is in the diagnosis of salivary and other tumours. Unfortunately, a comparison of the proteins in parotid saliva from parotid tumour patients as compared with controls revealed no differences which might be relevant for clinical diagnosis [138]. Interestingly though, it has recently been shown that different carcinoembryonic antigens are expressed by submandibular glands, a finding which might be useful in future studies of tumour markers [139].

4. CONCLUSIONS AND PERSPECTIVES

The clinical applications of electrophoretic analysis of saliva have been limited

by the methodology available and the lack of understanding of the nature of the polymorphisms present among the proteins. Detection methods have also been poor. With the development of new analytical procedures, especially the use of IPGs in 2D analysis, and immunoblotting, together with the new level of understanding of the nature of polymorphisms which has come from DNA studies, future prospects now look good.

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REFERENCES

1 J. O. Tenovuo (Editor), *Human Saliva: Clinical Chemistry and Microbiology*, Vols. I and II, CRC Press, Boca Raton, FL, 1989.
2 J. P. Waterhouse, J. A. Beeley and D. K. Mason, in J. H. Jones and D. K. Mason (Editors), *Oral Manifestations of Systemic Disease*, Baillière Tindall, London, 2nd ed., 1990, pp. 13–21.
3 A. Minaguchi and A. Bennick, *J. Dent. Res.*, 68 (1989) 2–15.
4 D. I. Hay, A. Bennick, D. H. Schlessinger, K. Minaguchi, G. Madapallimattam and S. K.Schluckebeir, *Biochem. J.*, 255 (1988) 15–21.
5 D. Kauffman, R. Wong, A. Bennick and P. Keller, *Biochemistry*, 21 (1982) 6558–6562.
6 D. Kauffman, T. Hofmann, A. Bennick and P. Keller, *Biochemistry*, 25 (1986) 2387–2392.
7 M. J. Levine, M. S. Reddy, L. A. Tabak, R. E. Loomis, E. J. Bergey, P. C. Jones, R. E. Cohen, M. W. Stinson and I. Al-Hashimi, *J. Dent. Res.*, 66 (1987) 436–441.
8 F. G. Oppenheim, in J. O. Tenovuo (Editor), *Human Saliva: Clinical Chemistry and Microbiology*, Vol. I, CRC Press, Boca Raton, FL, 1989, pp. 151–160.
9 R. F. Troxler, G. D. Offner, T. Xu, J. C. Vanderspek and F. G. Oppenheim, *J. Dent. Res.*, 69 (1990) 2–6.
10 J. J. Pollock, L. Denepitiya, B. J. McKay and V. J. Iacono, *Infect. Immun.*, 44 (1984) 702–707.
11 D. P. Dickinson, A. L. Ridall and M. J. Levine, *Biochem. Biophys. Res. Commun.*, 149 (1987) 784–790.
12 D. H. Schlessinger and D. I. Hay, *J. Biol. Chem.*, 252 (1977) 1689–1695.
13 B. D. Korant, J. Brzin and V. Turk, *Biochem. Biophys. Res. Commun.*, 127 (1985) 1072–1076.
14 A. Mellersh, A. Clark and S. Hafiz, *J. Vener. Dis.*, 55 (1979) 20–23.
15 J. R. Garrett, *J. Dent. Res.*, 66 (1987) 420–424.
16 H. Mehansho, L. G. Butler and D. M. Carlson, *Annu. Rev. Nutr.*, 7 (1987) 423–440.
17 J. H. Jones and D. K. Mason (Editors), *Oral Manifestations of Systemic Disease*, Baillière Tindall, London, 2nd ed., 1990.
18 J. C. Steiner and P. J. Keller, *Arch. Oral Biol.*, 13 (1968) 1213–1221.
19 Q. T. Smith, C. D. Runchey and B. L. Shapiro, *Arch. Oral Biol.*, 19 (1974) 407–410.
20 A. Y. Balekjian, T. S. Meyer, M. E. Montague and R. W. Longton, *J. Dent. Res.*, 54 (1975) 850–856.
21 B. J. Baum, J. L. Bird and R. W. Longton, *J. Dent. Res.*, 56 (1977) 1115–1118.
22 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–684.
23 L.C. Anderson, B. L. Lamberts and W. F. Bruton, *J. Dent. Res.*, 61 (1982) 393–396.
24 A. Shiba, K. S. Shiba and K. Suzuki, *J. Oral Rehabil.*, 13 (1986) 263–272.
25 T. Marshall, E. J. Kelso and K. M. Williams, *Biochem. Soc. Trans.*, 15 (1987) 507–508.
26 T. Marshall, R. C. Lynass and K. M. Williams, *Electrophoresis*, 10 (1989) 269–271.

27 S. G. Oberg, K. T. Izutsu and E. L. Truelove, *Am. J. Physiol.*, 242 (1982) G231–G236.
28 J. C. B. Lindsay and J. A. Beeley, in M. J. Dunn (Editor), *Electrophoresis '86*, VCH, Weinheim, 1986, pp. 222—225.
29 A. Shiba, K. Sano, M. Nakao, J. Yoshida, H. Cho and T. Hayashi, *J. Prosthet. Dent.*, 43 (1980) 385–391.
30 A. Shiba, J. Yoshida, M. Nakao, K. Sano, H. Cho and T. Hayashi, *J. Prosthet. Dent.*, 47 (1982) 246–251.
31 R. I. Henkin, R. E. Lippoldt, J. Bilstad, R. O. Wolf, C. K. L. Lum and H. Edelhoch, *J. Biol. Chem.*, 253 (1978) 7556–7565.
32 A. R. Shatzman and R. I. Henkin, *Biochem. Med.*, 29 (1983) 182–193.
33 M. G. Humphreys-Beher and D. J. Wells, *J. Appl. Biochem.*, 6 (1984) 353–360.
34 R. C. Duhamel, *Collagen Rel. Res.*, 3 (1983) 195–204.
35 R. C. Switzer, C. R. Merril and S. Shifrin, *Anal. Biochem.*, 98 (1979) 231–237.
36 M. E. Deh, J. K. Dzandu and G. E. Wise, *Anal. Biochem.*, 150 (1985) 166–173.
37 R. D. Freidman, *Anal. Biochem.*, 126 (1982) 346–349.
38 J. Heukeshoven and R. Dernick, *Electrophoresis*, 6 (1985) 103–112.
39 D. F. Hochstrasser, A. Patchornik and C. R. Merril, *Anal. Biochem.*, 173 (1988) 412–423.
40 C. Damerval, M. le Guilloux, J. Blaisonneau and D. de Vienne, *Electrophoresis*, 8 (1987) 158–159.
41 J. A. Beeley, D. Sweeney, J. C. B. Linday, M. L. Buchanan, K. S. Khoo and L. Sarna, *Electrophoresis*, (1991) in press.
42 P. G. Riches, B. Polce and R. Hong, *J. Immunol. Methods*, 110 (1988) 117–121.
43 B. D. Hames, in B. D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins*, IRL Press, Oxford, 1981, pp. 1–19.
44 J. A. Beeley, *Arch. Oral Biol.*, 14 (1969) 559–561.
45 J. A. Beeley, in N. Catsimpoolas and J. Drysdale (Editors), *Biological and Biomedical Applications of Isoelectric Focusing*, Plenum. London, 1977, pp. 1–28.
46 P. D. Eckersall, R. J. Mairs and J. A. Beeley, *Arch. Oral Biol.*, 26 (1981) 727–733.
47 K. Sano, M. Nakao, A. Shiba and K. Kobayashi, *Clin. Chim. Acta*, 137 (1984) 115–122.
48 A. Shiba, K. Sano, M. Nakao, K. Kobayashi and Y. Igarashi, *Arch. Oral. Biol.*, 28 (1983) 363–364.
49 M. P. Reinhart and D. Malamud, *Anal. Biochem.*, 123 (1982) 229–235.
50 K. S. Khoo and J. A. Beeley, *Electrophoresis*, 11 (1990) 489–494.
51 V. Neuhoff, R. Stamm, I. Pardowitz, N. Arnold, W. Erhardt and D. Taube, *Electrophoresis*, 11 (1990) 101–117.
52 M. Rimpilainen and P. G. Righetti, *Electrophoresis*, 6 (1985) 419–422.
53 P. G. Righetti, *J. Biochem. Biophys. Methods*, 16 (1988) 99–108.
54 K. Atland, A. van Eckardstein, A. Banzhoff, M. Wagner, U. Rossmann, R. Hackler and P. Becher, *Electrophoresis*, 8 (1987) 52–61.
55 C. S. Giometti and N. G. Anderson, in B. J. Radola (Editor), *Electrophoresis '79*, Walter de Gruyter, Berlin, 1980, pp. 395–404.
56 P. H. O'Farrell, *J. Biol.Chem.*, 250 (1975) 4007–4021.
57 T. Marshall, *Electrophoresis*, 5 (1984) 245–250.
58 M. Mogi, B. Y. Hiraoka, K. Fukasawa, M. Harada, T. Kage and T. Chino, *Arch. Oral Biol.*, 31 (1986) 119–125.
59 M. Mogi, B. Y. Hiraoka, M. Harada, T. Kage and T. Chino, *Arch. Oral Biol.*, 31 (1986) 337–339.
60 R. J. Mairs and J. A. Beeley, *Arch. Oral Biol.*, 32 (1987) 873–877.
61 J. A. Beeley, K. S. Khoo and P.-J. Lamey, *Electrophoresis*, 12 (1991) in press.
62 S. R. Porter, J. A. Beeley and C. M. Scully, *Med. Sci. Res.*, 15 (1987) 1251–1252.
63 M. J. Dunn, *Trends Anal. Chem.*, 10 (1991) 6.
64 E. A. Azen, in J. O. Tenovuo (Editor), *Human Saliva: Clinical Chemistry and Microbiology*, Vol. I, CRC Press, Boca Raton, FL, 1989, pp. 161–195.
65 A. D. Merritt and R. C. Karn, *Adv. Hum. Genet.*, 8 (1977) 135–234.

66 P. D. Eckersall and J. A. Beeley, *Biochem. Genet.*, 19 (1981) 1055–1062.
67 S. Tsuchida, and S. Ikemoto, *Forensic Sci. Int.*, 35 (1987) 159–163.
68 F. Boan and J. L. Caeiro, *Hum. Hered.*, 38 (1988) 83–90.
69 C. Scully, P. D. Eckersall, R. T. D. Edmond, P. Boyle and J. A. Beeley, *Clin Chim. Acta*, 113 (1981) 281–291.
70 D. M. Jenson, V. L. Royse, J. N. Bonello and J. Schaffner, *Dig. Dis. Sci.*, 32 (1987) 561–568.
71 T. E. Mifflin, G. Horton and D. E. Bruns, *Clin. Lab. Med.*, 6 (1986) 583–599.
72 T. E. Mifflin, R. W. Forsman and D. E. Bruns, *Clin. Chem.*, 35 (1989) 1651–1654.
73 T. Morgenstern, K. H. Niessen and M. Teufel, *Monatsschr. Kinderheilkd.*, 132 (1984) 661–665.
74 R. O. Wolf, V. S. Hubbard, B. K. Gillard and A. Kingman, *Clin. Chem.*, 32 (1986) 296–300.
75 F. Vinicor, L. M. Lehrner, R. C. Karn and A. D. Merritt, *Ann. Intern. Med.*, 91 (1979) 200–204.
76 A. L. Warshaw, E. R. Feller and K. H. Lee, *Lancet*, i (1977) 929–931.
77 D. M. Goldberg and R. J. Spooner, *Digestion*, 13 (1975) 56–75.
78 F. Piteira Barros, R. Espinheira, H. Geada and M. C. de Moura, *Am. J. Gastroenterol.*, 81 (1986) 261–265.
79 A. van Deun, C. Cobbaert, A. van Orshoven, G. Claeys and W. Lissens, *Ann. Clin. Biochem.*, 26 (1989) 422–426.
80 F. Lessard and R. Dion, *Clin. Chem.*, 35 (1989) 2116–2118.
81 I. Suehiro, M. Otsuki, A. Ohki, Y. Okabayashi, T. Oki and S. Baba, *Gastoenterol. Jpn.*, 19 (1984) 313–319.
82 N. Maeda, H.-S. Kim, E. A. Azen and O. Smithies, *J. Biol. Chem.*, 260 (1985) 11 123–11 130.
83 H.-K. Kim and N. Maeda, *J.Biol. Chem.*, 261 (1986) 6712–6718.
84 K. M. Lyons, E. A. Azen, P. A. Goodman and O. Smithies, *Genetics*, 120 (1988) 255–265.
85 K. M. Lyons, J. H. Stein and O. Smithies, *Genetics*, 120 (1988) 267–278.
86 H.-S. Kim, O. Smithies and N. Maeda, *Genomics*, 6 (1990) 260–267.
87 M. Shintani, K. Minaguchi, K. A. Lim, M. Hashimoto and K. Suzuki, *Hum. Hered.*, 40 (1990) 89–98.
88 M. Shintani, K. Minaguchi, K. Suzuki and K. A. Lim, *Biochem. Genet.*, 28 (1990) 173–184.
89 R. C. Karn, P. A. Goodman and P.-L. Yu, *Biochem Genet.*, 23 (1985) 37–51.
90 K. Minaguchi, T. Shirotani and K. Suzuki, *Ann. Hum. Genet.*, 52 (1988) 11–16.
91 K. Minaguchi and K. Suzuki, *Ann. Hum. Genet.*, 52 (1988) 177–187.
92 J. L. Caeiro, F. Boan and A. Carracedo, *Forensic Sci. Int.*, 33 (1987) 47–52.
93 J. C. vander Spek, G. D. Offner, R. F. Troxler and F. G. Oppenheim, *Arch. Oral Biol.*, 35 (1990) 137–143.
94 E. Saitoh, S. Isemura, K. Sanada, H.-S. Kim, O. Smithies and N. Maeda, *Biol. Chem. Hoppe Seyler*, 369 (Suppl. 6) (1988) 191–197.
95 L. C. Anderson and I. D. Mandel, *J. Dent. Res.*, 61 (1982) 1167–1168.
96 P.-L. Yu, D. Bixler, P. A. Goodman, E. A. Azen and R. C. Karn, *Genet. Epidemiol.*, 3 (1986) 147–152.
97 S. D. Obenhauf, R. A. Cowman and R. J. Fitzgerald, *Infect. Immun.*, 51 (1986) 440–444.
98 G. Madapallimattam and A. Bennick, *Biochem. J.*, 270 (1990) 297–304.
99 R. J. Gibbons, D. I. Hay, J. O. Cisar and W. B. Clark, *Infect. Immun.*, 56 (1988) 2990–2993.
100 R. J. Gibbons and D. I. Hay, *J. Dent. Res.*, 68 (1989) 1303–1307.
101 I. Al-Hashimi and M. J. Levine, *Arch. Oral. Biol.*, 34 (1989) 289–295.
102 R. B. Parekh, R. A. Dwek and T. W. Rademacher, *Br. J. Rheumatol.*, 27 (Suppl. II) (1988) 162–169.
103 C. J. Fischer, G. H. Wyshak and D. Wiesberger, *Arch. Oral Biol.*, 13 (1968) 257–270.
104 S. M. Herzberg, C. White and R. O. Wolf, *Oral. Surg.*, 36 (1973) 814–817.
105 E. Benedek-Spät, S. M. Puska and J. Rajki, *Otolaryngology*, 90 (1980) 275–282.
106 D. M. Chisholm, J. A. Beeley and D. K. Mason, *Oral Surg.*, 35 (1973) 620–630.
107 R. J. Mairs and J. A. Beeley, *Clin. Sci.*, 69 (1985) 727–735.
108 J. L. Chu, A. E. Gharavi and K. B. Elkon, *Clin. Exp. Immunol.*, 63 (1986) 601–607.
109 H. W. Trieshmann, G. N. Abraham and E. A. Santucci, *J. Immunol.*, 114 (1975) 176–181.
110 D. Greaves, J. T. Whicher, K. D. Bhoola, C. Scully, S. Flint, S. R. Porter, R. E. Chambers, P. A. Maddison, J. A. Beeley and R. Matthews, *Ann. Rheum. Dis.*, 48 (1989) 753–759.

111 N. Jezequel. F. Depasse, J. Jouquan, A. Lelong, S. Roncin, G. Pare, Y. L. Pennec and P. Youinou, *Clin. Exp. Rheumatol.*, 7 (1989) 123–125.
112 J. A. Beeley and P.-J. Lamey, unpublished results.
113 N. L. Rhodus, M. J. Schuh, Q. T. Smith and D. K. Johnson, *J. Dent. Res.*, 68 (1989) 928.
114 J. M. Rommens, M. C. Ianuzzi, B. S. Kerem, M. L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J. L. Cole, D. Kennedy, N. Hidaka, M. Zsiga, M. Buchwald, J. R. Riordan, L. C. Tsui and F. S. Collins, *Science*, 245 (1989) 1059–1065.
115 J. R. Riordan, J. M. Rommens, B. S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou, M. L. Drumm, M. C. Iannuzzi, F. S. Collins and L. C. Tsui, *Science*, 245 (1989) 1066–1073.
116 B. S. Kerem, J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald and L. C. Tsui, *Science*, 245 (1989) 1073–1080.
117 L. Sweeney and W. J. Warwick, *Arch. Pathol.*, 86 (1968) 413–418.
118 B. Tandler, C. R. Denning, I. D. Mandel and A. H. Kutscher, *J. Morphol.*, 127 (1969) 383–408.
119 R. G. Doggett, B. Bentinck and G. M. Harrison, *J. Clin. Pathol.*, 24 (1971) 270–282.
120 I. D. Mandel, *CRC Crit. Rev. Clin. Lab. Sci.*, 12 (1980) 321–366.
121 J. Blomfield, J. Dascula, E. W. van Lennep and J. M. Brown, *Gut*, 14 (1973) 558–565.
122 K. M. Doering, C. Arglebe, H. Lubany and R. Chilla, *Eur. J. Pediatr.*, 126 (1977) 185–188.
123 P. Meyer, R. R. Kleber and K. W. Mittenzwey, *Laryngol. Rhinol. Otol.*, 59 (1980) 274–278.
124 S. B. Bustos and L. Fung, in R. C. Allan and P. Arnaud (Editors), *Electrophoresis '81*, Walter de Gruyter, Berlin, 1982, pp. 317–328.
125 J. L. Gueant, M. Vidailhet, M. Djalali, J. Mickalski and J. P. Nicolas, *Clin. Chim. Acta*, 143 (1984) 217–223.
126 E. R. Hare and J. A. Verpoorte, *Pediatr. Res.*, 19 (1985) 938–943.
127 M. S. Reddy, M. J. Levine and A. Prakbphol, *J. Dent. Res.*, 64 (1985) 33–36.
128 P.-J. Lamey, B. M. Fisher and B. M. Frier, *Diabet. Med.*, 3 (1986) 537–540.
129 P.-J. Lamey, A. P. Savage, B. M. Fisher, S. R. Bloom and B. M. Frier, *J. Oral. Pathol. Med.*, 19 (1990) 351–354.
130 J. Tenovuo, O.-P. Lehtonen, J. Viikari, H. Larjava, P.Vilja and P. Tuohimaa, *J. Dent. Res.*, 65 (1986) 62–66.
131 Y. Foo, S. B. Rosalki, L. Ramdial, D. Mikhailidis and P. Dandona, *J. Clin. Pathol.*, 33 (1980) 1102–1105.
132 A. J. Finestone, G. R. Schacterle and R. L. Pollack, *J. Periodontol.*, 44 (1973) 175–176.
133 P.-J. Lamey, E. A. C. Follett, A. Nolan, T. W. McFarlane, I. Cooke and D. H. Kennedy, *J.Dent. Res.*, 69 (1990) 977.
134 P. Holmström, S. Syrjänen, P. Laine, S.-L. Valle and J. Suni, *J. Med. Virol.*, 30 (1990) 245–248.
135 D. Sun, D. W. Archibald and P. A. Furth, *AIDS Res. Hum. Retroviruses*, 6 (1990) 933–941.
136 M. B. Vasudevachari, K. Uffelman, P. F. Pierce, H. C. Lane and N. P. Salzman, *J. Clin. Microbiol.*, 27 (1990) 2384–2385.
137 A. Nolan, P.-J. Lamey and E. A. C. Follett, *J. Dent. Res.*, 70 (1991) 685.
138 C. Arglebe, J. David and R. Chilla, *Laryngol. Rhinol. Otol.*, 66 (1987) 104–106.
139 F. Zoubir, W. N. Khan and S. Hammerstrom, *Biochem. Biophys. Res. Commun.*, 169 (1990) 203–216.