Purification and Partial Characterization of Four Proteins from Human Parotid Saliva

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Four proteins, which have been designated A, B, C and D, have been purified from human parotid saliva. These proteins are the major constituents of parotid saliva which migrate rapidly to the anode in polyacrylamide electrophoresis at pH 9.5. Gel filtration and polyacrylamide electrophoresis were employed in the purification procedures. After purification all four preparations were tested for homogeneity by electrophoresis at pH 2.8 and 9.5, by isoelectric focusing in the pH range 3-10, by immunodiffusion, and by sedimentation in the analytical ultracentrifuge. None of the proteins showed significant activity in assays for amylase, acid and alkaline phosphatase, protease, lysozyme, ribonuclease, peroxidase, β -glucuronidase, β -galactosidase, iron-binding activity and esterase. No cross-reactions were detected with antisera specific for lactoferrin and 15 serum proteins. All four proteins were rich in glutamic acid, proline and glycine and were lacking completely the sulphur-containing amino acids. Proteins A and C contained no threonine or tyrosine. Carbohydrate could be demonstrated only in protein A at a concentration of 4% of the total protein.

Human parotid saliva contains a complex mixture of proteins. Polyacrylamide-gel electrophoresis has demonstrated as many as 30-40 bands stainable with protein dyes (Meyer & Lamberts, 1968). The most prominent protein is amylase, which has been isolated and characterized by Kauffmann, Zager, Cohen & Keller (1970). Lysozyme has also been purified (Balekjian, Hoerman & Berzinskas, 1969). Nakamura, Watanabe, Iwamoto & Tsunemitsu (1970) have isolated a phosphomonoesterase and Levine, Weill & Ellison (1969) have described a glycoprotein in this secretion. Recently a protein has been purified that has a strong tendency to adsorb on hydroxyapatite and dental enamel (Hay, 1970). Many other enzymic or biological activities have been demonstrated, but with the exception of peroxidase (Slowey, Eidelman & Klebanoff, 1968) none of these active agents has been isolated. Immunological techniques have shown the presence of some serum proteins, but they probably account for only a minor part of the protein (Stoffer, Kraus & Holmes, 1962; Tomasi & Zigelbaum, 1962). It is apparent that there are several proteins in parotid saliva that remain uncharacterized. This paper describes the isolation and characterization of four proteins from human parotid saliva.

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MATERIALS AND METHODS

Materials. Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. Ampholine, pH 3-10, was purchased from LKB-Produkter A.B., Stockholm, Sweden, and sodium *p*-nitrophenyl phosphate, casein, Bromophenol Blue and Methyl Green were from BDH Chemicals Ltd., Poole, Dorset, U.K. Coomassie Blue (Michrome No. 1137) was from E. Gurr Ltd., London S.W.14, U.K. Complete Freund's adjuvant and special Agar Noble were purchased from Difco Laboratories, Detroit, Mich., U.S.A., and uricase was from Worthington Biochemical Corp., Freehold 2, N.J., U.S.A. *o*-Nitrophenyl β -D-galactopyranoside and a dried preparation of *Micrococcus lysodeikticus* were obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. and soluble starch was purchased from Fisher Scientific Co., Toronto, Ont., Canada. Purified wheat embryo RNA was a gift from Dr B. G. Lane, Toronto, Ont., Canada. *N*-Benzoyl-L-arginine ethyl ester hydrochloride was purchased from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A., and phenolphthalein glucuronide from Calbiochem, Los Angeles, Calif., U.S.A. Bovine serum albumin was obtained from Armour Pharmaceutical Co., Kankakee, Ill., U.S.A., and [^{59}Fe]ferrous citrate from Charles Frosst Co., Montreal, Que., Canada. Antisera to the following human plasma proteins were purchased from Hoechst A.-G., Frankfurt/Main, Germany: prealbumin, α_1 -acid glycoprotein, α_1 -lipoprotein, α_1 -antitrypsin, group-specific component, caeruloplasmin, haptoglobin

β_2 -macroglobulin, haemopexin, β_1C/β_1A -globulin and β_2 -glycoprotein. In addition antisera to the following proteins were obtained from Hyland, Los Angeles, Calif., U.S.A.: human immunoglobulin A, immunoglobulin G, immunoglobulin M and albumin. Antiserum to human lactoferrin was donated by Dr J. Bienenstock, Hamilton, Ont., Canada. Antiserum to human transferrin was a sample previously prepared in this laboratory. All other chemicals were analytical grade.

Preparation of antisera and immunodiffusion. Rabbits were injected intradermally with mixtures of protein solution and complete Freund's adjuvant at weekly intervals of 2–3 weeks. This treatment was repeated two or four times and the animals were bled 1 week after the last injection. Antisera to proteins A, B and C were prepared in this way, but attempts to make antiserum to protein D were unsuccessful.

Double immunodiffusion was carried out in 1.5% agar gels in barbital buffer, pH 8.6, $I_0.025$. Wells of 1.5 mm diam. were cut in a gel made on microscopic slides like those commonly used in immunoelectrophoresis (Kabat & Mayer, 1964).

Polyacrylamide-gel electrophoresis. Analytical electrophoresis was carried out in cylindrical glass tubes. In all instances the same buffer was used in the gel as well as in the electrode vessels (continuous system). Unless otherwise indicated the buffer was 0.1 M-tris-glycine, pH 9.5, and anodally migrating components were separated. Different gel concentrations were used; the composition of the gels is indicated by the method of Hjertén (1962), giving the total acrylamide concentration (%T), and the bisacrylamide concentration (%C) as percentage of the total acrylamide concentration. The gels were polymerized with ammonium persulphate and NNN' -tetramethylethylenediamine. The sample was layered on top of the gel, and electrophoresis was continued until a tracking dye had reached the lower end of the gel. The gels were stained with Coomassie Blue according to the method of Chrambach, Reisfeld, Wyckoff & Zaccari (1967).

Preparative polyacrylamide-gel electrophoresis was carried out on the Buchler Poly Prep apparatus. A continuous buffer system of 0.1 M-tris-glycine, pH 9.5, was used in all separations. After polymerization of the gel solution with ammonium persulphate and NNN' -tetramethylethylenediamine, the current was passed through the gel for at least 2 h before sample application in order to avoid contact of the protein with residual persulphate in the gel. Persulphate was detected in the eluate as described by Bennick (1968). A constant potential of 600 V was maintained during electrophoresis of the protein sample. The anode was the lower electrode.

Analytical isoelectric focusing. Essentially the method of Dale & Latner (1968) was employed. Isoelectric focusing took place at 50 V and 3°C for 7 h. Each of the gels was fixed in two changes of 35 ml of 12.5% trichloroacetic acid for 24 h. The gels were then stained and destained by the method of Riley & Coleman (1968).

Assays. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard. β -Galactosidase was assayed according to the method of Hestrin, Feingold & Schramm (1955), with 0.2 M-sodium phosphate buffer, pH 6.0, and incubation for 15 min. Acid and

alkaline phosphatases were assayed as described by Bessey, Lowry & Brock (1963). For acid phosphatase the incubation period was 2 h. The method of Rick (1963) was used for protease assay. Peroxidase activity was determined by the method of Lück (1963). The E_{485} was read every $\frac{1}{2}$ h for 2 h after the addition of enzyme solution. β -Glucuronidase was assayed by the method of Fishman (1963). The incubation period was 5 h and the E_{540} of the solution was recorded. Ribonuclease activity was assayed as described by Helleiner (1955). Assays were performed in 0.5 M-tris-formate, pH 7.8, or 0.25 M-sodium phosphate buffer, pH 6.5, with incubation at 37°C for 1 h. Amylase was assayed by the method of Searcy, Hayashi & Berk (1966). The assay mixture was incubated at 25°C for $\frac{1}{2}$ h. Lysozyme activity was determined by the method of Jollés (1962). Iron-binding activity was evaluated by the method of Giblett, Hickman & Smithies (1959). Instead of a starch gel, columns (6 cm $\times$ 1 cm) of polyacrylamide gels with the composition 4.5% T–3% C were employed. A 0.1 M-tris-glycine buffer, pH 9.5, was used and experiments were performed with either anode or cathode as the lower electrode. The sample consisted of 175 μ l of concentrated parotid saliva containing approx. 7 mg of protein and 0.5 Ci of [^{59}Fe]ferrous citrate. After electrophoresis, the gels were sliced lengthwise; one half was stained and the other half was used to make a radioautograph. The esterolytic activity with *N*-benzoyl-L-arginine ethyl ester hydrochloride as substrate was measured as described by Trautschold & Werle (1961). The E_{253} was measured every 5 or 10 min for 30 min. The presence of blood-group substances was tested at the haematology laboratory of the Toronto General Hospital by Mrs M. Crookston.

Determination of sedimentation coefficients and molecular weight. The Beckman model E analytical ultracentrifuge was used. Duplicate determinations of the sedimentation coefficient were made for each protein at 20°C in 0.1 M-tris-glycine, pH 9.5. The molecular weight was determined by low-speed equilibrium centrifugation (Richards & Schachman, 1959). The partial specific volume was determined from the amino acid composition of the protein (Cohn & Edsall, 1965).

Amino acid analyses. Samples of the proteins A, B, C and D were dialysed at 0°C in six changes of 3.5 litres of water for 3 days. The samples containing approx. 0.4 mg of protein were dried and redissolved in 1 ml of 5.7 M-HCl. Samples were hydrolysed in evacuated sealed tubes at 110°C for 24, 48 and 72 h and analysed on the Beckman model 120C amino acid analyser.

Carbohydrate analyses. Total carbohydrates were determined by the anthrone method as described by Seifter, Dayton, Novic & Muntwyler (1950).

Collection of saliva for large-scale purification of salivary proteins. Carlson–Crittenden cups as modified by Curby (1953) were placed over the opening of the parotid ducts and lines from the cups connected to a vessel kept in crushed ice. The salivary flow was stimulated by sucking sour lemon drops. A total of 50 ml was collected twice daily. A solution of 0.2 M-EDTA, pH 7.0, was added to the saliva to a final concentration of 0.02 M. The sample was dialysed against water at 3°C for 24 h. The presence of uric acid in the dialysed saliva was tested by the enzymic method of Hawkins, Zipkin & Marshall (1963). Consistently it was found that all uric acid had been removed by dialysis. After dialysis the saliva was freeze-

dried and stored at -10°C . All saliva for preparative purposes was collected from the same donor.

Concentration of fractions. All fractions obtained in the purification procedures were concentrated by ultrafiltration in a Diaflo apparatus through UM2 membranes (Amicon). The fractions were quick-frozen and stored at -10°C .

Survey of salivary proteins. Saliva was collected from ten male and ten female white persons, age range 20–41 years. The collection and concentration procedure was the same as described for large-scale preparation except that 10 ml of saliva was collected separately from both the right and the left gland of each donor. The samples were redissolved in 0.5 ml of water and $5\mu\text{l}$ portions were used for analytical electrophoresis on polyacrylamide gels (4.5%T–3%C).

RESULTS

Survey of salivary proteins

The result of electrophoresis of saliva from 5 donors is illustrated in Plate 1(a). Gels containing saliva from the right and the left parotid gland of the same person were placed next to each other and appeared identical. Most of the variation in the band pattern was seen among the slow-moving components in the region labelled F. Region E, which contains amylase (Bennick & Connell, 1968), always showed one or more heavily stained bands, but their number and intensity varied. The bands labelled X, B, C and D were invariably present in all secretions. They appeared as distinctly stained bands, although there was some variation in the staining intensity from person to person. Both components X and C on occasion appeared as two bands. A visual impression of the staining intensity of components X, B, C and D suggests that they are major protein components of parotid saliva. On the basis of these observations, components X, B, C and D were selected for large-scale purification.

Purification of the salivary proteins

All procedures were carried out at 3°C . The freeze-dried residue from 400 ml of parotid saliva was redissolved in about 20 ml of 0.1M-tris-HCl buffer, pH 8.0, and applied to a column of Sephadex G-50. The result of the chromatography is shown in Fig. 1. The eluate was pooled in four fractions, I, II, III and IV. Analytical electrophoresis was performed on samples of each concentrated fraction. Plate 1(b) shows that components X and C were both present in fraction I. Component B was present in fraction III and component D in fraction IV.

Purification of components X and C

Fraction I was separated by preparative polyacrylamide-gel electrophoresis on a gel with the composition 4.5%T–3%C. The elution profile is presented in Fig. 2. The tubes were pooled as

Fig. 1. Fractionation of concentrated parotid saliva (985 mg of protein) on Sephadex G-50 (5 cm $\times$ 88 cm) in 0.1M-tris-HCl, pH 8.0. The flow rate was 60 ml/h; 20 ml fractions were collected. —, E_{280} ; ---, E_{220} . The E_{280} was read in a cell with a 1 cm optical path, and the E_{220} in a cell with a 1 mm path. The tubes were pooled into fractions I, II, III and IV as indicated on the diagram.

Fig. 2. Preparative electrophoresis of fraction I (143 mg of protein) on the Buchler Poly Prep in a polyacrylamide gel (12 cm long) (concentration 4.5%T–3%C). A continuous buffer system of 0.1M-tris-glycine, pH 9.5, was used and a constant potential of 600 V was maintained during electrophoresis. Elution buffer flow rate was 1.1 ml/min and 5.5 ml fractions were collected. —, E_{220} read in a cell with a 1 mm optical path; Δ , total *N*-benzoyl-L-arginine ethyl ester (PhCo-ArgEt) hydrolytic activity of the fractions. Tubes were pooled to give components X, C and C' as indicated on the diagram.

indicated in Fig. 2 and samples of the concentrated fractions were subjected to analytical electrophoresis on polyacrylamide gels (Plate 1c). Each of the gels containing a concentrated fraction shows a single

heavy band. These correspond to components X and C and a third component labelled C'. In the gel containing component C, a faint band anodal to the main band can be seen. This band was not always observed.

Component X was further purified by preparative electrophoresis on a polyacrylamide gel with the composition 15%T-3%C. The elution profile is presented in Fig. 3. The tubes were pooled as indicated in Fig. 3, yielding components A, A' and A''. A sample of component A was used for analytical electrophoresis. The stained gel is illustrated in Fig. 3 and shows a single band.

Purification of component B

Fraction III was separated on a preparative polyacrylamide gel, composition 4.5%T-3%C. The elution profile is presented in Fig. 4. A large peak was always observed followed by a second smaller peak. The amount of protein corresponding to the large peak was smaller than that represented by the second small peak. The protein representing the second peak was concentrated and 0.02mg of the protein was subjected to analytical electrophoresis. The stained gel is illustrated in Fig. 4 and shows the B band uncontaminated by other components, with the possible exception of a faint band just anodal to the B band.

Purification of component D

Fraction IV was purified by preparative polyacrylamide-gel electrophoresis on a gel with the composition 4.5%T-3%C. The elution profile is presented in Fig. 5. Two large peaks, which partly overlapped, were always seen, followed by a third small peak. The fastest migrating of the major peaks could be seen as a blue band on the gel and the other as a red band. The two major peaks only contained a small amount of protein compared with the third small peak. The protein corresponding to the small peak was concentrated and a sample used for analytical electrophoresis. The stained gel is presented in Fig. 5 and shows a single heavy band. The two faint bands anodal to the main band were not seen in other preparations.

Recovery of protein

From 100mg of parotid salivary proteins the following amounts were recovered: 57mg of fraction I, 26mg of fraction III and 10mg of fraction IV. From these amounts of protein it was possible to recover 2.6mg of component X, 1.7mg of component A, 3.3mg of component C, 0.6mg of component B and 0.4 mg of component D.

Criteria for purity

(a) *Polyacrylamide-gel electrophoresis.* Gels that varied in concentration from 8%T-3%C to 20%T-3%C were also used for analytical electrophoresis of components A, B, C, C' and D. Electrophoresis was performed with 0.1 M-tris-glycine buffer, pH 9.5, or with 0.05 M-formate, pH 2.8. In the latter instance cathodally migrating components were separated.

EXPLANATION OF PLATE 1

(a) Analytical electrophoresis of parotid saliva from five female donors. Polyacrylamide gels (6cm×5mm) (concentration 4.5%T-3%C) were used. A continuous buffer system of 0.1 M-tris-glycine, pH 9.5, was used. Gels containing saliva from the right and the left parotid gland of the same person are placed next to each other.

(b) Analytical gel electrophoresis of the fractions obtained in Fig. 1. Conditions of electrophoresis were the same as in Plate 1(a). I, II, III and IV refer to the fractions in Fig. 1. P.S. refers to a sample of the parotid saliva applied to the Sephadex G-50 column. A 0.1 mg sample was placed on each gel except for P.S., in which 0.2 mg was used. Electrophoresis was at 125 V. X, B, C and D refer to the bands that were selected for purification. Note the presence of components X and C in fraction I, component B in fraction III and component D in fraction IV.

(c) Analytical gel electrophoresis of the fractions illustrated in Fig. 2. Conditions of electrophoresis were the same as in Plate 1(a). Samples used were 0.02 mg except for fraction I, in which 0.1 mg was used.

EXPLANATION OF PLATE 2

(a) Isoelectric focusing on analytical polyacrylamide gels of the same dimensions as those used in analytical gel electrophoresis (gel concentration 7.1%C-2.6%T). B, C, C', D and A indicate the salivary protein samples present in the gel. P.S. is a sample of unfractionated parotid saliva. Blank indicates gels that contained no protein. Samples were 0.04mg of protein incorporated into the gels except for P.S., in which 0.4 mg was used. A pH gradient in the gels from pH 3.0 to 10.0 was employed and the potential was maintained at 50 V for 7 h. The six gels to the left were photographed after fixing of the gels for 24 h in two changes of 35 ml of 12.5% trichloroacetic acid. The two gels on the right were stained with Coomassie Blue after fixation.

(b) Double immunodiffusion in 1.5% agar gels containing barbital buffer, pH 8.6, I/0.025. The letters A, C, C', B and D at the margins of the photographs refer to the protein solutions placed in the corresponding outer wells. Anti A, Anti C and Anti B refer to the rabbit immune sera to A, C and B that were placed in the corresponding central well. Immunodiffusion took place at room temperature for 15 h with Anti A and Anti C and for 24 h with Anti B.

Fig. 3. Preparative electrophoresis of protein component X (7.9mg of protein) on a polyacrylamide gel (6cm long) (concentration 15%T-3%C). The experimental conditions were otherwise as described in Fig. 2. —, E_{220} ; Δ , total *N*-benzoyl-L-arginine ethyl ester hydrochloride (PhCO-ArgEt) hydrolytic activity (note the localization in component A'). The photograph inset illustrates analytical gel electrophoresis of component A under the same experimental conditions as in Plate 1(a), with the exception that the gel concentration was 15%T-3%C.

Fig. 4. Preparative electrophoresis of fraction III (50mg of protein) on polyacrylamide gel (12cm long) (concentration 4.5%T-3%C). —, E_{280} measured in a cell with a 1cm optical path. The experimental conditions were otherwise as described in Fig. 2. The photograph inset illustrates analytical gel electrophoresis of protein component B under the same experimental conditions as in Plate 1(a).

In alkaline gels of high concentration two bands could be seen in component C', of which the slowest appeared to contain two components. Apart from this and with the exception of a few instances in which minor faint bands could be seen all gels showed a single heavy band.

(b) *Isoelectric focusing*. Components A, B, C, C' and D were subjected to analytical isoelectric focusing. After fixing of the gels in 12.5% trichloroacetic acid, components B, C, C' and D each showed a single precipitated band (Plate 2a), although it is possible that there are two unresolved bands in component B. No band could be seen in the gel containing component A, but, upon staining, a single band appeared (Plate 2a). No additional bands were seen when the gels containing components B, C, C' and D were stained.

(c) *Double immunodiffusion*. Components C and C' reacted with a heavy continuous line with the antiserum to component C. A second fainter precipitation line developed later at the outer periphery of the heavy line. No other cross-reactions of the fractions were observed. Antiserum to component B reacted only with component B to give a single precipitation line, and antiserum to component A gave a single line with component A only. Component D did not react with the antiserum to components A, B or C. The results of the immunodiffusion are illustrated in Plate 2(b).

Enzyme assays

Under the conditions of the assays employed no β -galactosidase, alkaline phosphatase and protease could be detected in parotid saliva. The results of the assays of enzymes present in saliva are summarized in Table 1.

Amylase was found in fractions I, II, III and IV,

Fig. 5. Preparative electrophoresis of fraction IV (47 mg of protein) on a polyacrylamide gel (12 cm long) (concentration 4.5% T-3% C). —, E_{280} . The experimental conditions were otherwise the same as in Fig. 2. The photograph inset illustrates analytical gel electrophoresis of protein component D under the same conditions as in Plate 1(a).

but only negligible activity could be detected in components C, C' and B. Ribonuclease, β -glucuronidase, acid phosphatase, lysozyme and peroxidase could all be detected in unfractionated parotid saliva and one or more of fractions I, II, III and IV. None of these enzymes could be demonstrated in components X, A, B, C, C' and D. No binding of ^{59}Fe could be demonstrated by radioautography of gels containing these proteins. *N*-Benzoyl-L-arginine ethyl ester hydrolytic activity was located in fraction I. In subsequent separations of fraction I on polyacrylamide gel the *N*-benzoyl-L-arginine ethyl ester hydrolytic activities of the fraction were measured and revealed an irregular peak of enzyme activity in the region of component X (see Fig. 2). When component X was further purified on a more concentrated polyacrylamide gel the activity was now located in component A' (see Fig. 3).

Determination of secretor type

The donor of saliva for large-scale purification was found to be a secretor of blood-group substance A by examination of whole saliva. No blood-group substance could be detected in his parotid saliva.

Reactivity with antisera to substances other than salivary proteins

Only antisera to immunoglobulin A, immunoglobulin G, transferrin and albumin reacted with unfractionated parotid saliva in double immunodiffusion. Components X, B, C, C' and D did not react with antisera to immunoglobulin A, immunoglobulin G and transferrin, whereas antiserum to albumin reacted with component X. Further experiments with components A, A' and A'' demonstrated that albumin was present only in

Table 1. *Enzymes assayed that*

< Indicates that no activity was found in the sample. The number that follows this sign was obtained by dividing the lowest in the following units: amylase, $\Delta E_{540}/\frac{1}{2}$ h per mg of protein; ribonuclease, % acid-soluble RNA/h per mg of protein; β -glucuronidase, mg of protein; lysozyme, $\Delta E_{540}/\text{min}$ per mg of protein; peroxidase, $\Delta E_{485}/\text{h}$ per mg of protein. ΔE_n indicates change in extinction soluble in 5% trichloroacetic acid under the conditions of the assay. The lowest amount of activity detectable in the assay indicated in the table (last column).

Enzyme assayed	Protein									
	Parotid saliva		Sephadex fraction I		Sephadex fraction II		Sephadex fraction III		Sephadex fraction IV	
	Sp. activity (units/mg of protein)	Protein (mg)	Sp. activity (units/mg of protein)	Protein (mg)	Sp. activity (units/mg of protein)	Protein (mg)	Sp. activity (units/mg of protein)	Protein (mg)	Sp. activity (units/mg of protein)	Protein (mg)
Amylase	553	0.0004	137	0.0008	919	0.0002	1331	0.0001	44	0.0003
Ribonuclease, pH 7.8	88	0.16	<11	0.28	<27	0.11	<23	0.13	266	0.03
Ribonuclease, pH 6.5	32	0.20	<7	0.42	<18	0.17	<15	0.20	145	0.04
β -Glucuronidase	0.009	1.92	0.012	1.5	<0.007	0.6	0.009	0.6	<0.02	0.15
Acid phosphatase	0.67	0.50	0.45	0.18	<0.17	0.06	<0.07	0.14	<0.20	0.04
PhCO-ArgEt hydrolytic activity	0.084	4.52	0.11	2.56	<0.20	0.35	<0.07	0.96	<0.10	0.50
Lysozyme	0.088	0.25	<0.01	0.09	0.019	0.07	0.077	0.15	0.43	0.04
Peroxidase	0.16	0.15	0.23	0.12	0.07	0.18	<0.01	0.2	<0.12	0.04

Table 2. *Amino acid composition of components A, B, C and D*

Average values for 24, 48 and 72 h hydrolysate, except for threonine, serine, valine and isoleucine. Analysis was performed on a Beckman model 120C amino acid analyser, with a 6.6 mm cell optical path. Because of the small amount of component D analysed the values for this protein are approximate.

Amino acid	Component ...	Amino acids in hydrolysate (μmol)				% Amino acid of total residues recovered			
		A	C	B	D	A	C	B	D
Lys		0.061	0.059	0.060	0.024	1.0	1.3	1.9	5.7
His		0.057	0.074	0.010	0.037	0.9	1.7	0.3	8.7
Arg		0.108	0.153	0.124	0.022	1.7	3.4	3.9	5.2
Asp		0.977	0.335	0.174	0.035	15.5	7.5	5.5	8.3
Thr*		0.020	0	0.039	0.010	0.3	0	1.2	2.4
Ser*		0.359	0.153	0.284	0.031	5.7	3.4	8.9	7.3
Glu		1.950	1.339	0.828	0.065	30.8	30.0	26.1	15.3
Pro		0.932	1.131	0.332	0.037	14.7	25.3	10.4	8.7
Gly		0.979	0.924	0.222	0.052	15.5	20.7	7.0	12.3
Ala		0.079	0.036	0.039	0.011	1.3	0.8	1.2	2.6
Cys/2		0	0	0	0	0	0	0	0
Val†		0.283	0.090	0.113	0.007	4.5	2.0	3.6	1.7
Methionine		0	0	0	0	0	0	0	0
Ile†		0.158	0.055	0.073	0.020	2.5	1.2	2.3	4.7
Leu		0.250	0.090	0.171	0.015	4.0	2.0	5.4	3.5
Tyr		0.010	0	0.268	0.037	0.2	0	8.4	8.7
Phe		0.101	0.031	0.442	0.021	1.6	0.7	13.9	5.0
Total		6.324	4.470	3.179	0.424	100	100	100	100

* Result obtained by extrapolating to zero time.

† Values obtained from 72 h hydrolysate.

The negative results for β -galactosidase and alkaline phosphatase are in agreement with Chauncey, Henriques & Tanzer (1963), but alkaline phosphatase was found in parotid saliva by Cornish & Posen (1968). Kauffmann *et al.* (1970) did not find any proteolytic activity in salt-exchanged parotid saliva.

Peroxidase, β -glucuronidase and ribonuclease have all previously been found in human parotid saliva (Slowey *et al.* 1968; Chauncey, Lionetti, Winer & Lisanti, 1954; Eichel, Conger & Chernick, 1964). The virtual absence of amylase from components B, C and C' is in agreement with studies that have shown that under similar electrophoretic conditions amylase migrates slowly towards the anode (Steiner & Keller, 1968). Acid phosphatase has been demonstrated previously in human parotid saliva (Chauncey *et al.* 1954). A fraction that has acid phosphatase activity and that had a slower electrophoretic mobility than the component D fraction was isolated in this study.

Paper electrophoresis of parotid saliva at pH 8.6 has demonstrated that lysozyme migrates towards the cathode (Mandel, 1966). This is in agreement with the lack of activity in any of the purified fractions. Although we have not been able to demonstrate directly any iron-binding activity in

parotid saliva, it is possible that ^{59}Fe became bound to a component that did not move from the gel origin. Weinstein, Mandel & Brandstadter (1968) have described a kallikrein activity that would appear to have similar electrophoretic mobility to the *N*-benzoyl-L-arginine ethyl ester hydrolytic activity described in this study. *N*-Benzoyl-L-arginine ethyl ester hydrolytic activity has been used as a method for measuring kallikrein activity (Trautschold & Werle, 1961). The demonstration of transferrin and albumin in parotid saliva is in agreement with the results of Stoffer *et al.* (1962). They also found orosomucoid (α_1 -acid glycoprotein) and immunoglobulin M in parotid saliva. Neither of these could be demonstrated in this study. Immunoglobulin A has been demonstrated in parotid saliva by Tomasi & Zigelbaum (1962). The secretory immunoglobulin A found in parotid saliva is composed of serum immunoglobulin A and a so-called transport piece (T piece) (Tomasi, Tan, Solomon & Prendergast, 1965). The T piece has a sedimentation coefficient of 4.75S (Newcomb, Normansell & Stanworth, 1968) and it has been reported that free T piece may be present in normal parotid saliva (Brandtzaeg, Fjellanger & Gjeruldsen, 1968). The lower *s* values obtained for the purified salivary proteins and an amino acid composition of

the T piece (Tomasi & Bienenstock, 1968) markedly different from the composition of components A, B, C and D strongly suggest that none of these proteins is free T piece. Masson, Heremans & Dive (1966) demonstrated immunologically the presence of lactoferrin in concentrated (200-fold) human parotid saliva. Using a similar technique we were unable to demonstrate this protein in 20- and 100-fold concentrated parotid saliva.

The lack of reaction of protein components A, B, C, C' and D with any of the antisera to human plasma proteins indicates that they are specific salivary proteins. Components A, B, C and D appear to be highly purified on the basis of their behaviour in analytical polyacrylamide electrophoresis, isoelectric focusing and immunodiffusion. The last technique also demonstrated that only the C and the C' fraction have immunological similarities. Their sedimentation coefficients suggest that they are small molecules with molecular weights of the order of 10000. The amino acid composition of protein components A and C is remarkable because of the high contents of dicarboxylic acids, proline and glycine and small amounts or absence of threonine, tyrosine and sulphur-containing amino acids. The glycoprotein described by Levine *et al.* (1969) has an amino acid composition that in many respects is very similar to components A and C. In addition it contains approximately 32% carbohydrate, but only 4% could be found in component A and none appears to be present in component C. The protein isolated by Hay (1970) shows electrophoretic mobility of the same magnitude and direction as the proteins we have isolated. Amino acid analysis also shows a high content of glutamic acid and proline in this protein, but the tyrosine content is higher (156 residues per 1000) than in any of the proteins we have isolated. At present the relationship between the protein described by Hay (1970) and the proteins isolated by us remains unsettled.

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