CHROM. 5494

Heterogeneity of protein fractions isolated by preparative electrophoresis of human saliva*

Continuous flow, high voltage paper electrophoresis (CFPE) has been used in the preparative fractionation of proteins from a wide variety of complex biological fluids including milk[1], serum[2], and snake venom[3]. In addition, using a modified electrophoretic cell, APOSTOLOPOULOS[4] has described the separation of human mixed saliva into twelve fractions which is a higher resolution than that achieved by CALDWELL AND PIGMAN[5] with polyacrylamide disc gel electrophoresis.

Since the degree of homogeneity of the individual protein fractions was not reported[4], it becomes difficult to judge the usefulness of CFPE in comparison with other preparative methods (fractional precipitation, gel filtration, rivanol adsorption) available at this time for the isolation of human salivary proteins. The failure of CFPE as a preparative method in the isolation of human parotid fluid proteins has previously been noted[6,7].

The heterogeneity of protein fractions obtained by preparative electrophoresis of human mixed saliva is the subject of this report. The electrophoretic patterns shown are representative of results which have been obtained in the fractionation of over 200 individual (120–250 ml) saliva samples during our investigation of the hypocalcemic activity[7–9] in the human salivary secretion.

Materials

Collection of saliva. Unstimulated whole human saliva was obtained from healthy laboratory personnel, by expectoration into a cooled vessel containing Thymol crystals to inhibit bacterial growth.

To avoid excessive variation due to diurnal fluctuation, all samples were obtained in the evening (8.30–10.00 p.m.), 2 h after the evening meal following normal dental hygiene. Prior to sample collection the oral cavity was thoroughly rinsed with distilled water. Immediately after collection the samples were cleared by centrifugation at 27 000 g for 30 min in a refrigerated Sorvall RC2-B (S-34 Rotor) centrifuge and the supernatant was analyzed for protein by the method of LOWRY *et al.*[10] using crystalline bovine serum albumin as the standard.

In some instances, for comparative purposes, the cleared saliva was dialyzed (Visking cellulose tubing, pore size 24Å) against either deionized, glass-distilled water or the background electrolyte at 4° for 18 h prior to fractionation.

Sephadex G-100 and the chromatographic columns were products of Pharmacia Fine Chemicals Co.; the reagents for polyacrylamide electrophoresis were purchased from Canalco, Rockville, Md., U.S.A.

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* Supported (in part) by National Institutes of Health Special Research fellowship (1 FO3 DE45781-01) to C.A.B. from the Dental Research Institute and FIC grants (561 and 562) from Colorado State University. Portions of this work were carried out during the tenure of a postdoctoral fellowship (5-F2-DE-25, 879-02) to R. M. S. from the National Institute of Health, Dental Research Institute.

Methods

Preparative electrophoresis. For CFPE, two Spinco model CP continuous flow paper electrophoresis cells were used. All separations were carried out in a cold room (4°) and the two CP cells run in parallel, thus permitting careful observation of the effects of a given variable (voltage, sample load, electrolyte, etc.) on the resolving power of the preparative procedure. The first fifty-five samples were utilized in the development of a set of experimental conditions (described below) which produced the most efficient separations with either veronal* or borate buffer as background electrolytes.

Electrophoresis was carried out as described by BLOCK *et al.*[11] with the following instrumental settings: potential, 740 V (constant); current, 56–60 mA; sample flow, 1.4 ml/h; reservoir height, 8 cm; wick flow, balanced; sample tab, 10 cm from cathode side; electrolyte, borate buffer pH 9.0 (ionic strength 0.02).

When using veronal (pH 8.6, $\mu = 0.02$) as the electrolyte, optimal resolution was achieved under the following instrumental settings: potential, 960 V (constant); current, 86–90 mA; sample flow, 0.7 ml/h; reservoir height, 8 cm; wick flow, balanced; sample tab, 10 cm from cathode side. In all instances, a Schleicher and Schüll 470 paper curtain was used. The temperature within the electrophoretic cells was maintained constant (4 ± 0.05°) by recirculating both coolant and background electrolyte with Lauda (model TK-30SH) refrigerated circulators.

The relative distribution of protein in the various fractions was determined from the absorbancy at 280 mμ, using a Beckman DB-GT recording spectrophotometer. Individual protein peaks were pooled and concentrated by the addition of dry Sephadex G-25 (coarse)[12] or by lyophylization after a 24-h dialysis.

Gel filtration. Columns (2.5 × 45 cm) were packed to a bed height of approximately 38 cm with Sephadex G-100 which had been thoroughly equilibrated and defined (seven days) with 0.1 *M* Tris–HCl, 0.1 *M* NaCl buffer, pH 8.6. Fractions were collected with an ISCO (Lincoln, Nebr.) fraction collector model 327 and continuously monitored at 254 mμ by means of a UV recording analyzer (ISCO, UA-2).

Polyacrylamide electrophoresis. Pooled fractions were identified and compared with whole human saliva by means of gel electrophoresis. This procedure was carried out according to the method of ORNSTEIN AND DAVIS[13] at a pH of 8.2–8.4 with 7.5% acrylamide in the gel preparation; 40 μl of each fraction were subjected to a current of 3 mA for approximately 60 min. The gels were stained with Amido Schwarz and destained with 15% acetic acid. With the exception of salivary immunoglobulins, no attempt was made to characterize individual protein components in this study. However, qualitative tests for lysozyme[14] and α-amylase[15] were carried out on pooled fractions obtained after CFPE or gel filtration.

Immunoelectrophoresis. The salivary immunoglobulins were identified according to the method of GRABAR AND WILLIAMS[16] applying the microtechnique of SCHEIDEGGER[17] without further modification.

Results and discussion

Preparative electrophoresis. As shown in Fig. 1, continuous flow electrophoresis in borate buffer resolved the cleared saliva into six major protein fractions: (1) a

* Abbreviations used in this work: veronal = sodium diethyl barbiturate–barbituric acid buffer, pH 8.6; borate = sodium tetraborate–boric acid buffer, pH 9.0; I_GA, I_GG and I_GM = immunoglobulins A, G and M, respectively.

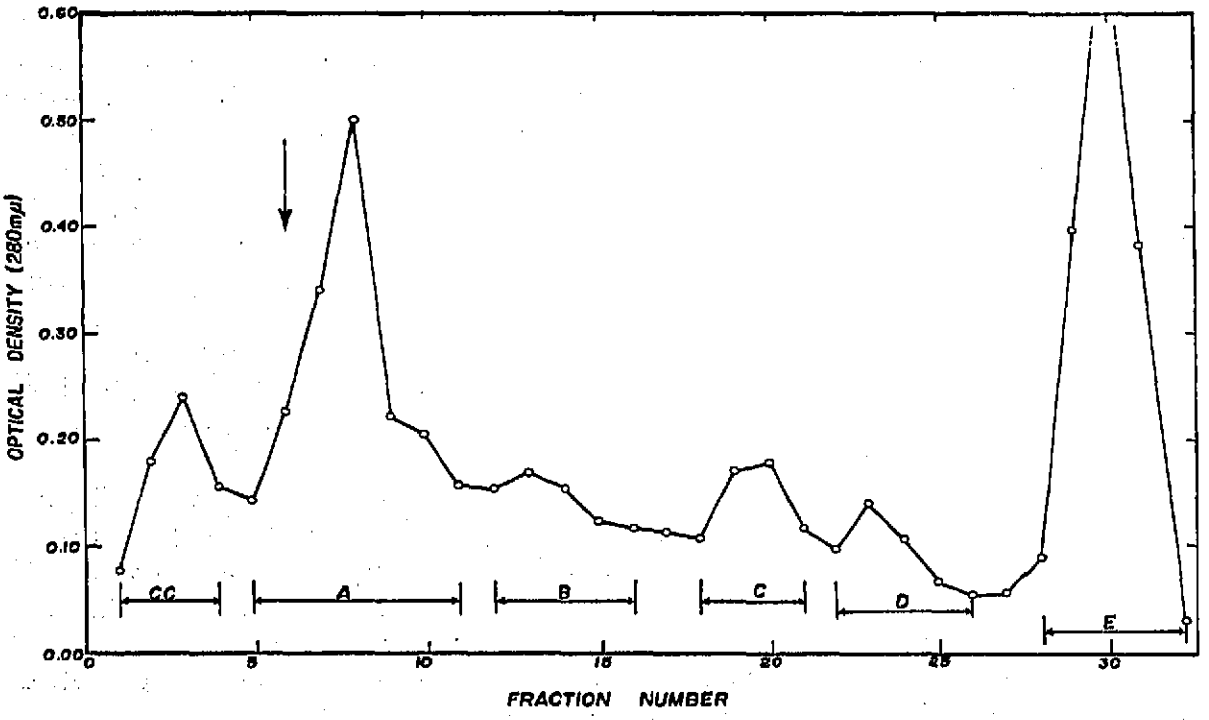

Fig. 1. Preparative electrophoresis of whole human saliva in borate buffer, pH 9.0. 120 ml of the cleared saliva (101 mg protein) were applied to the curtain at the point indicated by the arrow.

cathodic component (CC) (tubes 1–4); (2) fraction A (tubes 5–11), a very large component with mobility similar to serum proteins in the gamma region constituting about 10–20% of the total salivary proteins; (3) fraction B (tubes 12–16), containing proteins with mobility comparable to that of the beta components in serum; (4) fraction C (tubes 18–21), proteins with alpha-2 mobility; (5) fraction D, proteins exhibiting mobilities comparable to both albumin and alpha-1 region of serum;

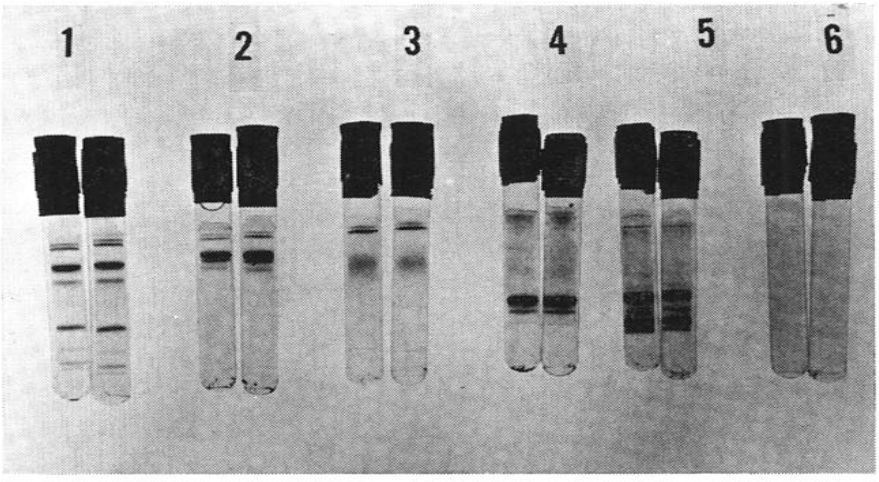

Fig. 2. Polyacrylamide electrophoresis of the relative distribution of protein fractions obtained by continuous flow electrophoresis of cleared human saliva, borate buffer, pH 9.0, μ = 0.02. 1 = whole human saliva; 2 = fraction A; 3 = fraction B; 4 = fraction D; 5 = fraction D; 6 = fraction E.

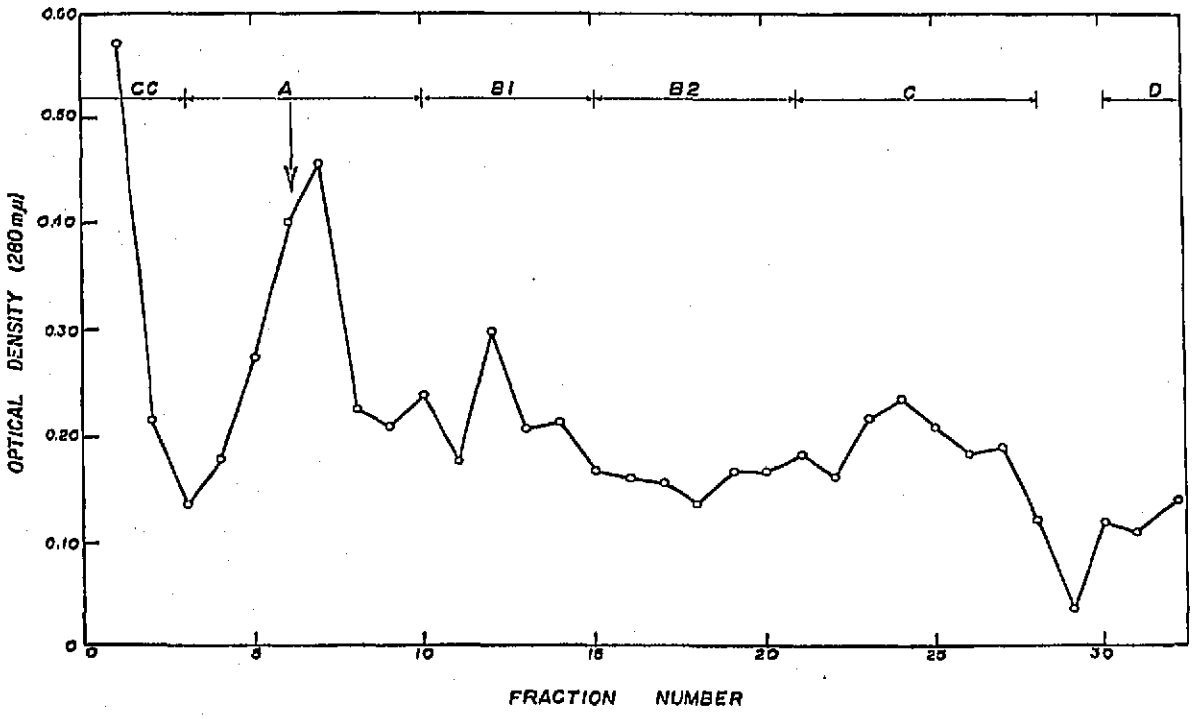

Fig. 3. Preparative electrophoresis of whole human saliva in veronal buffer, pH 8.6, $\mu = 0.02$. 120 ml of the cleared saliva (120 mg protein) were applied to the curtain at the point indicated by the arrow.

and (6) fraction E, an anodal, histidine-rich, non-dialyzable component with pre-albumin mobility the nature of which was not been elucidated.

While lysozyme actitivy was restricted to the cathodic component (CC), amylase activity was present in fractions A, B and C, decreasing in that order. The salivary immunoglobulins were present in both fractions A and B and a great deal of overlap was also seen in the albumin and pre-albumin components, fractions C and D.

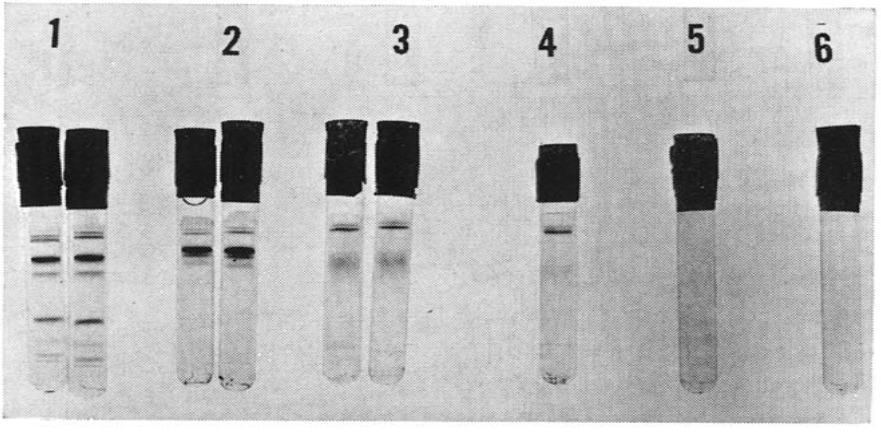

Fig. 4. Polyacrylamide electrophoresis of the relative distribution of protein fractions obtained by continuous flow electrophoresis of cleared human saliva, veronal buffer, pH 8.6, $\mu = 0.02$. 1 = whole human saliva; 2 = fraction A; 3 = fraction B_1; 4 = fraction B_2; 5 = fraction C; 6 = fraction D.

Polyacrylamide electrophoretic patterns (Fig. 2) obviate the fact that even under optimal conditions for separation, a high degree of heterogeneity and cross-contamination occurred in all the fractions obtained by CFPE.

Very poor recovery of cathodic, as well as albumin, pre-albumin and components with alpha-mobility (fraction D) could be achieved (Figs. 3 and 4)using veronal buffer due to severe loss of protein to the side wicks, even under optimal equilibrium conditions. A drop in potential (to 650 V) avoided this protein loss but then no effective separation was obtained. The resolution of fractions A from B (B_1 in veronal) was, however, not affected by the use of either veronal or borate buffers and the electrophoretic patterns were essentially identical. Therefore, for the separation of these fractions either of the two electrolytes can be effectively used.

As pointed out by APOSTOLOPOULOS[4], it is likely that better separations with borate buffer are due to formation of charged complexes between the carbohydrate moiety of salivary glycoproteins and the ionic forms of borate. Another possibility for the increased angular displacement in borate buffer would be the formation of multiple zones by borate–protein interactions as described by CANN[18].

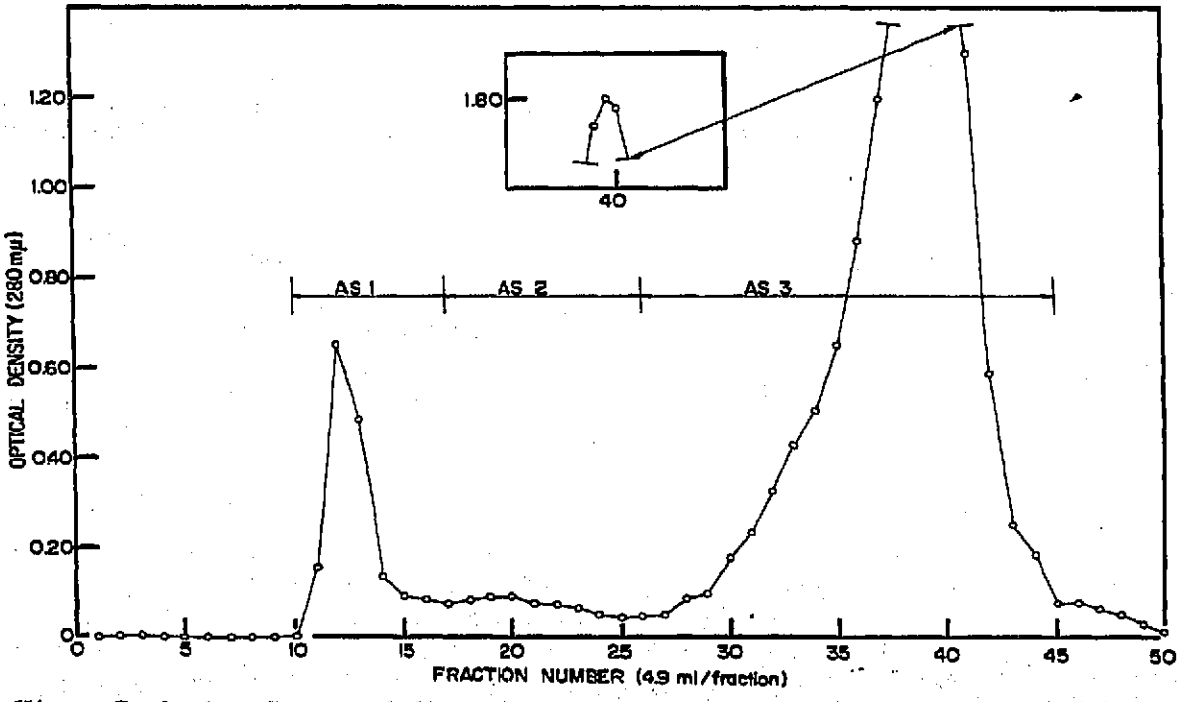

Fig. 5. Sephadex G-100 gel filtration of fraction (A see Fig. 1), partly purified by preparative electrophoresis. Column dimensions and conditions for elution are reported in the text.

Gel filtration. Exclusion chromatography of fraction A on a 2.5 × 38 cm column of Sephadex G-100 revealed three different components (Fig. 5) (1) fractions AS_1 (tubes 11–17), containing proteins completely excluded from the gel matrix; (2) fraction AS_2 (tubes 18–26); and (3) fraction AS_3 (tubes 17–48), containing the bulk of the protein in fraction A. While fractions AS_2 and AS_3 had amylase activity, the salivary immunoglobulins were restricted to the excluded fraction (AS_1). The gel electrophoretic patterns are shown in Fig. 6. Similar elution patterns were obtained when fraction B was chromatographed on Sephadex G-100.

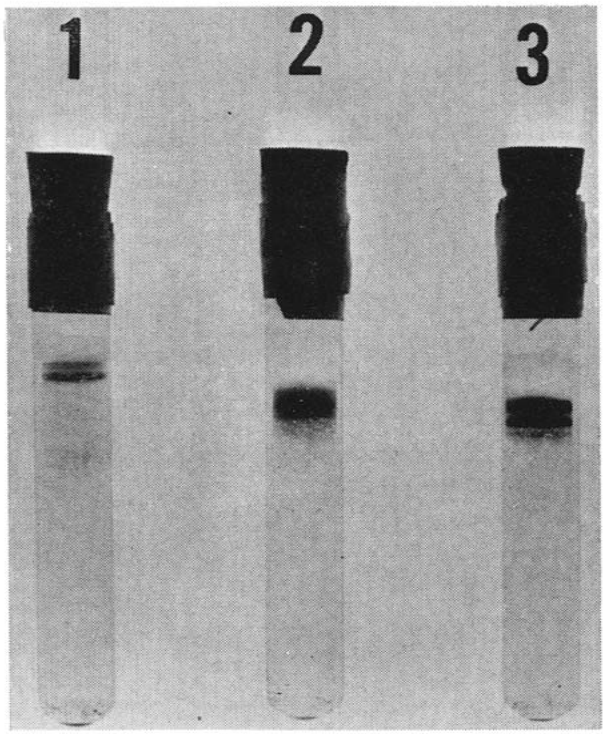

Fig. 6. Polyacrylamide electrophoresis of fractions obtained by purification of fraction A after gel filtration chromatography on Sephadex G-100. 1 = fraction AS_1; 2 = fraction AS_2; 3 = fraction AS_3.

Immunoelectrophoresis. Results of immunoelectrophoretic analysis of fraction AS_1, obtained by gel filtration of fraction A, are shown in Fig. 7. Both I_GA and I_GG immunoglobulins were found in this fraction. Although not shown in Fig. 7, I_GM was also found, consistently, in the mixed saliva of two normal individuals.

Conclusions

When using CFPE as a preparative procedure for the fractionation of human mixed saliva, the following points should be considered.

Disadvantages. (1) Equipment is bulky, expensive. (2) Temperature control of background electrolyte and coolant is an absolute necessity. (3) Resolution is poor, even under maximal equilibration conditions. (4) Flow rates must be kept low (*i.e.* 1.4 ml/h) for optimal separation; consequently, considerable amounts of time are required to process large sample volumes. (5) Not useful for the fractionation of parotid saliva.

Advantages. (1) Mixed saliva can be fractionated in its native state without prior concentration (avoids lyophilization or negative pressure dialysis). (2) Dialysis against distilled water or background electrolyte for up to 18 h does not significantly improve the separation; therefore, the dialysis step is not necessary. (3) Mucin clot formation is avoided by the continued dilution of the salivary fluid with the background electrolyte. (4) Electrophoresis is carried out under very mild conditions and protein denaturation is minimal. (5) CFPE in either veronal or borate buffers when followed by Sephadex G100 or G150 gel filtration allows very efficient isolation of the

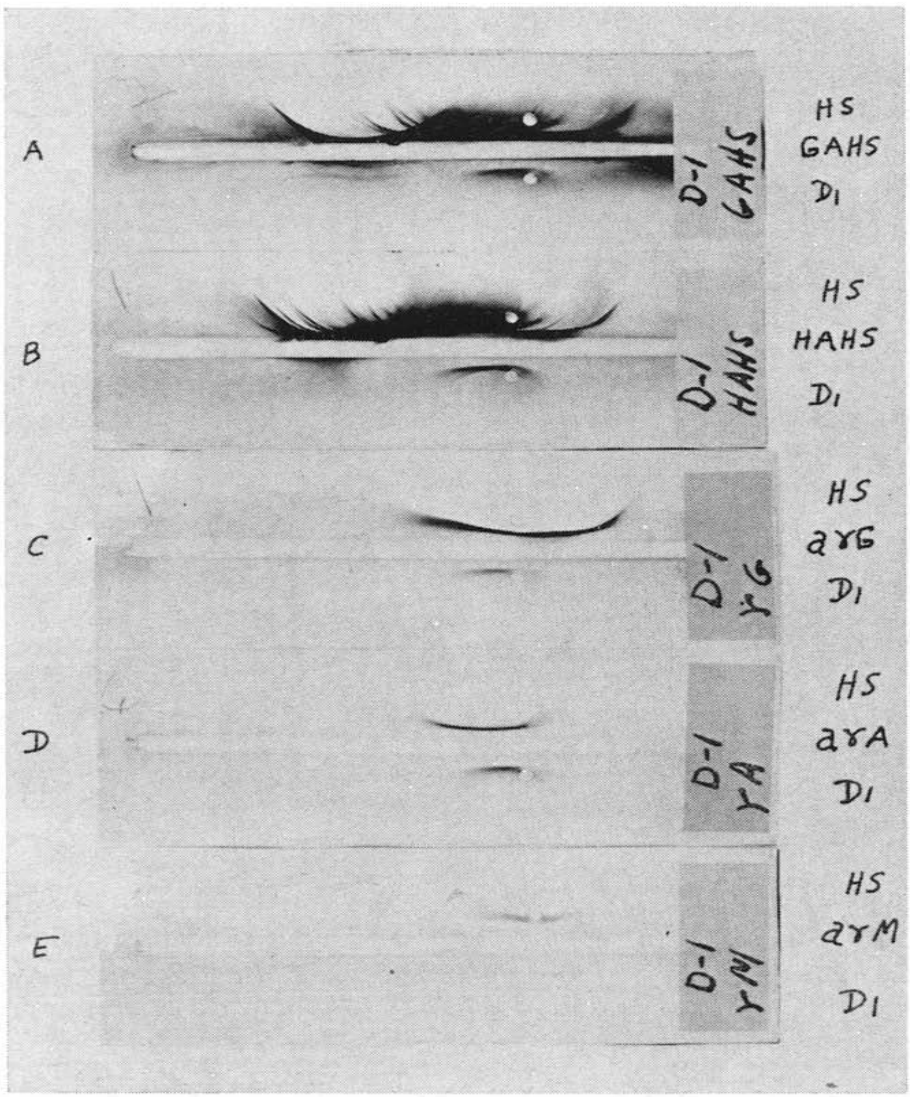

Fig. 7. Immunoelectrophoretic analysis of fraction AS_1. A: HS = normal human serum, GAHS = goat anti-human serum antiserum, D_1 = first peak eluted after exclusion chromatography of fraction A on Sephadex G-100. B: HAHS = horse anti-human serum antiserum. C: aγG = goat anti-G globulin antiserum. D: aγA = goat anti-A globulin antiserum. E: aγM = goat anti-M globulin antiserum.

salivary immunoglobulins, I_GA and I_GG. (6) CFPE using borate buffer (but not veronal) achieves a rapid and efficient preliminary purification and separation of components with albumin and pre-albumin mobility from the amylases, immunoglobulins and the components with mobilities comparable to the B fraction in serum.

In conclusion, the advantages of CFPE for the preparative fractionation of the human mixed salivary fluid far outweigh its disadvantages. In particular, the avoidance of mucin clot formation, the mildness of the procedure and the ability to par-

tially fractionate the saliva in its native state without prior dialysis or lyophilization make this a useful, if not convenient, method of separation.

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Received June 3rd, 1971

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