Am J Hum Genet 27:304–314, 1975

Partial Purification and Characterization of a Polymorphic Protein (Pa) in Human Parotid Saliva

ROBERT D. FRIEDMAN[1] AND A. DONALD MERRITT[2]

INTRODUCTION

A number of human parotid salivary proteins appear to be under genetic control according to electrophoretic evidence [1]. Azen [2] recently detected a genetic polymorphism associated with parotid basic proteins in an acid-urea starch gel electrophoretic system. Utilizing a similar system, Friedman et al. [3] observed an acidic salivary protein, Pa, present in whole parotid and submandibular saliva of certain individuals.

Electrophoretic, population, and genetic studies of the Pa protein have demonstrated it to be inherited as an autosomal dominant trait with a Caucasian gene frequency of .21 [4]. Similarities between the products of the Pr and Pa loci infer an association [4] which can be studied through biochemical comparisons of the respective gene products. This report deals with the purification and partial characterization of the Pa protein.

MATERIALS AND METHODS

Collection and preparation of saliva samples. Parotid saliva samples (5 ml) were collected in glass test tubes, rapidly shell frozen, lyophilized, and resuspended at 2.5 to five times the original concentration [4]. Large parotid saliva samples for biochemical studies were frozen in 25 ml portions, lyophilized, and stored at −20° C.

Acid-urea starch gel electrophoresis. The gel system of Sung and Smithies [5] was used [4]. In order to increase the resolution of anodal protein bands, the electrophoretic running time was extended. It was discovered that cobalt nitrate could be eliminated from the staining solution without affecting resolution of the Pa band.

Ion-exchange chromatography. DEAE-cellulose (Eastman Organic Chemicals, Rochester, N.Y.) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.5; a 50-ml buret was used as a supporting column. Lyophilized samples of 50–100 ml of parotid saliva were dissolved in 10–20 ml buffer, dialyzed against the buffer, and centrifuged at 1,000 *g*. The clear supernatant was applied to the column. Small portions of the variant protein were lost in the precipitate. This was ameliorated by dialyzing freshly collected parotid saliva

Received April 3, 1974; revised December 6, 1974.

Publication no. 74-02 from the Department of Medical Genetics. This work was supported by U.S. Public Health Service grants P01 GM 21054 and 19178.

[1] Department of Biochemistry, Temple University School of Dentistry, Philadelphia, Pennsylvania 19140.

[2] Department of Medical Genetics, Indiana University Medical Center, Indianapolis, Indiana 46202.

against 0.05 M Tris-HCl buffer (pH 7.5) and applying the sample directly to the column during column preparation to reduce the clogging effect of mucous proteins [6]. The first method was generally simpler and was used since the loss of variant protein was not substantial. Following sample application, the column was exhaustively eluted with equilibration buffer.

A combination stepwise and linear ionic gradient was then applied. The first step was performed with 0.05 M Tris-HCl (pH 7.5) and 0.15 M NaCl until negligible absorbance could be recorded at 222 nm. Then the continuous gradient consisting of two 100-ml cylinders of 0.05 M Tris-HCl (pH 7.5), one of which had 0.4 M NaCl as the source of the ionic gradient, was initiated. Samples of 5.5 ml were collected.

Gel filtration. Pooled samples from DEAE-cellulose chromatography were dialyzed overnight at room temperature against a constantly changing distilled water supply. Ascending chromatography on Sephadex G-200 (Pharmacia, Sweden) prepared in a 0.5 M ammonium bicarbonate buffer, pH 7.9, was employed using a 2.5 × 84-cm column. Fractions of approximately 5.5 ml were collected at a flow rate of 20 ml/hr. The void volume, determined with Blue Dextran 2000, was 129 ml.

Analytical disc gel electrophoresis. The anionic system described by Davis [7] was employed using 7.5% polyacrylamide gels. Samples were subjected to electrophoresis for about 35 min at 2 mA per gel and then for another 45 min at 3 mA per gel. The gels were stained with 1% Amido Black in 7% acetic acid and destained in a solution of 5% acetic acid for a few days.

Isoelectric focusing. LKB column 8101 (LKB Instruments, Inc., Chicago, Ill.) with a sucrose density gradient was prepared according to the manufacturer's directions; 2.5 ml ampholyte, pH 3–10 (Ampholine, LKB), was used. Parotid saliva (42 ml) was dialyzed against distilled water at 4° C and loaded onto the column during preparation. Focusing was carried out at 4° C to a final maximum voltage of 900 V (48 hr). Fractions of 3.8 ml were collected at a flow rate of 1–2 ml/min; pH was measured at 4°C with a type TTT 1c Radiometer pH meter with scale expander.

Analytical assays. Protein was estimated by the method of Lowry and co-workers [8] with bovine serum albumin as standard. Examination for carbohydrate was performed by the method of Dubois et al. [9].

Amino acid analysis. Dialyzed and dried protein samples were hydrolyzed at 100° C in distilled 6 N HCl for 18–24 hr in evacuated sealed tubes. Amino acid analyses were performed on a Phoenix amino acid analyzer. Acid hydrolyses at 48 and 72 hr were not performed due to the limited amount of material isolated.

RESULTS

Concentrated parotid saliva samples collected at intervals during the day from a Pa(+) individual were tested by electrophoresis on acid-urea starch gels (fig. 1). All Pa(+) individuals invariably secrete the Pa protein; its concentration relative to other salivary proteins varies slightly as the proportion of the total protein secreted. Saliva collected from Pa(—) subjects at different times of the day and on different days never revealed Pa protein.

Parotid saliva from a Pa(+) individual was applied to a Sephadex G-200 column prior to ion-exchange chromatography on DEAE-cellulose (fig. 2). Pooled chromatographic eluates were then tested by electrophoresis in an acid-urea starch gel (fig. 3). The Pa protein was enriched in fraction 40–47. This pooled sample also contained glycoproteins and other more basic proteins which could be excluded by purification procedures on DEAE-cellulose.

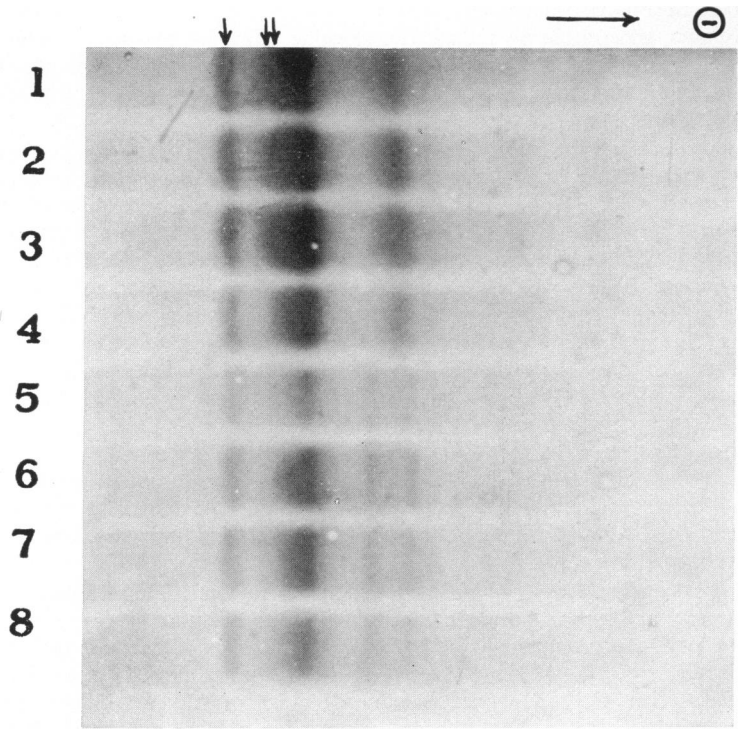

FIG. 1.—Acid-urea starch gel electrophoresis (pH 2.4) of parotid saliva from two Pa(+) individuals, J. F. (*samples 1–4*) and L. C. (*samples 5–8*), collected at different times of the day. *Samples 1 and 5,* 9 A.M.; *samples 2 and 6,* 11:30 A.M.; *samples 3 and 7,* 1 P.M.; *samples 4 and 8,* 4 P.M. Single and double arrows indicate positions of Pa(+) and Pa-II bands, respectively.

Acid-urea starch gel electrophoresis of DEAE-cellulose fractions identified the Pa protein in the pooled continuous gradient region (approximately 0.2 M NaCl) along with another more cathodally migrating protein designated Pa-II (figs. 4 and 5).

Separation of the Pa(+) and Pa-II proteins was achieved by gel filtration on Sephadex G-200 as shown in figure 6. Acid-urea starch gel electrophoresis of pooled fractions S-I and S-II are enriched for Pa and Pa-II proteins (fig. 7).

Anionic polyacrylamide disc gel electrophoresis of these pooled fractions was also performed (fig. 8). The least retarded Sephadex fraction, S-I, showed a single discrete protein at a position similar to protein observed in disc electrophoresis of Pa(+) parotid saliva. Fraction S-II showed two faster migrating proteins on disc gel electrophoresis, while S-II appeared to be a single band on electrophoresis in an acid-urea starch gel.

The amino acid analyses of Pa and Pa-II proteins are shown in table 1. Similarities are apparent in the types of amino acids present in both proteins. A high

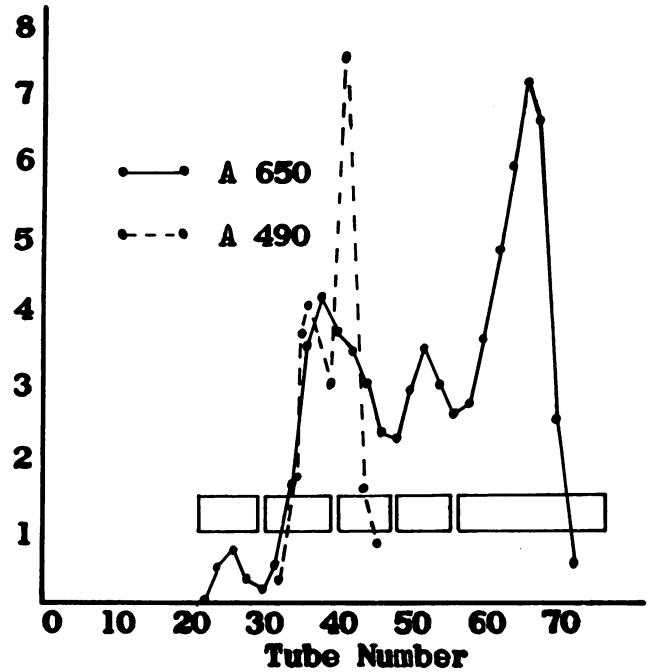

FIG. 2.—Gel filtration chromatography on Sephadex G-200 of Pa(+) parotid saliva from individual G. K. Buffered parotid saliva (50 ml) was applied to column, and 5.5 ml per tube was eluted. *Solid line,* Protein measured by method of Lowry et al. [8]; *dashed line,* total carbohydrate estimated by method of Dubois et al. [9]; *open rectangles,* fraction on which acid-urea starch gel electrophoresis performed (see fig. 3). Recorded as absorbance per milliliter.

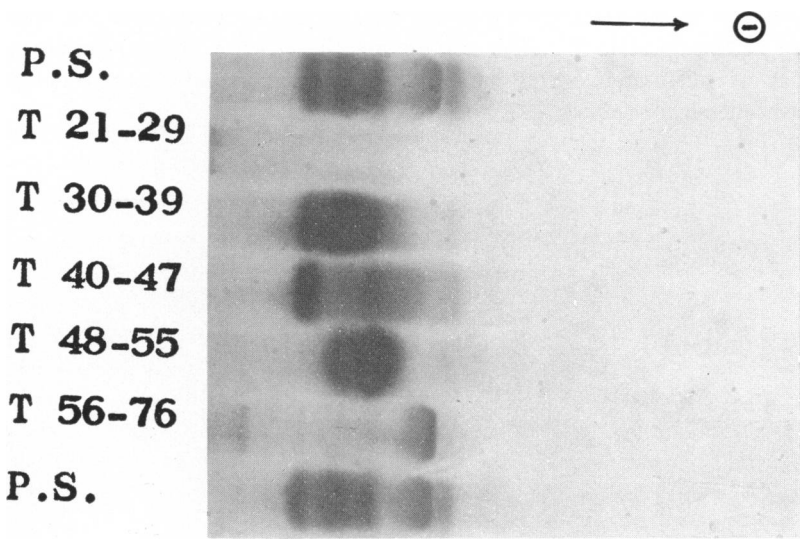

FIG. 3.—Acid-urea starch gel electrophoresis performed on pooled fractions shown in fig. 2. P.S. = Pa(+) parotid saliva prior to fractionation.

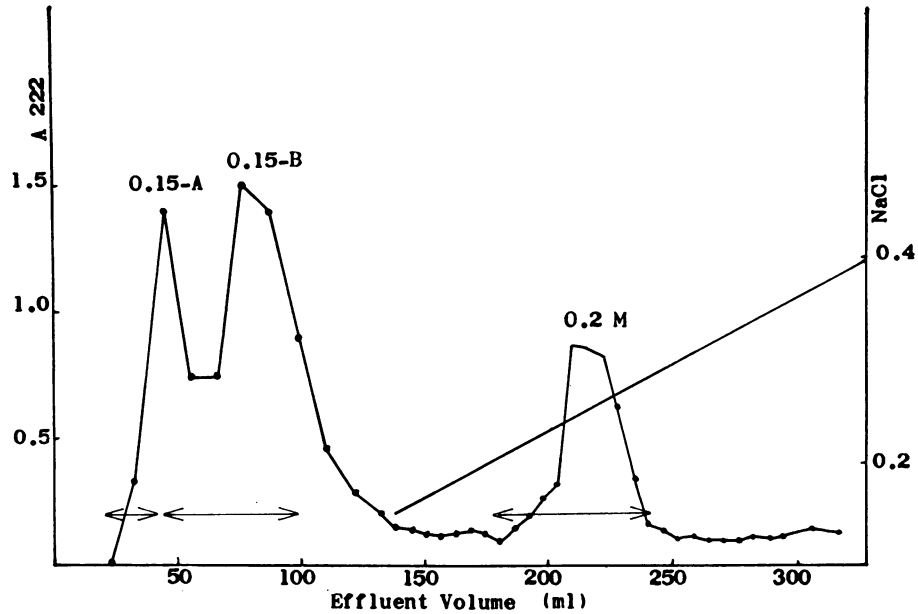

FIG. 4.—Stepwise and continuous linear ionic-gradient chromatography on DEAE-cellulose. Buffered parotid saliva (45 ml) was applied to column. Pooled regions are designated 0.15 M—A, 0.15 M—B, and 0.2 M NaCl.

proportion of proline, glutamic acid (includes glutamine), and glycine is present in both proteins. Differences between these two proteins include the relatively high amount of aspartic acid (includes asparagine) in Pa-II and the very small amount of threonine in Pa. Also notable are the absence of hexosamines and the presence of small amounts of carbohydrate (approximately 1.6%) detected by sensitive chemical analyses [9].

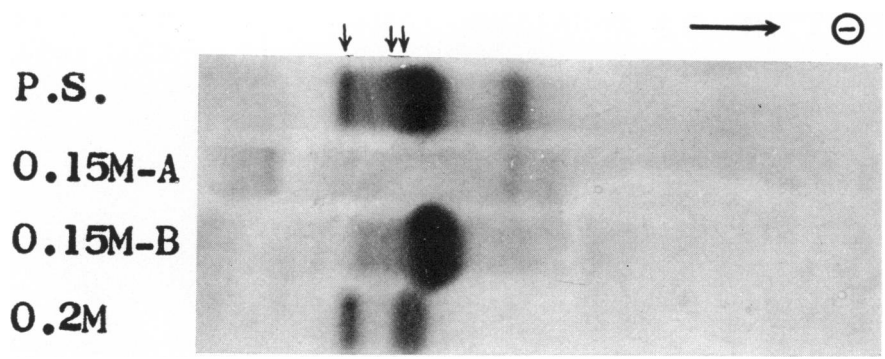

FIG. 5.—Acid-urea starch gel electrophoresis of pooled DEAE-cellulose fractions (see fig. 4). Single and double arrows indicate positions of Pa(+) and Pa-II bands, respectively.

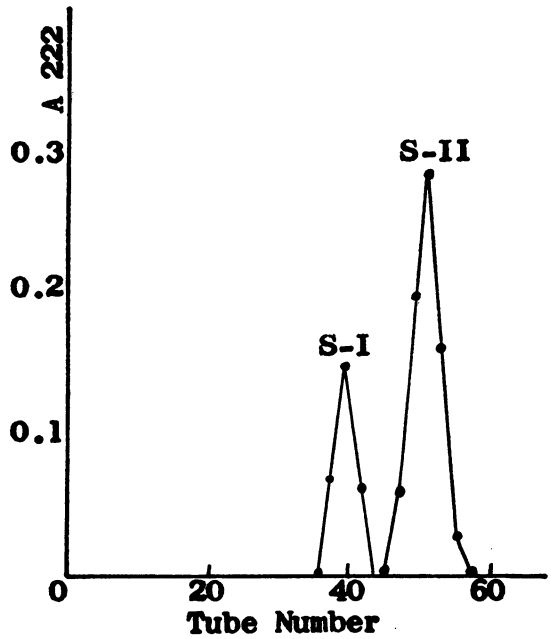

FIG. 6.—Gel filtration chromatography on Sephadex G-200 of pooled continuous linear gradient DEAE-cellulose fraction (0.2 M NaCl).

Isoelectric focusing of Pa(+) parotid saliva (fig. 9) confirmed the anionic character of the Pa protein. Acid-urea starch gel electrophoresis of pooled fractions from isoelectric focusing shows that Pa protein is primarily in one sample (tubes 7–9, fig. 10). The isoelectric point then lies within the pH range 3.9–4.5.

DISCUSSION

Using the acid-urea starch gel system of Sung and Smithies [5], an autosomal dominant polymorphism of an acidic salivary protein (Pa) was noted as the prominent anodal fraction on electrophoresis [3]. Genetic studies of this polymorphism

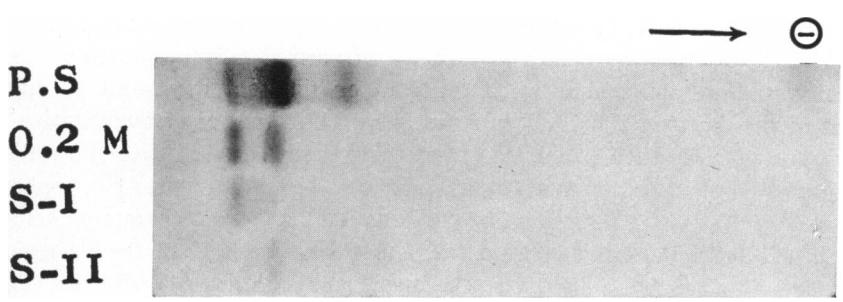

FIG. 7.—Pooled Sephadex fractions S-I and S-II after acid-urea starch gel electrophoresis (see fig. 6). P.S. = Pa(+) parotid saliva.

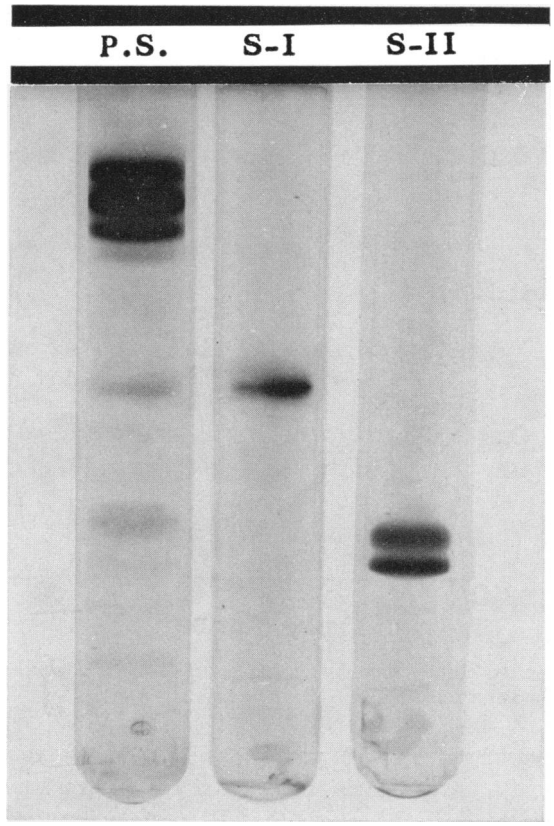

FIG. 8.—Anionic polyacrylamide disc gel electrophoresis of Sephadex G-200 pooled samples S-I and S-II. Sample P.S. contains approximately 350 μg of parotid fluid protein from a Pa(+) individual; S-I and S-II contain 60 and 110 μg protein, respectively.

and a comparison to the Pr protein system described by Azen and Oppenheim [10] and Azen and Denniston [11] have been reported [4]. While Pa may be detected in whole, submandibular, and parotid saliva, the latter was used for the present studies. The Pa region from individuals phenotypically designated as Pa(+) is physiologically reproducible when typed by acid-urea starch gel electrophoresis. Constituting approximately 9% of parotid salivary proteins, it shows minor variations in amount when collected on different days and at different times of the day (see fig. 1). While possible genetic control of quantitative variation has not yet been evaluated, samples from individuals classed as Pa(—) never show protein in this most anodal position when subjected to electrophoresis.

Isolation of Pa was performed by Sephadex G-200 chromatography. Acid-urea starch gel electrophoresis of pooled fractions showed protein in the Pa region in tubes 30–47 (fig. 3). Subsequent chromatography on DEAE-cellulose (fig. 4) provided a fraction at 0.2 M NaCl enriched in two proteins, one migrating in the Pa region and another migrating more anodally, Pa-II. The 0.2 M NaCl fraction

TABLE 1

AMINO ACID COMPOSITION OF Pa AND Pa-II PROTEINS

Amino Acid	Pa (S-I)	Pa-II (S-II)
Asp*	21	128
Thr	2	47
Ser	72	52
Pro	223	172
Glu*	197	228
Gly	185	178
Ala	22	29
Val	63	28
Met	Trace	0
Ile	34	13
Leu	55	42
Phe	27	15
Tyr	0	0
Lys	18	16
His	26	21
Arg	53	34

NOTE.—Composition given in residues per 1,000.
* Includes deamidated and free amino acids.

from DEAE-cellulose was then chromatographed on Sephadex G-200, yielding two peaks designated S-I and S-II. In acid-urea starch gels each of the two protein regions, Pa (S-I) and Pa-II (S-II), contained what appeared to be a single species (fig. 7). Subsequent disc electrophoresis of the S-I and S-II peaks compared with unfractionated parotid saliva showed S-I to consist of a single band and S-II to consist of two faster migrating proteins (fig. 8). It is important to note that ion-exchange chromatography neither affected the electrophoretic behavior nor the elution profile obtained with Sephadex G-200 separation of Pa proteins.

The behavior of Pa (S-I) and Pa-II (S-II) on acid-urea starch gels, disc electrophoresis, and Sephadex G-200 gel chromatography suggests that Pa and Pa-II are different. Amino acid analysis reveals a relatively higher amount of aspartic acid in Pa-II and only a small amount of threonine in Pa (see table 1). On the other hand, a high proportion of proline, glutamic acid, and glycine is present in both proteins.

A comparison of the amino acid composition of Pa and Pa-II with the results obtained by Oppenheim et al. [12] shows that Pa-II most closely resembles their proteins III or IV, more recently defined by Azen and Oppenheim [10] as a genetic polymorphism, Pr (proline-rich salivary protein). Since Pa-II produces two bands on disc electrophoresis, it is possible that Pa-II and Pr 3 and 4 are the same. Their electrophoretic positions are certainly similar.

Disimilarities between Pa and Pr 1, 2, 3, and 4 (previously referred to as Pr I, II, III, and IV [12]) have now been shown in acid-urea starch gels and polyacrylamide [4]. We currently do not have a satisfactory explanation for the quan-

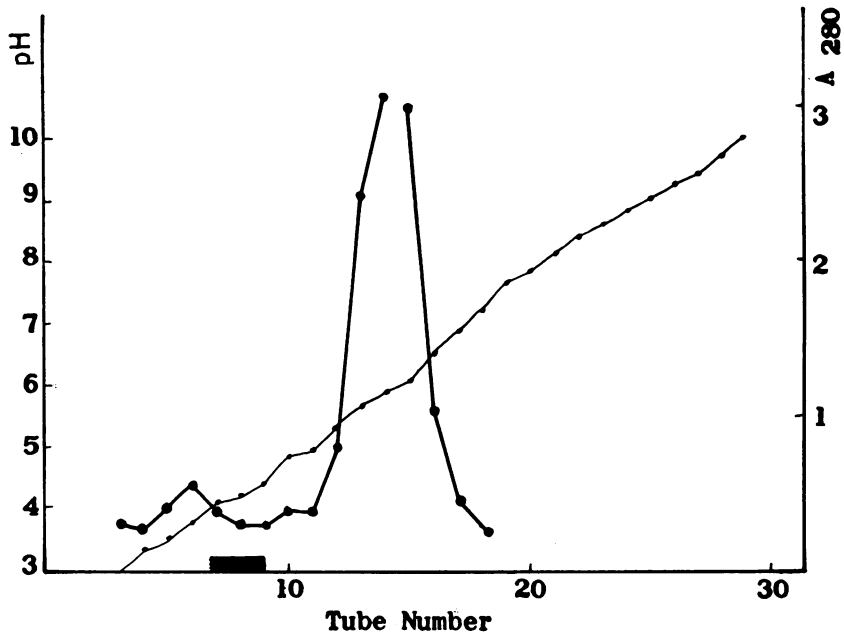

FIG. 9.—Isoelectric focusing of 40 ml of Pa(+) parotid saliva from subject J. F. *Light line,* pH gradient; *heavy line,* absorbance at 280 nm; *solid rectangle,* protein-enriched fractions.

titative discrepancy in threonine found between the S-II (Pa-II) and Pr 3 or 4 proteins. According to Oppenheim et al. [12], the Pr 3 and 4 proteins are very similar in amino acid composition, although Pr 4 may be somewhat higher in threonine.

Substantial similarities in amino acid composition between Pa(+) and the four "proline-rich" proteins are notable, especially the very small amount of threonine. An essential difference is the presence of some carbohydrate in our samples. Such variable carbohydrate findings are not uncommon when different techniques are used for detection. The slight retardation of Pa on Sephadex G-200 chromatography provided some indication of its molecular weight.

An important distinction between Pa (S-I) and Pa-II (S-II) is their apparent difference in molecular size (fig. 6). Fraction 40–47 contains most of the Pa material, while fraction 48–55 contains little Pa but a great deal of Pr material (S-II).

Following isoelectric focusing of Pa(+) saliva (figs. 9 and 10), only fraction 3–6 showed predominantly S-II (Pr 3 and 4) material, suggesting that these proteins are more acidic. Fraction 7–9 showed three bands, indicating closeness in their isoelectric points. Therefore, Pa and Pr 1 and 2 have similar isoelectric points but appear to differ in molecular size; Pa and S-II (Pr 3 and 4) differed appreciably in both size and charge. It is interesting to speculate whether molec-

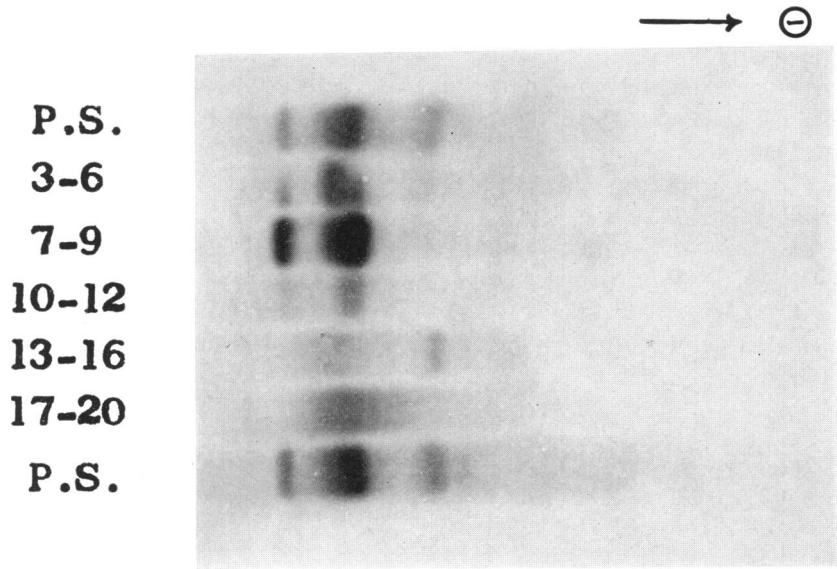

FIG. 10.—Acid-urea starch gel electrophoresis of pooled samples from isoelectric focusing designated by tube number (see fig. 9). Pa is proximal protein. P.S. = parotid saliva from Pa(+) individual prior to treatment.

ular size differences may be more apparent than real, since these salivary proteins may have an unusual molecular configuration that is predominantly linear causing early elution from Sephadex G-200. Accordingly, the apparent molecular weight of Pa is in the range of 50,000–150,000 daltons from our Sephadex G-200 results. On the other hand, Oppenheim et al. [12] have suggested a range in molecular weight of 30,000–50,000 for proline-rich proteins based on Sephadex G-75 studies; however, lower values of 12,300 (Pr 1) and 6,100 (Pr 3) were obtained by sedimentation equilibrium. Aside from the possibility of aggregation, the discrepancies in their results were not explained [12].

It now appears that the Pa proteins isolated in these studies are nonrandomly associated with the Pr 1, 2, 3, and 4 proteins [4]. Further, Pa is similar to, or identical with, new Pr proteins at position "X" [11], migrating cathodal to Pr 1, 2, 3, and 4 in an anionic polyacrylamide gel.

As yet, the functions of these proline-rich salivary proteins have not been elucidated. Although some of these proteins appear to differ in molecular size, charge, and immunological properties [12, 13], their physiological and genetic relationships are worthy of careful consideration. It is difficult to distinguish between the existence of multiple alleles producing many closely related proteins and multiple loci arising from gene duplication producing products of similar function. Consequently, complete understanding of the functional properties of these and other related salivary proteins must await further biochemical and genetic studies.

SUMMARY

A polymorphic acidic protein (Pa) has been isolated from human parotid saliva by the use of ion-exchange and gel filtration chromatography. Following these purification procedures, analytical anionic polyacrylamide disc gel electrophoresis revealed a single stainable band. Amino acid analysis demonstrated a protein particularly rich in proline, glutamic acid, and glycine, but with reduced amounts of threonine and no tyrosine. Only a very small percentage of carbohydrate was detected. Isoelectric focusing at pH 3–10 verified the acidic character of this protein with an isoelectric point in the range pH 3.9–4.5. Other salivary proteins called Pa-II, possibly related physiologically and genetically to the Pr system, were also partially purified and studied. Differences were noted between Pa and Pa-II proteins in molecular size and amino acid composition.

ACKNOWLEDGMENTS

We thank Mr. Earl Pearson for performing the amino acid analysis in this study. We are also grateful to Dr. Ed Kravitz for reading and editing the manuscript.

REFERENCES

1. Burgess RC, Connell GE, MacLaren CM, Williams TA: Genetic control of salivary protein composition. *J Dent Res* 45:613–623, 1966
2. Azen EA: Genetic polymorphism of basic proteins from parotid saliva. *Science* 176:673–674, 1972
3. Friedman RD, Newell R, Merritt AD: A new polymorphism in human saliva detected on starch-urea gel electrophoresis (abstr.). *Am J Hum Genet* 24:42a, 1972
4. Friedman RD, Merritt AD, Rivas ML: Genetic studies of human acidic salivary protein (Pa). *Am J Hum Genet* 27:292–303, 1975
5. Sung M, Smithies O: Differential elution of histones from gel-trapped nuclei. *Biopolymers* 7:39–58, 1969
6. Masson PL, Carbonara AO, Heremans JF: Studies on the proteins of human saliva. *Biochim Biophys Acta* 107:485–500, 1965
7. Davis BJ: Disc electrophoresis: inverted method. *Ann NY Acad Sci* 121:404–427, 1964
8. Lowry OM, Rosenbrough N, Farr AL, Randall R: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
9. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F: Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356, 1956
10. Azen EA, Oppenheim FG: Genetic polymorphism of proline-rich human salivary proteins. *Science* 180:1067–1069, 1973
11. Azen EA, Denniston CL: Genetic polymorphism of human salivary proline-rich proteins: further genetic analysis. *Biochem Genet* 12:109–120, 1974
12. Oppenheim FG, Hay DI, Franzblau C: Proline-rich proteins from human parotid saliva. I. Isolation and partial characterization. *Biochemistry* 10:4233–4238, 1971
13. Bennick A, Connell GE: Purification and partial characterization of four proteins from human parotid saliva. *Biochem J* 123:455–464, 1971