Proline-Rich Proteins from Human Parotid Saliva.
I. Isolation and Partial Characterization*

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ABSTRACT: Four proline-rich proteins have been isolated from human parotid gland saliva. Although easily detected on polyacrylamide gel electrophoretograms of gland saliva, these proteins are not readily detectable on gel patterns obtained from whole saliva. All four proteins appear to have similar chemical compositions. Quantitative amino acid analysis of each protein revealed a predominance of the following amino acids: 22–27% proline, 20–22% glycine, and 26–36% glutamic acid and aspartic acid. Amide content was extremely high as determined by a microdiffusion method. Sulfur-containing amino acids could not be detected and of the aromatic residues only phenylalanine was found. Only small amounts of alanine were detected as well. Neither hexosamines nor neutral hexoses are present in significant amounts. The isoelectric points, obtained by isoelectric focusing ranged from 4.1 to 4.7. Analytical ultracentrifugation gave molecular weights of 12,000 for one of these proteins and 6000 for another. All four parotid proteins are susceptible to proteolysis by purified collagenase A from *Clostridium histolyticum*.

Several studies on human salivary proteins employing various electrophoretic techniques have indicated the complexity of this body fluid (Mandel and Ellison, 1963a,b). Because of the difficulties encountered in the isolation and characterization of specific salivary proteins, many of these investigations were aimed at the identification of certain serum proteins in saliva (Fischer *et al.*, 1968; Claman *et al.*, 1967). It is well known that parotid and submandibular secretions are the major contributors to whole saliva. The electrophoretic pattern of human whole saliva on paper as well as acrylamide gels, however, differs distinctly from that of gland saliva (Ellison *et al.*, 1960; Hay, 1969). A group of proteins present in the electrophoretogram of both parotid and submandibular secretions are not readily detected electrophoretically after their release into the oral cavity. The present communication describes the isolation and partial characterization of four chemically similar proteins from parotid saliva which are not detected in the electrophoretogram of whole saliva. These highly soluble proteins exhibit unusual biochemical characteristics. They are rich in the amino acids glycine and proline. Aspartic acid and glutamic acid are also present in high concentration in their amide form. All four proteins are degraded by bacterial collagenase. Certain glycoprotein and mucoprotein preparations from parotid gland saliva have revealed similar amino acid compositions (Levine *et al.*, 1969). In contrast to these results, the isolated proteins described in this communication contain little or no carbohydrate.

Experimental Section

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed according to the method described by Davis (1964) using a separating gel of 7.5% acrylamide. The sample for electrophoresis was prepared by mixing the protein solution, stacking gel buffer, and 40% sucrose in the ratio 1:1:2 and 0.2 ml of this was layered carefully on top of the spacer gel instead of using a sample gel. The proteins were detected by staining with 0.5% Amido-Schwarz in 7% acetic acid for 1 hr, followed by an 18- to 24-hr destaining period in 7% acetic acid. This technique was used following all chromatographic procedures to locate the proteins in the effluent fractions.

Amino Acid Analysis. Protein samples were hydrolyzed in 6 N HCl at 110° for 24 hr in tubes sealed after evacuation. The HCl was removed by evaporation in a vacuum desiccator under reduced pressure over concentrated H_2SO_4 and solid NaOH. Amino acid analyses were performed on a Technicon amino acid analyzer.

Total Nitrogen, Amide Nitrogen, and Protein Determinations. Protein samples were incinerated with H_2SO_4 (concentrated) at 450° for 30 min, diluted with water five times, and total nitrogen content was determined by a modification of the microdiffusion method of Conway (1963). Amide nitrogen was also determined by the microdiffusion procedure after hydrolysis of the protein was carried out in 2 N HCl at 100° for 2 and 8 hr (Wilcox, 1967). Protein concentration was determined by the method of Lowry *et al.* (1951). All analyses were done in duplicate.

Carbohydrate Analyses. Neutral sugars were determined by the anthrone procedure described by Van Handel (1967) using α-1-acid glycoprotein as a standard and amino sugars (glucosamine, galactosamine, and mannosamine) were determined by means of the amino acid analyzer.

Isolation of Proline-Rich Proteins from Parotid Gland Saliva. COLLECTION OF SALIVA. Parotid saliva was collected in ice-chilled containers in 50-ml lots from students (18- to 25-yr old) using the device described by Curby (1953). Salivary flow was stimulated by using lozenges containing citric acid. The saliva was pooled, dialyzed, and freeze-dried. Freeze-dried parotid saliva protein (5 g) was obtained from 1 l. of saliva.
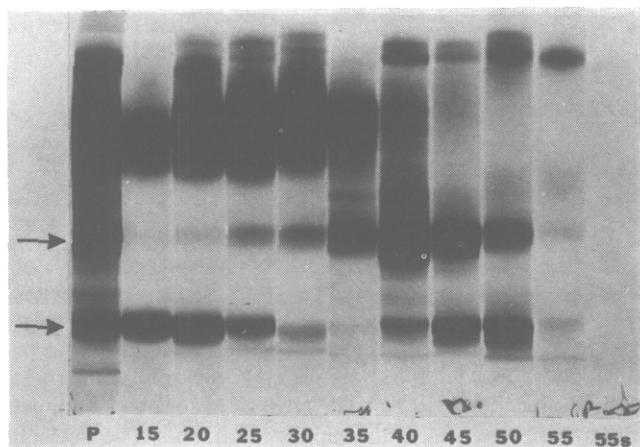
FIGURE 1: Sequential ammonium sulfate fractionation of parotid saliva (P). Previously dialyzed and lyophilized parotid saliva (0.5 g) was dissolved in 5 ml of 0.05 M Tris-HCl buffer (pH 6.8). Solid $(NH_4)_2SO_4$ was added sequentially as indicated in the figure. The precipitates formed at the various $(NH_4)_2SO_4$ concentrations (15–55%) were dissolved in the same buffer and aliquots assayed by polyacrylamide gel electrophoresis. The fraction soluble in 55% $(NH_4)_2SO_4$ (55 S) was dialyzed exhaustively before electrophoresis. The arrows indicate the protein regions containing the proline-rich components.

FIGURE 2: Gel filtration of 35–50% $(NH_4)_2SO_4$ fraction of parotid saliva. See text for details.

This preparation was used for the isolation of the proteins to be described below. All additional procedures were carried out at room temperature.

AMMONIUM SULFATE FRACTIONATION. Freeze-dried parotid saliva protein (5 g) was dissolved in 250 ml of 0.05 M Tris-HCl buffer (pH 8.0) and stirred for 2 hr. A small insoluble residue was removed by centrifugation at 27,000g for 10 min. The supernatant was brought to 35% saturation with solid $(NH_4)_2$-SO_4, equilibrated for 2 hr with stirring, and then centrifuged at 27,000g for 30 min. The clear supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and treated identically. The 35–50% insoluble fraction so obtained was dissolved in 40 ml of 0.05 M Tris-HCl buffer (pH 6.8) and dialyzed against the same buffer, which was used for the gel filtration step.

GEL FILTRATION. Portions (10 ml) of the 35–50% $(NH_4)_2SO_4$ fraction were chromatographed on a Sephadex G-75 column, 70 × 2.5 cm, using a flow rate of 30 ml/hr. Fractions (10 ml) were collected, each fraction being monitored by disc gel electrophoresis. The fractions containing the proline-rich proteins were pooled, concentrated using polyethylene glycol, and dialyzed in the cold against the Tris-HCl buffer employed in the ion-exchange chromatography step.

ION-EXCHANGE CHROMATOGRAPHY. Aliquots (10 ml) of these fractions containing the proline-rich proteins obtained from the gel filtration step were placed on a DEAE-Sephadex A-25 column of dimensions 90 × 1.5 cm employing 200 ml of 0.05 M Tris-HCl buffer (pH 8.5) with a 0.025–0.60 M NaCl linear gradient. The flow rate was 12 ml/hr and 5-ml fractions were collected and monitored both by disc gel electrophoresis and by chloride determination using a Cotlove automatic titrator (American Instruments). This step separated the proline-rich proteins into two regions designated A and B. The fractions constituting region A were pooled, dialyzed, and rerun on another DEAE-Sephadex A-25 column of dimensions 75 × 2.5 cm using an even shallower gradient of 600 ml of 0.14–0.24 M NaCl and a flow rate of 40 ml/hr. Fractions (12 ml) were collected and again monitored by disc gel electrophoresis as well as by chloride determinations. Similarly, those fractions
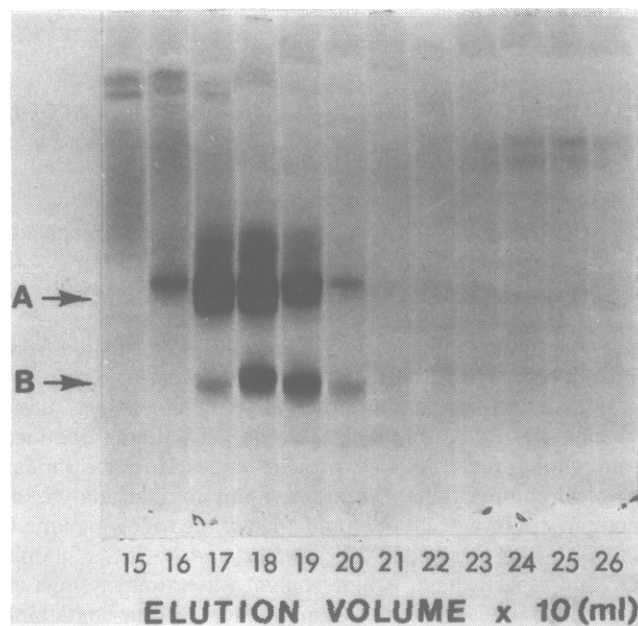
containing region B were rerun using a linear gradient of 600 ml of 0.20–0.30 M NaCl and monitored using the same procedures. Fractions judged to be pure by disc gel electrophoresis were pooled and rechromatographed three to five times until a product giving only a single band on disc gel electrophoresis was obtained.

Analytical Ultracentrifugation. The molecular weight determinations were carried out using the sedimentation equilibrium method of Yphantis (1964) in a Beckman Spinco Model E analytical ultracentrifuge using standard schlieren optics. Solutions of protein (10 mg/ml) were centrifuged for 4 hr at 20° at 20,000 and 18,000 rpm, respectively. The partial specific volume was calculated from the amino acid composition of the proteins (Cohn and Edsall, 1943, 1965).

Determination of Isoelectric Points. The LKB Uniphor equipment was used to determine the isoelectric points of the proteins using either an Ampholine mixture ranging in pH from 3 to 6 or 3 to 5. The column contained 2–3 mg of protein and at a constant voltage of 800 V and a temperature of 2.5° stabilization was reached after 48 hr. The column was drained and the effluent was collected in 3-ml fractions, of which aliquots were subjected to disc gel electrophoresis. pH determinations of fractions containing protein were carried out at the same temperature. It was not necessary to remove the Ampholines before electrophoresis since these migrate with the tracking dye.

Proteolytic Digestion. The action of a purified preparation of collagenase A from *Clostridium histolyticum* (Harper *et al.*, 1965) was examined in the following manner. Two-hundred micrograms of each protein (I, II, III, or IV) was dissolved in 200 μl of water and mixed with 50 μl of buffer (0.05 M Tris-HCl containing 0.005 M $CaCl_2$, pH 7.4). To this was added 26 μg of collagenase A dissolved in 50 μl of the same buffer. The reaction mixtures were allowed to incubate at room temperature. At time intervals of 15, 30, and 60 min, aliquots of 100 μl of the reaction mixture were removed and acidified with 50 μl of glycine–acetate buffer (0.375 M, pH 4). The enzymatic reaction was monitored by disc gel electrophoresis. Fifty microliters of 40% sucrose was added to the acidified reaction mix-
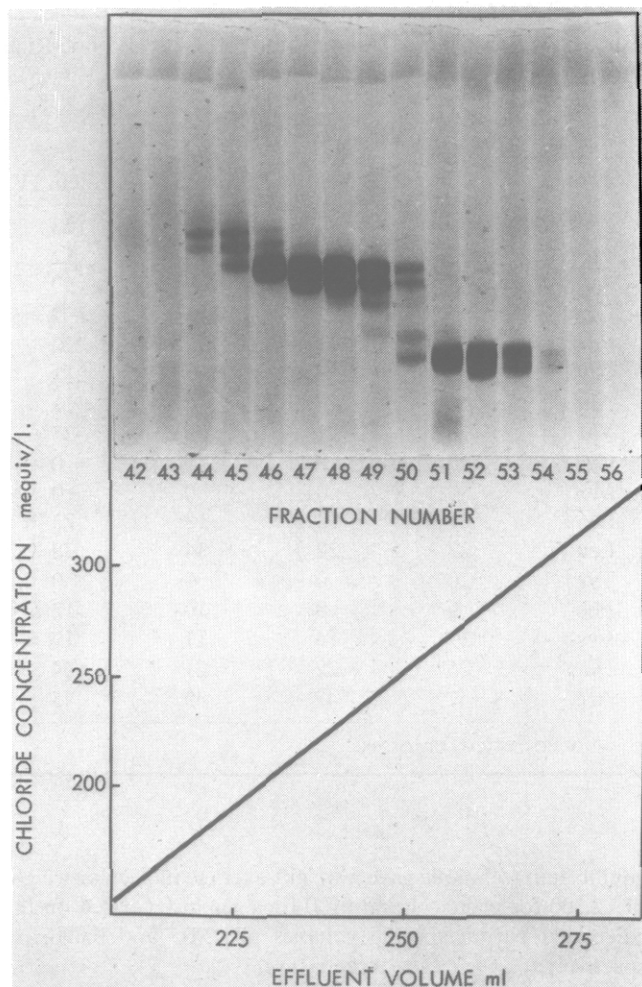
FIGURE 3: Elution profile of partially purified parotid proteins on DEAE Sephadex A-25. The individual fractions were monitored by both disc gel electrophoresis and chloride determinations. For experimental details, see text. Region A can be seen in fractions 46–49 and region B in fractions 51–53.

FIGURE 4: Final purification of protein I on DEAE-Sephadex A-25. Note the traces of protein II in fractions 49–55. Only fractions 44–47 were judged pure. See text for details.

ture and the resulting solution (0.2 ml) was subjected to disc gel electrophoresis.

Results

Isolation. AMMONIUM SULFATE FRACTIONATION. The $(NH_4)_2$-SO_4 fractionation profile of 0.5 g of parotid saliva protein is shown in Figure 1. The precipitates were dissolved in 5 ml of 0.05 M Tris-HCl buffer (pH 6.8) and 0.05 ml was applied per gel. The proteins to be isolated are located in two distinct regions visible on acrylamide gels (arrows). Using only the fraction insoluble in 35–50% $(NH_4)_2SO_4$ a substantial purification is achieved by eliminating the bulk of the slowly moving components. Another component traveling with the faster of the two protein regions is insoluble in 15–25% $(NH_4)_2SO_4$.

GEL FILTRATION. To eliminate the possibility of contamination with high molecular weight protein not readily detected by polyacrylamide electrophoresis, we employed gel filtration. The elution profile of the 35–50% $(NH_4)_2SO_4$-insoluble fraction on Sephadex G-75 containing the proline-rich proteins is shown in Figure 2. The two protein regions (A and B) elute close to each other. In addition, the data suggest that each region contains two individual protein components. At this protein load per gel at least two contaminating compo-
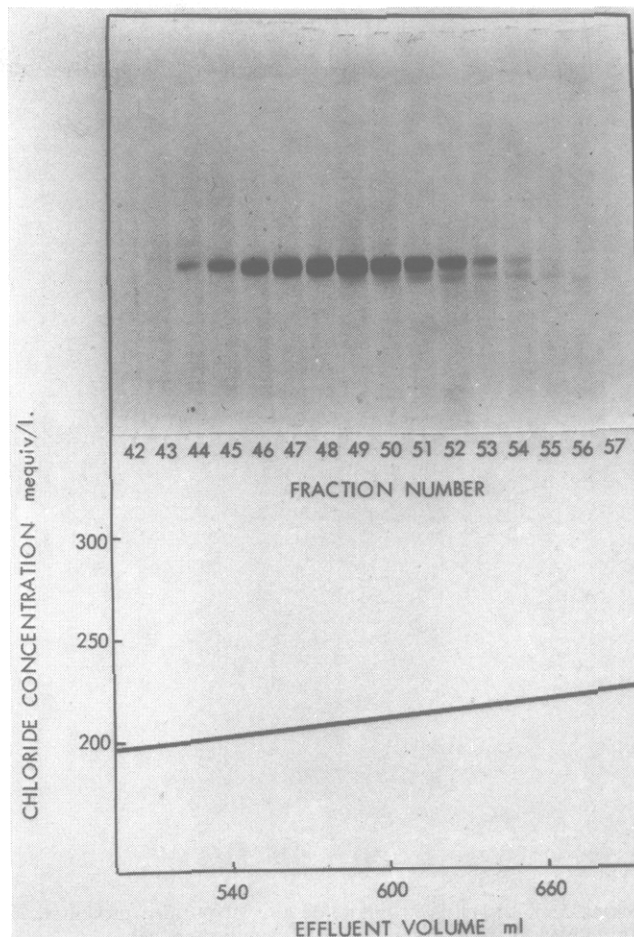
nents with slightly lower electrophoretic mobility coincide in their elution volume with the two protein regions. Since the above data indicated that these four proteins differ only slightly in their molecular size and shape, it was felt that gel filtration would not serve as a suitable method for further separation. The difference in electrophoretic mobility between region A and region B suggested the use of ion-exchange chromatography.

ION-EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX A-25. Separation of region A from region B was achieved by employing a linear gradient of 0.025–0.6 M NaCl on DEAE-Sephadex A-25 (Figure 3). The two proteins comprising region A are referred to as protein I and protein II; region B consists of protein III and protein IV. Careful inspection of the gel patterns of region A revealed slight differences in the elution volume of protein I and II. The same appeared to be true for protein III and IV constituting region B. Region A was resolved into protein I and protein II by applying a shallow gradient ranging from 0.14 to 0.24 M NaCl. The elution profile of protein I which had been recycled through the ion-exchange column three times (each time with the same gradient) is shown in Figure 4. Traces of protein II can be seen in the later fractions (fraction 49–55). Only the first four fractions (fraction 44–47) were judged as pure and pooled. A similar result was achieved by applying the same procedure for protein II. With a different gradient, namely 0.20–0.30 M NaCl clear resolution of protein III and IV was obtained. The elec-
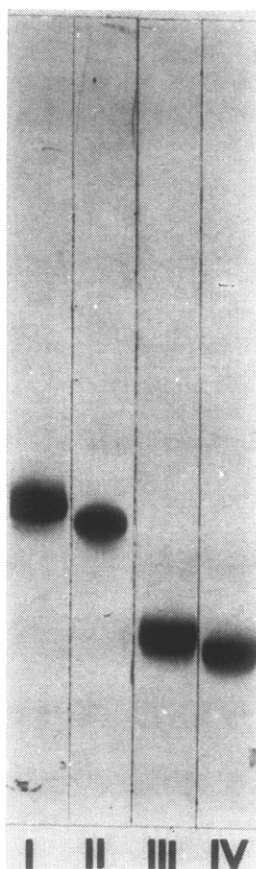
FIGURE 5: Disc gel electrophoretograms of purified protein I, II, III, and IV. Of each protein, 65 μg was applied per gel.

TABLE I: Amino Acid Composition of Four Proline-Rich Proteins from Human Parotid Saliva.

	Residues/1000[a]			
Residue	Protein I	Protein II	Protein III	Protein IV
Asp	76	77	107	103
Thr	12	3	5	12
Ser	43	39	49	59
Glu	194	255	256	195
Pro	271	261	212	220
Gly	220	213	197	211
Ala	10	8	12	11
Val	28	21	29	35
Cys	0	0	0	0
Met	0	0	0	0
Ile	21	13	18	23
Leu	27	22	34	38
Tyr	0	0	0	0
Phe	9	8	10	12
Lys	17	16	11	12
His	25	22	21	24
Arg	47	42	39	45

[a] Not corrected for losses.

trophoretogram of all four protein preparations are shown in Figure 5. The final yields for protein I, protein II, protein III, and protein IV were 21, 8, 22, and 10 mg, respectively.

Characterization of Proline-Rich Proteins. AMINO ACID COMPOSITION. Quantitative amino acid analysis clearly revealed a similarity among all four proteins (Table I). The most essential finding is the predominance of glycine, proline and acidic amino acids, which account for 75% of all the residues. Sulfur-containing amino acids and tyrosine were not found; of the aromatic residues only phenylalanine was detected at low levels. The latter result was further confirmed by spectral analyses, yielding no evidence for an absorption maximum at 280 mμ. Recovery of nitrogen from the amino acid analyses varied between 88 and 98% for all four proteins.

TOTAL NITROGEN, AMIDE NITROGEN, AND PROTEIN DETERMINATION. Approximately 2.0 mg each of protein I, II, III, and IV was dissolved in 5.0 ml of H_2O. Aliquots were removed for determining total nitrogen, amide nitrogen, and protein concentration by the Lowry procedure employing bovine serum albumin as a standard. As can be seen in Table II, a large percentage of the acidic residues in all four proteins are in their amide form. The Lowry protein values for the salivary proteins are significantly lower than the protein value one would expect from the nitrogen determination. Similar results are seen with other proline-rich proteins such as collagen (Franzblau, 1962).

CARBOHYDRATE CONTENT. No evidence for significant amounts of neutral sugars nor hexosamines could be found in any of the four proteins. A value of less than 0.5% was determined for neutral sugar (glucose equivalents).

ANALYTICAL ULTRACENTRIFUGATION. The sedimentation

equilibrium technique gave a weight-average molecular weight of 12,300 for protein I and 6100 for protein III based on the calculated partial specific volumes of 0.705 and 0.696, respectively.

ISOELECTRIC POINTS. The isoelectric points were determined in two separate experiments, using a preparation containing protein I and protein II with an Ampholine mixture ranging in pH from 3 to 6 and another fraction containing protein III and protein IV using a pH range of 3–5. The isoelectric points so obtained for protein I, II, III, and IV were 4.71, 4.59, 4.14, and 4.09, respectively.

COLLAGENASE TREATMENT. Treatment of these parotid proteins with collagenase A from *Cl. histolyticum* (Harper and Kang, 1970) indicated proteolytic cleavage of all four components as shown in Figure 6. Incubation for only 60 min at room temperature resulted in a marked loss of stain intensity when compared with the control incubation (no enzyme). In a separate experiment, incubation for 6 hr caused complete disappearance of the starting material. Furthermore, in the case of protein I and protein II a cleavage product with a mobility similar to the components of region B can be detected.

Discussion

The four secretory proteins described in this communication constitute about 4% of total parotid saliva protein. They contain relatively large amounts of proline (22–27%), and glycine (20–22%) while cysteine or cystine and tyrosine are not present. In addition, the amino acid analyses display the presence of extremely high levels of the acidic amino acids, glutamic acid and aspartic acid. The latter amino acids, however, exist in the isolated protein almost entirely in their amide form, namely, glutamine and asparagine.

Although protein preparations from parotid saliva with a similar amino acid composition have been previously reported (Mandel *et al.*, 1965; Levine *et al.*, 1969; Weill, 1965)

TABLE II: Properties of Protein I, II, III, and IV.

	Protein			
	I	II	III	IV
Total nitrogen (μmoles/ml)	5.65	5.14	6.07	6.57
Amide nitrogen (μmoles/ml)	1.03	0.82	1.08	1.18
Total protein^a (μg/ml)	195	253	275	320
Total protein^b (μg/ml)	385	373	441	458
Amide-N/total-N (%)	18.3	15.9	17.8	17.9
Gln + Asn/Glu + Asp + Gln + Asn (%)	>95	68	70	88

^{a} Based on Lowry, using bovine serum albumin as standard.
^{b} Calculated from total nitrogen employing a factor which was determined for each protein from its amide nitrogen content and amino acid composition; the factors for protein I to IV were 4.87, 5.18, 6.19, and 4.98.
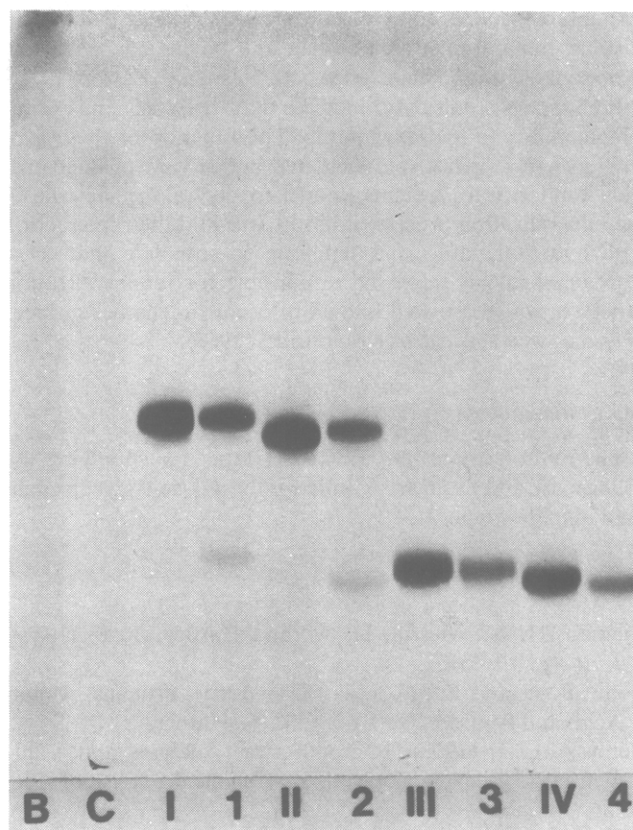

FIGURE 6: Incubation of proline-rich proteins with purified collagenase A from *Cl. histolyticum* carried out for 60 min at room temperature. I–IV = control incubation of protein I–IV (no enzyme added). 1–4 = incubation mixtures of protein I–IV; B = buffer blank; C = collagenase alone.

the proline-rich proteins isolated in this study differ markedly. Levine and coworkers (1969) found a glycoprotein with a molecular weight of 35,000 which migrates cathodically in slab acrylamide electrophoresis and contains 47% carbohydrate. Subjecting the proteins reported in this study to disc gel electrophoresis, according to the procedure of Reisfeld *et al.* (1962), gave further evidence for their acidic character by not even penetrating the stacking gel. Tests for neutral sugars and hexosamines gave no evidence for the presence of substantial amounts of carbohydrate. The glycoprotein preparations reported by Weill (1965) and Mandel *et al.* (1965) contain up to 90% carbohydrate. It is tempting to suggest that the proteins isolated in this study represent the polypeptide chain of the glycoprotein reported by the above mentioned investigators.

The gel filtration studies require some clarification. The elution behavior of these four proline-rich proteins on Sephadex G-75 suggests a molecular weight range for these proteins of 30,000–50,000. Their electrophoretic mobility on polyacrylamide gels suggests a similar conclusion, since protein I and protein II migrate slower than human serum albumin while protein III and protein IV slightly faster. The molecular weights determined by sedimentation equilibrium are significantly lower than one would have expected for the observations just alluded to. One cannot explain this discrepancy on the basis of isoelectric points since that of albumin and these proline-rich proteins are similar. It is possible that the apparent higher molecular weights observed with polyacrylamide gel electrophoresis and gel filtration studies may be due to aggregation. It should be pointed out that the minimum molecular weight calculated from the amino acid composition agree well with those values obtained by sedimentation equilibrium.

The dye binding characteristics of these proteins is of some interest. Amido-Schwarz seems to be the only stain which gives sharp bands on polyacrylamide gels. The widely used staining procedures employing coomassie blue and Woolfast blue (Meyer and Lamberts, 1965, 1968) afforded no detectable protein when applied on gels containing the proline-rich proteins. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate could not be run according to the procedure of Weber and Osborn (1969) since one is required to stain the proteins with coomassie blue. Use of Amido-Schwarz in this procedure would require much higher protein

concentrations for the standards and accuracy would, therefore, be greatly diminished. Even, the intensity of the Amido-Schwarz stain fades rather rapidly. The optimal destaining time was 12–24 hr. Destaining for 48 hr results in completely translucent gels, whereas an albumin band retains most of its color intensity for months. The chemical basis of this unusual staining behavior is not yet elucidated but this observation adds to the uniqueness of these parotid proteins and might provide a useful tool to probe structural aspects.

It is interesting to point out that the amino acid compositions of these proteins suggest some similarity to known structural proteins. The high content of proline and glycine with the concomitant sparcity of sulfur-containing amino acids is suggestive of collagen-like material. However, there does not appear to be any hydroxyproline or hydroxylysine. Perhaps the most significant relationship to collagen resides in the fact that these secretory proteins are readily cleaved by Clostridial collagenase A. This enzyme has been shown to be highly specific in its attack on native collagen; its specificity has been shown to require the sequence Pro-X-Gly-Pro-Y with splitting occurring at the peptide bond between the residues X and glycine (Seifter and Gallop, 1966). This would suggest that similar, if not identical, peptide sequences as those found in collagen, occur in these proteins. The nature of the resulting peptides from collagenase treatment is presently being investigated.

Comparison of the amino acid composition of these parotid proteins with that of another structural protein isolated from enamel matrix of bovine teeth is also striking. High concen-

trations of proline, glycine, and glutamic acid are found in various preparations from human and bovine enamel, while hydroxylated amino acids appear to be sparse (Eastoe, 1971).

Preliminary studies also indicate that these proteins exhibit a high affinity to hydroxylapatite. The function of these proteins in nature is not yet elucidated but in view of their possible similarity to proteins known to play a specific role in the mineralization process of hard tissue and their interaction with hydroxylapatite it is tempting to speculate that these secretory proteins might be responsible for remineralization processes which are well known to occur on enamel surfaces *in vivo* as well as *in vitro* (Koulourides, 1968).

Acknowledgments

References

Claman, H. N., Merrill, D. A., and Hartley, T. F. (1967), *J. Allergy 40*, 151.

Cohn, E. J., and Edsall, J. T. (1943, 1965), Proteins, Amino Acids and Peptides, New York, N. Y., Hafner.

Conway, E. J. (1963), Microdiffusion Analyses and Volumetric Errors, New York, N. Y., Chemical Publishing Co., Inc.

Curby, W. A. (1953), *J. Lab. Clin. Med. 41*, 493.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Eastoe, J. E. (1971), *Compr. Biochem. 26*, 785.

Ellison, S. A., Mashimo, P. A., and Mandel, I. D. (1960), *J. Dent. Res. 39*, 892.

Fischer, C. J., Wyshak, G. H., and Weisberger, D. (1968), *Arch. Oral Biol. 13*, 257.

Franzblau, C. (1962), Ph.D. Thesis, A. Einstein College of Medicine, New York, N. Y.

Harper, E., and Kang, A. H. (1970), *Biochem. Biophys. Res. Commun. 41*, 482.

Harper, E., Seifter, S., and Hospelhorn, V. D. (1965), *Biochem. Biophys. Res. Commun. 18*, 627.

Hay, D. I. (1969), *J. Dent. Res. 48*, 806.

Koulourides, T. (1968), *Ann. N. Y. Acad. Sci. 153*, 84.

Levine, M. J., Weill, J. C., and Ellison, S. A. (1969), *Biochim. Biophys. Acta 188*, 165.

Lowry, D. H., Rokbrough, N. A., Tarr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mandel, I. D., and Ellison, S. A. (1963a), *Ann. N. Y. Acad. Sci. 106*, 271.

Mandel, I. D., and Ellison, S. A. (1963b), *Ann. N. Y. Acad. Sci. 131*, 802.

Mandel, I. D., Thompson, R. H., and Ellison, S. A. (1965), *Arch. Oral Biol. 10*, 499.

Meyer, T. S., and Lamberts, B. L. (1965), *Biochim. Biophys. Acta 107*, 144.

Meyer, T. S., and Lamberts, B. L. (1968), *Arch. Oral Biol. 13*, 839.

Oppenheim, F. G. (1971), Abstracts, 49th General Meeting of the International Association of Dental Research, Chicago, Ill., Abstract 844, p 263.

Reisfeld, R. A., Lewis, H. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.

Seifter, S., and Gallop, P. M. (1966), *Proteins 4*, 293.

Van Handel, E. (1967), *Anal. Biochem. 19*, 193.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Weill, J. C. (1965), M. S. Thesis, State University of New York at Buffalo.

Wilcox, P. E. (1967), *Methods Enzymol. 11*, 65.

Yphantis, D. A. (1964), *Biochemistry 3*, 297