ISOLATION OF A "PROLINE-RICH" PROTEIN FROM RAT PAROTID GLANDS FOLLOWING ISOPROTERENOL TREATMENT

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SUMMARY: Prolonged treatment of rats with isoproterenol induces both hypertrophy and hyperplasia of the parotid glands. Daily injections of 5 mg dl-isoproterenol resulted in a 4-6 fold increase in gland wet weight within 7 days. The protein composition of parotid gland homogenates and saliva, as monitored by disc gel electrophoresis, was markedly altered. In particular, the concentration of a proline-rich protein increased dramatically. This protein was purified by Sephadex G-100 chromatography and preparative disc gel electrophoresis and was found to be comprised mainly of four amino acids (moles/100 moles): Pro, 29.5; Glx, 19.3; Gly, 17.1; Asx, 11.9. The molecular weight as determined by sedimentation equilibrium is about 25,000. When [^{14}C] glucosamine was injected intraperitoneally, a small amount of label was incorporated into the proline-rich protein. Upon stopping the isoproterenol treatment, the parotid glands and the polyacrylamide disc gel patterns of the soluble proteins both return to normal within 10 to 14 days.

INTRODUCTION: Several reports on proline-rich proteins and glycoproteins isolated from human parotid gland secretions have appeared (1-4). Mandel, Thompson and Ellison (1) found hexose, hexosamine, fucose and sialic acid in a glycoprotein which contained 34 moles of proline and 20 moles each of glycine and glutamic acid (per 100 moles amino acids). Levine, Weill and Ellison (2) isolated a glycoprotein which had the following analysis: 62.5% protein, 1.9% sialic acid, 14.2% glucosamine, 4.0% fucose and 11.8% neutral hexose. The amino acid composition (moles/100 moles) consisted of 33.6 proline, 20.5 glycine, 20.9 glutamic acid and 6.6 aspartic acid. Aromatic and sulfur-containing amino acids were not detected. Subsequently, Bennick and Connel (3) and Oppenheim, Hay and Franzblau (4) isolated and partially characterized a series of proline-rich proteins from human parotid saliva which, with one possible exception, did not contain carbohydrate. The amino acid compositions of the proteins isolated by both of these laboratories are essentially identical, and are very similar to those reported by the previous investigators.

We wish to report on the isolation and partial characterization of a proline-rich protein from the parotid glands of rats treated with isoproterenol. The amino acid composition and molecular weight have been determined. Evidence is presented that small amounts of [^{14}C] glucosamine are incorporated into this proline-rich protein.

MATERIALS AND METHODS: Female Sprague-Dawley rats weighing approximately 180 grams were used. Animals born on the same day were separated into control and Ipr-treated[1] groups. The animals in the experimental group received daily subcutaneous injections of 5 mg dl-isoproterenol-HCl dissolved in 0.5 ml of isotonic saline. Animals were fed ad libitum. Food, but not water, was removed after the seventh injection. After an 18 hr fast, the animals were anesthetized by injection of 5 mg nembutal and sacrificed by exanguination. The parotid glands were quickly excised and dissected free from adhering tissue. Glands of the Ipr-treated animals increased 4-6 fold in wet weight.

Isolation of the Proline-Rich Protein. The glands were sliced into thin sections and 0.5 to 1.5 g were homogenized with 4 volumes (w/v) of isotonic saline at 4° in a glass-teflon homogenizer. The homogenate was centrifuged at 35,000 x g for 30 min and the supernatant fluid (crude extract) was stored at -20°. Portions (4 to 5 ml) of the crude extract containing a total of about 16 mg protein were chromatographed on Sephadex G-100. Fractions from the column were analyzed by disc gel electrophoresis. The fractions containing the proline-rich protein were pooled, dialyzed against distilled water, lyophilized, and dissolved in distilled water to give a final protein concentration of 4 mg/ml. Preparative disc gel electrophoresis was performed on a 2 cm x 25 cm gel column with acrylamide solutions prepared as described by Davis (5). Aliquots containing approximately 2 mg protein were electrophoresed at 5 ma for 22 hours at 4°. The gel column was removed, and 1 cm segments were macerated and eluted in the cold for 36 hrs with 2 ml distilled water. Each solution was dialyzed against distilled water, lyophilized, and an aliquot monitored by analytical disc gel

[1] Ipr-treated refers to isoproterenol-treated animals.

electrophoresis. The proline-rich protein migrated approximately 14 cm into the preparative gel, and diffused over a distance of 3 cm. This procedure was repeated several times to collect sufficient material for subsequent studies. Recoveries from the preparative gels ranged from 75 to 85%.

Analytical disc gel electrophoresis was performed according to the procedure of Davis (5) in a Buchler Polyanalyst apparatus at 4°. The gels were stained with 0.2% naphthol blue-black for detecting protein. Duplicate gels were stained for carbohydrate with the periodic acid-Schiff's reagent (6). Sedimentation equilibrium ultracentrifugation studies were carried out according to the procedure of Yphantis (7). Amino acid analysis was performed on a Beckman Model 119-HP with areas computed by an Infotronics CRS 110 integrator, as described previously (8).

[^{14}C] Glucosamine incorporation into the proline-rich protein was studied in both control and experimental animals. Animals from both groups, following the 18 hr fast, were injected intraperitoneally with 5 mg isoproterenol.[2] One hr later they were injected with 5 mg propranolol in 0.5 ml saline. Within 5 min after the propranolol injection, 5 μc [^{14}C] glucosamine (10 μc/μmole) in 0.2 ml saline was injected intraperitoneally. One hr later, the animals were sacrificed and the parotid glands were treated as described previously. Radioactivity was counted using the Triton-toluene system (9). Gel slices were digested with 30% H_2O_2 at 50° overnight before adding the counting solution.

RESULTS AND DISCUSSION: The elution patterns from Sephadex G-100 column chromatography of crude extracts from control and Ipr-treated animals are shown in Fig. 1. While qualitative differences in the two extracts are not evident from the Sephadex G-100 patterns, obvious differences are demonstrated by disc gel electrophoresis of the crude extracts (Fig. 2A and B). Of particular interest was a protein band which was not detectable in control animals, but appeared in gland extracts and saliva of Ipr-treated animals. Analytical disc gel electro-

[2] Better results were obtained by first stimulating secretion (to empty the gland) and then blocking secretion to cause accumulation of newly synthesized substances within the gland.

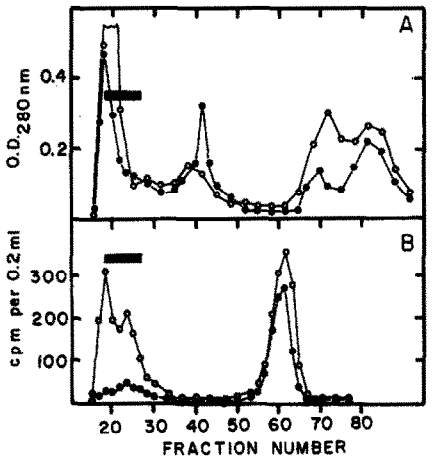

Fig. 1. Column chromatography on Sephadex G-100. A. The sample of crude extract (about 16 mg protein) was applied to a 1.5 cm x 80 cm column of Sephadex G-100 (fine) and eluted with 0.9% NaCl at a flow rate of 0.9 ml per min. Fractions of 1.5 ml were collected and assayed by 280 nm absorption. Control rats, ●—●; Ipr-treated, o—o. B. Crude extracts of rat parotid glands prepared following injection of [^{14}C] glucosamine were treated as described in A. Aliquots of 0.2 ml were counted. Control rats, ●—●; Ipr-treated rats, o—o. (The second radioactive peak comprising Fractions 51-65 has not been investigated.)

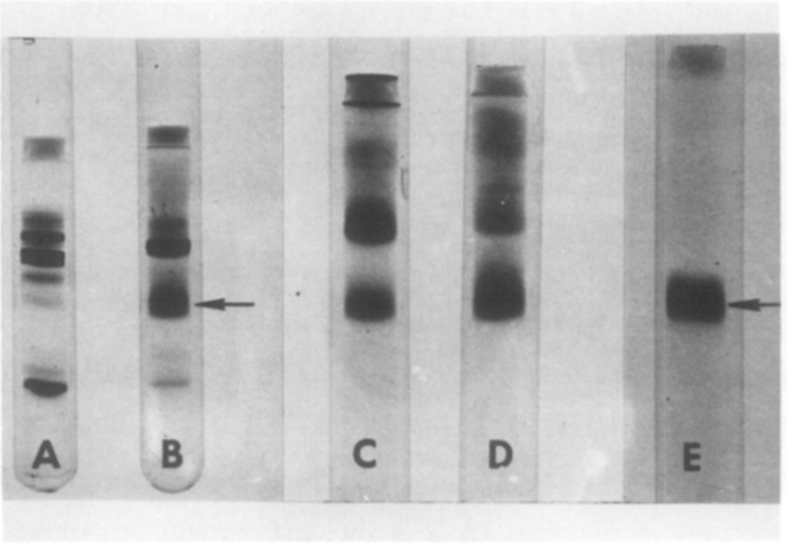

Fig. 2. Polyacrylamide disc gel electrophoresis. A constant current of 2 mA/column was applied until the tracking dye reached the bottom of the gel. Gels were stained for protein by immersion for 20 min in a 0.2% solution of naphthol blue-black. Destaining was accomplished by soaking the gels in 7.5% acetic acid at room temperature. The following amounts of protein (μg) were applied per gel: A, 80; B, 70; C, 80; D, 80; E, 40. A = crude extract, control; B = crude extract, Ipr-treated; C = fraction from Sephadex G-100, crude extract; D = fraction from Sephadex G-100, saliva; E = purified proline-rich protein. The proline-rich protein is indicated by the arrows

phoresis was used to follow this protein during purification. This protein was found in fractions 20-28 from the Sephadex G-100 column. It was further purified by preparative disc gel electrophoresis. The electrophoretic pattern of the purified protein is shown in Fig. 2E. Only one relatively diffuse protein band was detected. The stain in this band gradually faded over a period of 2-3 weeks, when the gels were stored in 7% acetic acid. The fading occurred in a consistent manner across the diffuse band. Periodic acid-Schiff (PAS) reagent stained the identical area in a separate gel, and like the naphthol blue-black stain, faded rapidly and evenly. Very light staining was obtained by PAS treatment even with 10 times the amount of protein usually applied to the gels. Rat parotid saliva, collected by cannulation of the parotid gland ducts,[3] also contained the proline-rich protein (Fig. 2D). As with the crude extracts, the proline-rich protein was detected only in the saliva of the Ipr-treated animals. The electrophoretic patterns, after purification of the crude extract and the parotid saliva on Sephadex G-100, are very similar (Fig. 2C and D).

Results of sedimentation velocity studies on the purified proline-rich proteins were consistent with disc gel electrophoresis in that one component was detected (Fig. 3). The sedimentation equilibrium procedure gave a weight-average molecular weight of 25,000 $\pm$ 2,000 for the proline-rich protein, calculated on an assumed partial specific volume of 0.74. This molecular weight value is inconsistent with the apparent molecular size obtained from Sephadex G-100, which suggests a molecular weight of greater than 60,000. The high content of proline (30%) may be, in part, the cause of this apparently anomalous result.

The amino acid composition (Table I) of this proline-rich protein is strikingly similar to analyses reported by previous workers for proline-rich substances from human parotid secretions and rat pancreas zymogen granule membranes.

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[3]The authors wish to express their appreciation to Ms. Ann Taylor, Department of Pediatrics, for performing the cannulations.

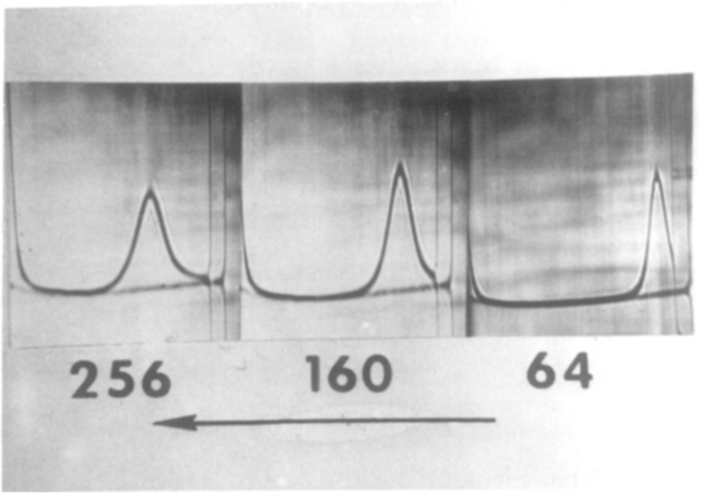

Fig. 3. Sedimentation velocity studies. Protein concentration was 2.0 mg/ml in 0.05 M sodium phosphate buffer, pH 7.0. Sedimentation was carried out in a 12 mm cell with a Spinco Model E ultracentrifuge operating at 60,000 rpm, a temperature of 25°, and a bar angle of 60°. Time in minutes (64, 160, 256) is given.

The results of injecting [^{14}C] glucosamine into control and Ipr-treated rats are shown in Fig. 1. While the amount of label incorporated in both experiments was low, there was an increase of [^{14}C] incorporation into the fractions corresponding to the proline-rich protein from the Ipr-treated rat. Purification of the proline-rich protein by analytical disc gel electrophoresis resulted in the coincidence of naphthol blue-black staining and [^{14}C] incorporation.

A single treatment of isoproterenol is known to cause the stimulation of secretion of rat parotid glands with subsequent synthesis of protein, RNA and DNA.[4] Prolonged treatment results in the hypertrophy and hyperplasia of the salivary glands with little, or no effect, on other tissues. When the treatments are stopped, the changes in the gland are reversible and the gland apparently returns to its normal state. Data obtained from the Ipr-treated rats studied in this investigation are consistent with these reports. When Ipr-treatment was stopped, the proline-rich protein decreased and was not detectable after two weeks. Confirming observations of others (4), Coomassie

[4] Reference 10 is a review on the effects of isoproterenol treatment on rat parotid glands.

TABLE I. COMPARISON OF AMINO ACID COMPOSITIONS OF PROLINE-RICH PROTEINS

	Source of Proteins			
	Rat Parotid Glands	Human Parotid Saliva		Zymogen Granule Membrane
	moles per 100 moles			
Asp	11.9[a]	7.5[b]	7.6[c]	4.4[d]
Thr	1.2	0	1.2	2.1
Ser	4.7	3.4	4.3	2.9
Glu	19.2	30.0	19.4	17.5
Pro	29.5	25.3	27.1	29.8
Gly	17.1	20.7	22.0	17.2
Ala	2.0	0.8	1.0	2.9
Val	1.1	2.0	2.8	2.8
Ile	0.4	1.2	2.1	2.1
Leu	1.4	2.0	2.7	3.7
Tyr	0.6	0	0	1.4
Phe	0.4	0.7	0.9	1.9
Lys	2.3	1.3	1.7	3.2
His	5.0	1.7	2.5	1.3
Arg	2.7	3.4	4.7	5.9

[a]Results of amino acid analysis of the proline-rich protein isolated in this study. Values given are the averages of three determinations, and corrected for losses. Methionine and cystine were not detected following performic acid oxidation.

[b]Results reported by Bennick and Connell, Component C (3).

[c]Results reported by Oppenheim, Hay and Franzblau, Protein I (4).

[d]Results reported by Amsterdam, Schramm, Ohad, Salomon and Selinger (11).

blue gave no detectable stain when applied to gels containing the proline-rich protein and the intensity of the naphthol blue-black stain faded rapidly.

While a biological role for the proline-rich proteins has not been defined, these soluble, proline-rich proteins may be involved as structural subunits in membrane biogenesis, specifically in the formation of zymogen granule membranes.

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