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Induction of Protein and Glycoprotein Synthesis in Rat Submandibular Glands by Isoproterenol*

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A family of proline-rich proteins which contain over 40% proline and a glycoprotein were isolated from submandibular glands of isoproterenol-treated rats by extraction with 10% trichloroacetic acid and fractionation of the acid-soluble portion on Bio-Gel A-1.5m. The proline-rich proteins were subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were detected by formaldehyde fixation and Coomassie staining. After elution from the gels, amino acid compositions were determined. The family of proline-rich proteins (about six) appears to be qualitatively identical with a family of proteins from parotid glands of isoproterenol-treated rats (Muenzer J., Bildstein, C., Gleason, M., and Carlson, D. M. (1979) *J. Biol. Chem.* 254, 5623–5628, 5629–5634), except for a substantial change in the relative amount of each protein.

An acid-soluble glycoprotein (GP-158) was not detected in extracts of submandibular glands of normal rats, but GP-158 was highly induced by isoproterenol treatment. This glycoprotein has an apparent M_r = 158,000 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The major amino acids were Asx, 13%; Glx, 18%; Pro, 29% and Gly, 11%. GP-158 contained about 37% carbohydrate, and its sugar composition on a molar basis was mannose, 3.0; galactose, 2.1; *N*-acetylglucosamine, 3.5; fucose, 1.2; and sialic acid (*N*-glycolyl(4-*O*-acetyl)neuraminic acid), 0.9. *N*-Acetylgalactosamine was not detected. A glycopeptide isolated from GP-158 had the same sugar composition as GP-158. The amino acid sequence of the glycopeptide was shown to be Asp-Gly-(Asn)-Gln-Thr-Gln-Pro-Arg-Pro-(Gly-Pro). Only the parotid and submandibular glands of rats responded in this dramatic fashion to isoproterenol. The isoproterenol-treated rat is considered an appropriate model system for studying the overall effects of catecholamine β-agonists on gene expression in these secretory tissues.

The presence of PRPs[1] in salivary glands and salivary secretions of various species is well established (2–6). Generally two families of these unusual proteins are isolated, acidic proteins which may contain sugars and phosphate and are about 25% proline, and a group of basic proteins (pI > 10) which contains about 43% proline, but which usually contains no sugars or phosphate. The peptide sequence of the acidic PRP from human parotid gland secretions has been determined by Wong *et al.* (7, 8) and is comprised in part of a highly conserved repeating sequence of 21 amino acids. The amino acid sequence of human basic proline-rich proteins has been reported recently (9), and the results also show a repetitive partial sequence. Muenzer *et al.* (6, 10) found a dramatic increase in PRPs in rat parotid glands after prolonged isoproterenol treatment, and the basic PRPs made up more than 50% of total glandular proteins. Cell-free translations of these proteins has recently been reported (11). In addition, a new glycoprotein with an apparent M_r = 220,000, induced by isoproterenol treatment, has been isolated from parotid gland extracts of isoproterenol-treated rats (12).

This study extends previous findings by describing a simple and rapid procedure for isolating and identifying proline-rich proteins and describes for the first time the induction of a glycoprotein in the submandibular glands of isoproterenol-treated rats. This glycoprotein has an apparent M_r = 158,000 on SDS-polyacrylamide gels, is clearly different in carbohydrate and amino acid compositions from glycoproteins isolated from parotid glands, and appears to contain a repeating glycopeptide unit.

EXPERIMENTAL PROCEDURES

Materials—All materials were of highest purity available and were purchased from commerical sources unless otherwise indicated. The following substances were purchased from the respective companies: L-[3,4(*n*)-^{3}H]proline (40–60 Ci/mmol), Amersham Radio Chemicals, Arlington Heights, IL, L-proline, neuraminidase from *Clostridium perfringens*, pronase (type VI), DL-isoproterenol HCl, and 1-anilino-8-naphthalensulfonate, Sigma; Sephadex G-50, Pharmacia Fine Chemicals Inc., Piscataway, NJ: molecular weight standards and Bio-Gel A-1.5m and P-2, Bio-Rad; Earle's balanced salt solution, Gibco Laboratories, Grand Island, NY; and transferrin (human plasma), Calbiochem-Behring.

Isolation of Proline-rich Proteins and Glycoproteins—Sprague-Dawley male rats (200–225 g), fed Purina laboratory chow *ad libitum*, were used as tissue donors. Isoproterenol treatment and *in vivo* labeling of proteins with [^{3}H]proline were performed as described by Muenzer *et al.* (6). Unless otherwise indicated, isoproterenol was administered for 10 days.

Rats were anesthetized with sodium pentobarbital and killed by exsanguination. The salivary glands and other organs were excised, stripped of connective tissue, and placed in 5 volumes of ice-cold 10% trichloroacetic acid unless otherwise indicated. Tissues were homogenized in an Omni-mixer at top speed for 60 s. After centrifugation at 17,000 × *g* for 20 min, trichloroacetic acid was removed from the soluble fraction by extraction with water-saturated ether for four times, 4 volumes each time. The aqueous phase, designated the trichloroacetic acid-soluble fraction, contains the proline-rich pro-

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[1] The abbreviations used are: PRPs, proline-rich proteins; SDS, sodium dodecyl sulfate; GP-158, glycoprotein of apparent M_r = 158,000. PRP-38 and PRP-34, for example, refer to proline-rich proteins with apparent molecular weights on SDS-polyacrylamide gels of 38,000 and 34,000, respectively. PRP-38SM and PRP-38P represent M_r = 38,000 PRP from submandibular and parotid glands.

teins and GP-158. Subsequent fractionations were achieved by gel filtration with Bio-Gel A-1.5m and by preparative SDS-polyacrylamide gel electrophoresis (13). Proteins were detected on gels by the fluorescent probe 1-anilino-8-naphthalenesulfonate (14) and were eluted by the procedure of Chan *et al.* (15). Sodium dodecyl sulfate and salts were removed by precipitation of the proteins four times with acetone and then by dialyzing against 0.1 M acetic acid.

In Vitro Labeling with [^{3}H]Proline—Radioactive labeling was performed as described by Kemper *et al.* (16) with modification. Tissue slices of about 20–30 mg of either parotid or submandibular glands were incubated at 37 °C in 60 μl of Earle's balanced salt solution containing 50 μCi of [^{3}H]proline in an atmosphere of 5% CO_2 and 95% air. The gland pieces were rinsed in Earle's salt solution and disrupted in 0.1 ml SDS-containing sample buffer (13) by sonication. After centrifugation, the supernatant fluid was either heated to boiling for 5 min or made 10% with trichloroacetic acid. In both cases, precipitates were discarded. Trichloroacetic acid was removed by using ether, and both soluble fractions were analyzed by SDS-polyacrylamide gel electrophoresis (13). Pulse-chase experiments were performed with about 20 mg of tissue and labeled with [^{3}H]proline as mentioned above for 1 h. Tissue slices were then incubated in the same volume of media containing 0.01 M proline. Samples were prepared for electrophoresis by sonication in SDS-containing buffer.

Amino Acid Analysis—Protein samples (0.2–0.5 mg) were hydrolyzed in 1 ml of 6 N HCl at 110 °C for 24 h, as described previously (10). Analysis was performed on a Durrum autoanalyzer.

Carbohydrate Analysis—Neutral sugars were assayed by gas chromatography of the alditol acetates essentially as described previously (17). In these studies, a 25-m capillary column, SP2100, was used and programmed for 210 °C for 13.5 min, increase of 5 °C/min for 2 min, constant at 220 °C for 8 min, increase of 5 °C/min for 2 min, constant at 230 °C for 5 min. Amino sugars were estimated by using the amino acid analyzer after hydrolysis of samples (0.5 mg) in 1 ml of 4 N HCl for 6 h at 100 °C, and transferrin was used as a standard. Sialic acid was assayed by the thiobarbituric acid procedure (18), both prior to and after acid hydrolysis (0.1 N H_2SO_4, 80 °C, 1 h). Glycolic acid was determined as described (19). Sialic acid was identified by thin layer chromatography (20).

Phosphorus Determination—Total phosphorus was assayed as described by Ames and Dubin (21).

Neuraminidase Treatment—Glycoproteins (2–5 mg) were dissolved in 0.5 ml of 0.05 M sodium acetate buffer, pH 5.5, and were treated with 5 milliunits of neuraminidase from *C. perfringens*. Incubation was carried out at 37 °C for periods up to 24 h. Alkaline hydrolysis of the *O*-acetyl groups was performed according to the method of Pepper (22).

Proteolytic Digestion of GP-158—Glycopeptides were prepared from GP-158 by pronase digestion as described by Zinn *et al.* (23). The pronase digest was fractionated on a Sephadex G-50 column. The glycopeptide peak was identified by the phenol-sulfuric acid reaction (23) and fluorescamine (24). The peak which contained carbohydrate was pooled and lyophilized. Desalting was performed by using a Bio-Gel P-2 column that was equilibrated with H_2O.

Protein Determinations—Proline-rich proteins generally do not have aromatic amino acids and were quantitated by absorbance at 230 nm; $E^{1\%}_{1\,cm} = 25$.

SDS-Polyacrylamide Gel Electrophoresis—Gel electrophoresis was carried out as described by Laemmli (13) and modified by others (25). Proteins precipitated with acetone were dissolved in sample buffer and boiled for 5 min. Electrophoresis was performed on 12% polyacrylamide gel unless otherwise stated. Proteins were fixed in the gel and stained by the procedure of Steck *et al.* (26), except that formaldehyde concentrations of the second staining solution and destaining solution were increased by 5- and 2.5-fold, respectively. Destained gels were soaked in 10% acetic acid and 1% glycerol for about 20 min before drying. Glycoproteins were detected by the periodic acid-Schiff staining procedure (27). Radioactivity on gels was detected either by fluorography (28) or by utilizing EN^{3}HANCE from New England Nuclear.

RESULTS

Isolation of Trichloroacetic acid-soluble Components—The initial purification step took advantage of the unusual acid solubility of these proline-rich proteins and GP-158. Salivary glands from DL-isoproterenol-treated rats were homogenized in 10% trichloroacetic acid. Components of the trichloroacetic acid-soluble fractions from both parotid and submandibular glands were resolved by gel filtration on Bio-Gel A-1.5m and SDS-polyacrylamide gel electrophoresis into a glycoprotein fraction and into several proline-rich proteins (PRP-38 to PRP-24) (Fig. 1). Peak III apparently contains low molecular weight peptides not detected on the gels. When parotid glands from DL-isoproterenol-treated rats injected with [^{3}H]proline were used, over 90% of the ^{3}H label incorporated into protein was in the 10% trichloroacetic acid-soluble fraction (data not shown).

Tissue Slice and Pulse-Chase Studies—To ensure that the series of proline-rich proteins did not arise from the trichloroacetic acid treatment or from proteolysis, tissue slices were labeled with [^{3}H]proline and pulse-chased (Fig. 2). Proline-rich proteins were rapidly and highly labeled in the tissue and appeared in increasing amounts in the medium with time when pulse-chased. This series of proteins appeared to be

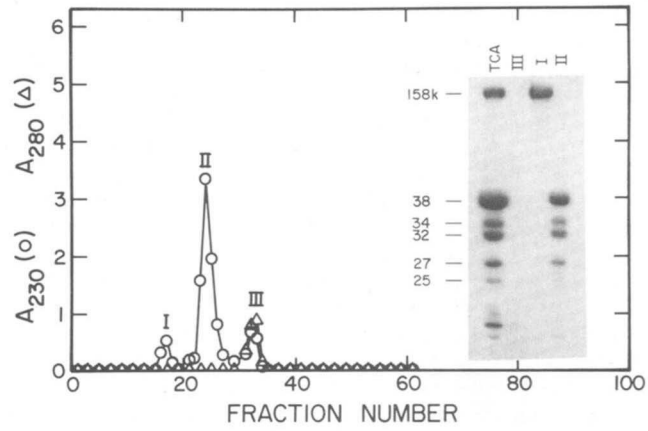

FIG. 1. **Chromatography of the trichloroacetic acid-soluble fraction from rat submandibular glands on Bio-Gel A-1.5m.** Five ml (about 10 mg/ml, based on 230 nm absorption) of the trichloroacetic acid (*TCA*)-soluble extract of submandibular glands of isoproterenol-treated rats were chromatographed on a column (2 × 110 cm) of Bio-Gel A-1.5m. The column was equilibrated and eluted with 25 mM Tris-HCl, pH 7.4, containing 0.14 M NaCl. Five-ml fractions were collected. An identical elution pattern was obtained with extracts of rat parotid glands. Analysis of the trichloroacetic acid extract and three peaks from the column by SDS-polyacrylamide gel electrophoresis is shown in the *insert*. *158k*, for example, M_r = 158,000.

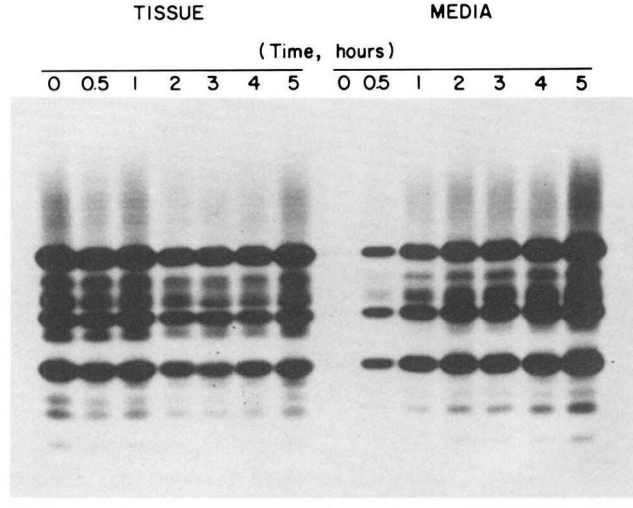

FIG. 2. **Fluorographs of pulse-chase experiments.** See "Experimental Procedures" for details. Tissues and media were analyzed by SDS-polyacrylamide electrophoresis.

synthesized and secreted in concert with no apparent changes in either the appearance of new protein bands or in the disappearance of protein bands. Tissue slices labeled with [^{3}H]proline and either extracted with 10% trichloroacetic acid or homogenized in sample buffer (13) or buffer A gave identical ^{3}H-labeled protein profiles on SDS-polyacrylamide gel electrophoresis (figure not shown). Similarly, tissue slice experiments with submandibular glands showed neither detrimental effects of trichloroacetic acid extraction nor a product-precursor relationship for the proline-rich proteins.

Tissue Survey for Trichloroacetic Acid-soluble Components—Various tissues of rats treated with isoproterenol were extracted with 10% trichloroacetic acid and were assayed for proline-rich proteins by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, only parotid and submandibular glands had appreciable acid-soluble material. Chromatography on Bio-Gel A-1.5m and amino acid analysis of the acid-soluble components of tissues other than the salivary glands did not indicate any proteins corresponding to proline-rich proteins.

Changes in Trichloroacetic Acid-soluble Components with Time of Isoproterenol Treatment—Only small amounts of trichloroacetic acid-soluble materials were present either in the parotid or submandibular glands of normal rats (Fig. 4, *A* and *B*). With injection of isoproterenol dramatic changes in both proline-rich proteins and GP-158 occur. The proline-rich proteins of both glands seem to be increasing at a corresponding rate. The parotid gland appears to respond more rapidly to isoproterenol treatment than the submaxillary glands. However, PRP-38P constitutes the major proline-rich protein in submandibular glands. PRP-38 has been obtained in apparently pure form from both parotid and submandibular glands by preparative SDS-polyacrylamide gel electrophoresis.

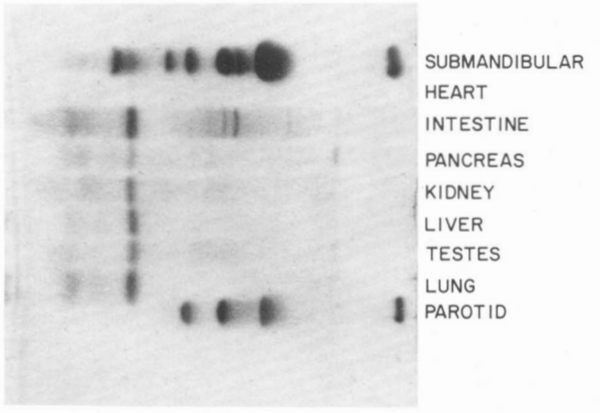

FIG. 3. **Tissue survey for proline-rich proteins.** Trichloroacetic acid extracts were analyzed as described under "Experimental Procedures."

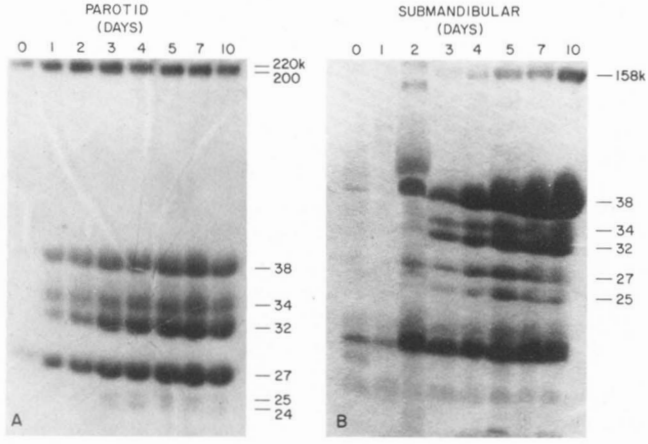

FIG. 4. **Changes in proline-rich proteins in parotid and submandibular glands with duration of isoproterenol treatment.** See "Experimental Procedures" for details. Trichloroacetic acid extracts were analyzed at various days of isoproterenol treatment. *220k*, for example, M_r = 220,000.

TABLE I
Amino acid compositions

	PRP-38		GP-158	Glycopeptide
	Parotid	Submandibular		
	mol/100 mol			
Aspartic acid	2.9	2.9	13.4	15.8
Threonine	0.0	0.54	7.8	8.6
Serine	1.6	2.1	1.5	0.6
Glutamic acid	23.9	23.2	18.4	18.8
Proline	41.8	41.3	28.9	29.2
Glycine	23.2	21.6	11.0	11.2
Alanine	0.0	0.8	0.3	0
Histidine	0.0	0.0	10.2	7.7
Lysine	1.0	0.9	0.0	0
Arginine	4.7	4.7	6.8	7.5

TABLE II
Carbohydrate compositions

Sugars	GP-158		Glycopeptide	
	μmol/g	*molar ratio*	*μmol/g*	*molar ratio*
Fucose	260	1.2	362	1.1
Mannose	662	3.0	971	3.0
Galactose	470	2.1	664	2.0
N-Acetylglucosamine	772	3.5	1165	3.6
Sialic acid	209	0.9	293	0.9
Glycolic acid	196	0.8		
% carbohydrate	37		58	
Mannose:aspartate			2.8:1	

Compositional Analyses—Amino acid analysis of the major component of the proline-rich proteins of submandibular glands (PRP-38SM) is essentially identical with that from the parotid glands (PRP-38P) (Table I). We assume that PRP-38SM and PRP-38 are identical. These proteins completely lack aromatic and sulfur-containing amino acids (10). Unlike the proline-rich protein family, GP-158 contains appreciable amounts of aspartic acid, threonine, and histidine. About 37% of the dry weight of GP-158 is carbohydrate (Table II). *N*-Acetylgalactosamine was not detected and presumably only glycosidically *N*-linked oligosaccharide chains are present.

Neuraminidase Treatment—The results of neuraminidase treatment both before and after base treatment to remove *O*-acetyl groups are shown in Fig. 5. Quantitative removal of sialic acid from fetuin was observed in about 1 h, whereas, after 24 h with GP-158, less than 10% of sialic acid was removed. After alkaline hydrolysis, neuraminidase completely removed sialic acid from GP-158.

Glycolic acid analysis (Table II) shows that the sialic acid is *N*-glycolylneuraminic acid, and this was confirmed by chromatographic analysis (Fig. 5).

Glycopeptide Isolation and Analysis—GP-158 was subjected to pronase treatment, and products were separated by Sephadex G-50 chromatography (Fig. 6). The molecular weight of the glycopeptide fraction on Sephadex G-50 was approximated at 3500, or close to the molecular weight of fetuin glycopeptide which was prepared in the same manner. Sugar recoveries exceeded 85%. The molar ratios of amino acids

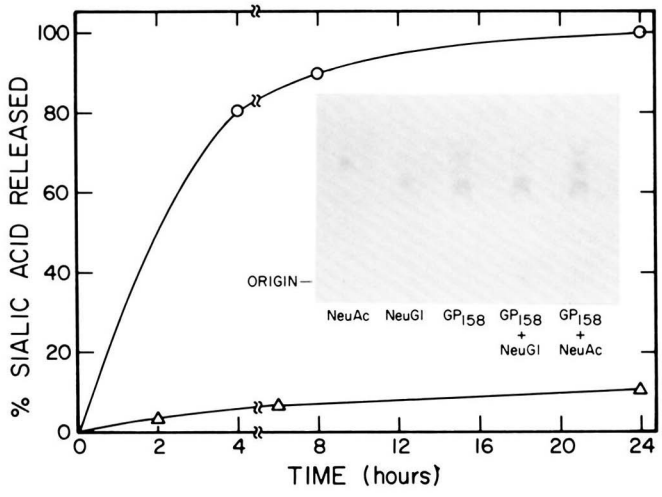

FIG. 5. **Release of sialic acid from GP-158 by *C. perfringens* neuraminidase.** Free sialic acid was assayed by the thiobarbituric acid procedure. △——△, GP-158 treated with neuraminidase; ○——○, GP-158 treated first with 0.1 M NaOH at 22 °C for 72 h and then with neuraminidase. Chromatography of sialic acid from GP-158 is illustrated in the *inset*. *NeuGl*, *N*-glycolylneuraminic acid; *NeuAc*, *N*-acetylneuraminic acid.

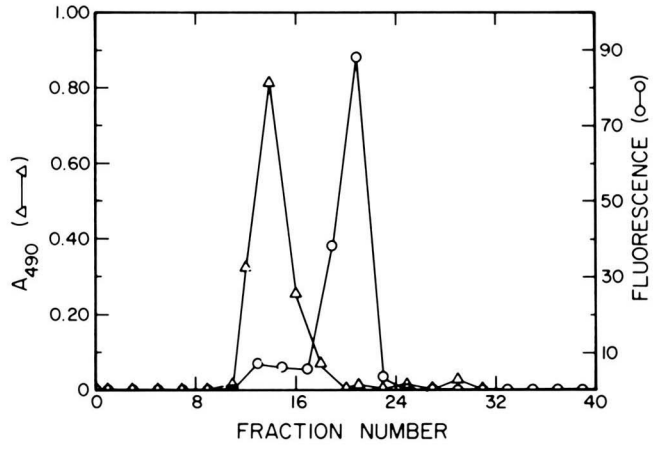

FIG. 6. **Chromatography of glycopeptide fraction on Sephadex G-50.** Pronase-treated GP-158 was applied to a column (2 × 65 cm) of Sephadex G-50, equilibrated and eluted with 50 mM sodium phosphate, pH 7.4. Five-ml fractions were collected. Carbohydrate was detected by the phenol-sulfuric acid procedure (△——△) and peptides and amino acids by the fluorescamine reaction (○——○).

(Table I) and sugars (Table II) in GP-158 and the glycopeptide fraction were very similar. Estimated from the amino acid and carbohydrate compositions, assuming one carbohydrate chain, the molecular weight of the glycopeptide is 3300. This is in agreement with the apparent molecular weight obtained by Sephadex G-50 chromatography. Peptide sequence analysis showed amino acid residues in the order of Asp-Gly-(Asn)-Gln-Thr-Gln-Pro-Arg-Pro-(Gly-Pro). The carbohydrate is presumed to be attached at the Asn in the third position as evidenced by no observable residue at that position during sequencing and by the presence of Thr 2 residues to the carboxyl side. Aside from the first Asp, which was present on about 30% of the peptide chains from two different glycopeptide preparations, the remainder of the peptide chain showed total homogeneity at each amino acid position. This clearly shows that the glycopeptide is present as a repeating peptide unit in the glycoprotein. About 55% of the peptide chain is removed by pronase treatment.

DISCUSSION

Results obtained by these studies demonstrate that the proline-rich proteins and the glycoprotein GP-158 which are induced by isoproterenol treatment can be isolated by two simple purification steps. The solubility properties of these proteins together with the amino acid analysis serve to characterize these proline-rich proteins from the parotid and submandibular glands of DL-isoproterenol-treated rats. Tissue surveys by the procedures outlined here, or by labeling *in vivo* with [^{3}H]proline (6), have shown that these unusual proteins were not detected in other tissues of either normal or DL-isoproterenol-treated rats. The addition of 1% phosphotungstic acid to 10% trichloroacetic acid quantitatively preciptates the proline-rich proteins and GP-158, and is necessary for assaying cell-free translations of these proteins (11).

Difficulty in detecting the proline-rich proteins on polyacrylamide gels was noted previously (6, 10). The addition of formaldehyde, used by Steck *et al.* (26) for cross-linking small basic proteins to the polyacrylamide matrix, has allowed for the fixing and staining of these proteins. Identification of the proline-rich proteins was confirmed by 1) identical electrophoretic profiles comparing Coomassie blue staining and [^{3}H] proline incorporation and fluorography, 2) 230 nm absorption and a total lack of 280 nm absorption, and 3) amino acid compositions. Based on these parameters, the 38,000-dalton proline-rich proteins from both parotid and submandibular glands (PRP-38P and PRP-38SM, respectively) are considered to be identical. However, the regulatory systems in each gland appear to differ since the relative amounts of the proline-rich proteins from the two glands are dramatically different. PRP-38SM comprises about 60–70% of the proline-rich protein from submandibular glands, whereas PRP-38P constitutes only about 30% of the total protein complement found in parotid glands. Analysis of the cell-free translation products of mRNA from parotid glands of isoproterenol-treated rats showed multiple protein bands when labeled with either [^{35}S]methionine or [^{3}H]proline (11). Similar experiments with mRNA from submaxillary glands also gave multiple bands,[2] and in both instances the proteins translated *in vitro* were similar in relative amounts and in number of components to the proteins isolated from the glands or synthesized by tissue slices. In the cell-free translations, [^{35}S] methionine is incorporated only as the NH_2-terminal amino acid (11), whereas in cell culture experiments there was no incorporation of [^{35}S]methionine.

Amino acid sequences of human proline-rich proteins have established that both the acidic (7, 8) and the basic (9) proline-rich proteins have defined repeating peptide segments with a very high degree of homology. This repetitive nature of these human proline-rich proteins appears to be true for glycoprotein GP-158 in that the glycopeptide fraction contains a sequence of about 10 amino acids with absolute homology. The molar ratios of amino acids in GP-158 and in the glycopeptide fraction are very similar, which show that the amino acids released by pronase digestion are essentially the same as those remaining in the peptide chain of the glycopeptide. Possibly GP-158 is composed of a repeating peptide unit of about 25 amino acids.

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[2] H. Mehansho and D. M. Carlson, unpublished results.

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