Chem. Pharm. Bull. 29(5)1365—1372(1981)

Studies on LM Protein Appearing in Submandibular Glands of Isoproterenol-treated Rats. I. Purification and Characteristics of Its Appearance

Yukio Naito

Daiyūkai Institute of Medical Sciences, The 2nd Division,[1] 7-28-1, Matsufuri-dori, Ichinomiya, 491, Japan

(Received August 13, 1980)

A large mobile (LM) protein that is accumulated in saliva of rats upon chronic administration of isoproterenol (IPR), a β-adrenergic drug (L. Menaker, *et al.*, *Ala. J. Med. Sci.*, **11**, 356—358 (1974)), has been purified to electrophoretic homogeneity. Rabbit anti-LM protein antibody was prepared, and used for determination of the LM protein by a single radial immunodiffusion method. The increase in the amount of this protein in the soluble fraction of submandibular glands was roughly proportional to the increase in the wet weight of glands and the most effective dose of IPR was found to be 40 mg/kg.

The LM protein which accumulated in the submandibular glands upon chronic administration of IPR was secreted as a result of IPR stimulation, whereas methoxamine, an α-adrenergic drug, did not induce significant secretion of the accumulated LM protein. Apparently the protein newly synthesized in submandibular glands upon chronic administration of IPR is secreted through the action of β-adrenergic receptors.

Keywords——rat submandibular glands; isoproterenol; methoxamine; hypertrophy and hyperplasia; saliva proteins; LM protein; proline-rich proteins; protein synthesis

The prolonged treatment of rats[2] or mice[3] with isoproterenol (IPR), a β-adrenergic drug, is known to result in enlargement of the salivary glands. These macroscopic changes are the consequence of hypertrophic and hyperplastic changes which are relatively specific to the acinar cells.[4–8] In addition, structural changes of secretory granules in the acinar cells were observed after IPR stimulation.[8,9]

Chronic administrations of IPR induce changes in the amount of salivary protein and in the concentrations of inorganic components in rat saliva.[10–12] Menaker *et al.* (1974)[13,14] first reported that a large mobile (LM) protein appeared in the saliva of IPR treated rats as determined by polyacrylamide gel electrophoresis of whole saliva. This protein was localized in the secretory granular fraction in the homogenized submandibular glands.[13,14]

On the other hand, a proline-rich protein was found in parotid gland homogenates and saliva of IPR-treated rats.[15] This protein was not detectable in normal glands, but increased dramatically upon IPR treatment.

The mechanisms of secretion of proteins from salivary glands have been studied by many investigators. Amylase in the secretory granules of acinar cells in parotid glands was released by the action of β-adrenergic agonists.[16–18] On the other hand, the secretions of epidermal growth factor and nerve growth factor, which are localized in the tubles of submandibular glands, were regulated by α-adrenergic agonist.[19–21] However, it remains to be clarified how the salivary proteins are produced in large amounts upon IPR administration, while they are not detectable in normal rat submandibular glands.

In an attempt to elucidate the secretory mechanism, LM protein was isolated from the IPR-treated rat submandibular saliva, and a method for quantitation of the LM protein was established with rabbit antibody against the purified LM protein. By using these methods, some of the characteristics of the appearance of LM protein in rat submandibular saliva in response to IPR treatment were clarified.

NII-Electronic Library Service

Materials and Methods

Animal——Male Sprague-Dawley rats (250—300 g weight) were used in all experiments. The temperature and relative humidity of the animal quarters were maintained at 22°±2° and 60±10%, respectively. A 12 hr light/dark cycle was maintained in the room by an automatic timing device with the lights turned off at 6 p.m. The animals had access to chow (Oriental Yeast Co., Tokyo) and water *ad libitum*. Rats were fasted for 18 hr before collections of saliva and serum, and before excision of salivary glands.

IPR Administration——The animals in experimental groups received injections (0.1 to 100 mg/kg) of *dl*-isoproterenol-HCl (Nakarai Chemicals, Kyoto) dissolved in sterilized saline, while control rats were given an equal volume of sterilized saline. Injections were given once a day at 6 p.m. for a week or for three days.

Saliva Collection——The rats were anesthetized by intraperitoneal injection (40 mg/kg of body weight) of sodium pentobarbital (Nembutal, Abbott Laboratories), placed supine and tracheotomized. Both submandibular ducts were cannulated intraorally with polyethylene tubing (PE 10, Clay Adams) using the method of Yoshida *et al.*[22] Submandibular saliva was collected 24 hr after the final IPR injection, and methoxamine-HCl (Nippon Sinyaku Co., Kyoto), an α-adrenergic drug, or isoproterenol-HCl (a β-adrenergic drug) was used as a secretory stimulant at a dose of 20 mg/kg of body weight (intraperitoneally). Parotid saliva was collected similarly for 90 min. The collected saliva was kept ice-cold and trisodium edetate was added (final concentration; 0.04 M) to avoid subsequent precipitation of protein. The mixture was lyophilized and stored in a desiccator.

Preparation of Soluble Protein Fraction of Submandibular Glands——Rats were sacrificed by exsanguination. Submandibular glands were quickly excised and weighed before or after the saliva collection. The glands were sliced into thin sections with a razor and homogenized in 10 volumes (w/v) of 0.01 M phosphate buffer (pH 7.2) at 4° with a glass-Teflon homogenizer. The homogenate was centrifuged at 15000×g for 30 min and the supernatant fluid was separated. A soluble protein fraction, obtained from the supernatant fluid after centrifugation at 100000×g for 1 hr, was stored at −20°.

Protein Determination——Protein concentration was determined by the method of Lowry *et al.*[23] using bovine serum albumin (Sigma Chemical) as a standard.

Analytical Polyacrylamide Gel Electrophoresis——This was carried out essentially as described by Davis[24]; 1.5 ml of running gel solution (11.25% acrylamide–0.3% N,N′-methylenebisacrylamide solution, pH 6.7) was placed in a glass tube (0.4×12 cm) and water was layered on the solution to effect polymerization. On the polymerized 11.25% gel, 0.2 ml of stacking gel solution was placed and photopolymerized. Bromophenol blue (B.P.B.) was used as a marker dye. Tris (hydroxymethyl)aminomethane–glycine buffer (pH 8.3, μ: 0.05) was used as an electrophoretic buffer. The gels were run with 3 mA/tube until B.P.B. had migrates 8 cm. After electrophoresis, the gels were stained with 0.5% wool-fast-blue BL and destained with 1% acetic acid according to the method of Mayer and Lamberts.[25] Relative mobility of proteins was calculated from the mobility of B.P.B. taken as unity. The electrophretic pattern of proteins in the gel was scanned at 575 nm with an Ozumar SD-92 autodensitometer (Asuka Mfg., Tokyo).

Purification of LM Protein——i) Gel Chromatography on a Sephadex G-75 Column: Sephadex G-75 superfine (Pharmacia Fine Chemicals) was equilibrated with 0.02 M phosphate buffer (pH 7.2) and packed in a column (2×79 cm). Lyophilized submandibular saliva was dissolved in the buffer to give a concentration of 30 mg/ml and the solution was loaded on the column. Elution was performed by the descending method at 10 cm water pressure. The void volume of the column was measured by the use of Blue dextran 2000 (Pharmacia Fine Chemicals). B.P.B. was used as a marker for the internal volume of the column. The eluates were combined into three pools (G-I—G-III) on a basis of distribution coefficient. Each fraction was dialyzed against distilled water and lyophilized.

ii) Preparative Polyacrylamide Gel Electrophoresis: This was performed according to the method of Mizutani *et al.*[26] The running gel solution (150 ml) was placed in a glass tube (4×40 cm) and polymerized. On the polymerized gel, 15 ml of stacking gel solution was placed and photopolymerized. The partially purified fraction from Sephadex G-75 column chromatography (G-II, 20 mg) was dissolved in 20% sucrose containing 0.001% B.P.B. and the solution was loaded on the gel. Electrophoresis was carried out in a cold room at 30 mA for about 12 hr. The gel was taken out and sliced into thin sections. The LM protein was extracted from the slices with 0.03 M Tris (hydroxymethyl) aminomethane–HCl buffer, pH 8.9. The extract was dialyzed against distilled water and lyophilized (fraction G-II.DE).

iii) Second Gel Chromatography on a Sephadex G-75 Column: The fine polyacrylamide gel particles in the fraction G-II.DE were removed by second gel chromatography on a Sephadex G-75 column (2×79 cm). The purified protein fraction was dialyzed against distilled water and lyophilized (fraction G-II.DE.S-II).

Double Radial Immunodiffusion——Antibody against LM protein was prepared by inoculating rabbits in the toe pad with 500 μg of purified LM protein in Freund's incomplete adjuvant (Difco Laboratories) once a week for four weeks. The blood removed from the carotid artery was allowed to clot and serum was prepared by centrifugation at 500×g for 10 min. The serum was incubated at 56° for 30 min and allowed to stand overnight at 4°. Then, it was centrifuged at 3000×g for 20 min and the supernatant fluid was

NII-Electronic Library Service

separated and stored at −20°. The double radial immunodiffusion was carried out using anti-LM protein antibody according to the method of Ouchterlony.[27]

Single Radial Immunodiffusion——The concentration of LM protein in the sample solutions was determined by the single radial immunodiffusion method reported by Macini *et al.*[28] Agarose-I (Wako Pure Chemical Ind., Osaka) was melted in 0.001 M phosphate buffer (pH 7.2) containing 0.01% NaN_3 and 0.15 M NaCl to make a 2% solution. A melted agarose-I solution was mixed with an equal volume of prewarmed (56°) antibody diluted to 1/37 with the same buffer. The mixture of antibody and agarose-I was poured into a glass plate (7.5×7.5 cm) to make a 1.5 mm thick layer. Antigen wells (3 mm diameter) were punched on each antibody-agarose-I plate and were filled with 10 μl of sample solution. The plates were incubated in a humid atmosphere at room temperature. The area of precipitin rings formed on the plate was measured accurately.

The concentration of LM protein in sample solutions was calculated from the calibration curve. The purified LM protein was used as a standard antigen.

Results

The elution patterns from Sephadex G-75 of submandibular saliva proteins from control and IPR-treated rats are shown in Fig. 1. The saliva proteins in the control group were separated mainly into two peaks, whereas three major peaks were observed in the IPR-treated groups. The eluates of both groups were combined into G-I (Kav 0—0.4), G-II (Kav 0.4—0.7) and G-III (Kav 0.7—1.0) fractions as shown in Fig. 1. The proteins recovered in G-I, G-II and G-III fractions accounted for 78, 10 and 9.5% of the total, respectively, in the control group, and 52.5, 35 and 10.5% in the experimental group. In the IPR-treated group, the amount of the first fraction was less than that in the control group. However, the amount of second fraction was 3.5-fold more than that of the control group.

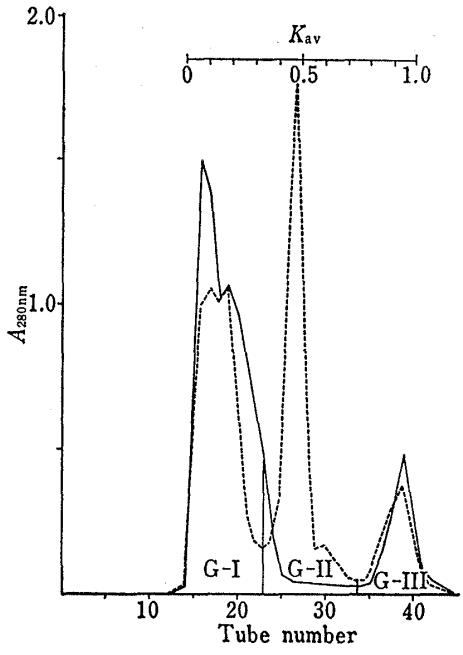

Fig. 1. Separation of Saliva Proteins by Sephadex G-75 Column Chromatography

Submandibular saliva (200 mg protein) was applied to a 2.0×79 cm column of Sephadex G-75 (superfine) and proteins were eluted with 0.02 M phosphate buffer, pH 7.2, at a flow rate of 6 ml/hr. Fractions of 6.9 ml were collected and the absorbance at 280 nm was measured. The eluates were combined into three pools (G-I—III). Saliva from control rat, ——; saliva from IPR-treated rats, ----.

The electrophoretic patterns of the saliva proteins of both control and experimental groups are shown in Fig. 2. The saliva proteins of the control group were separated into about 24 protein bands by polyacrylamide gel electrophoresis. Among the protein bands, the one with a relative mobility of 0.86 (a) tended to decrease upon IPR treatment (Fig. 2-a). The saliva from IPR-treated rats contained five proteins that were not observable in the control group (Fig. 2-b). The relative mobilities of these proteins were 0.07 (No. 1), 0.78 (No. 2), 0.79 (No. 3), 0.92 (No. 4) and 0.95 (No. 5). The protein band with a relative mobility of 0.92 increased most on IPR treatment and was assumed to be the LM protein reported by Menaker *et al.*[12] The following experiments were focussed mainly on this protein. The protein (a) which decreases on IPR treatment and the LM protein had similar relative mobilities on polyacrylamide gels. However, the two proteins were eluted separately from a Sephadex G-75 column in the G-I and G-II fractions, respectively. Only small amounts of proteins in fraction G-III of both groups were fixed on the gels or stained by the dye used.

Fraction G-II in the experimental group was further purified by preparative polyacrylamide gel electrophoresis. After electrophoresis, the gel was sliced and the LM protein in the

NII-Electronic Library Service

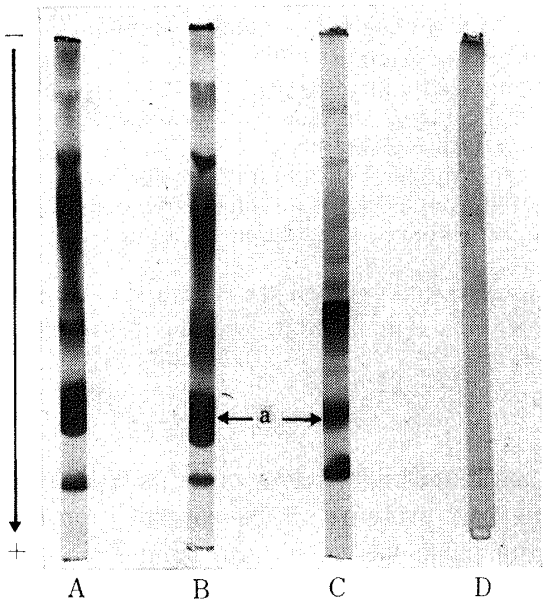

Fig. 2-a. Polyacrylamide Gel Electrophoresis of Control Submandibular Saliva Protein

Saliva proteins separated by Sephadex G-75 column chromatography (G-I, G-II and G-III described in Fig. 1) were analyzed. A, submandibular saliva protein; B, fraction G-I; C, fraction G-II; D, fraction G-III.

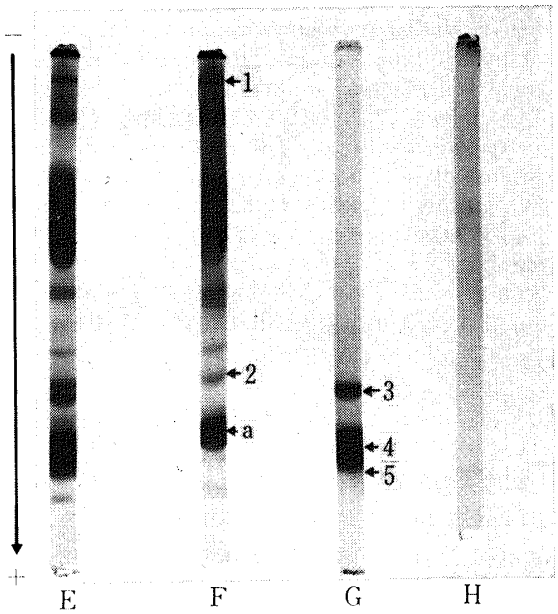

Fig. 2-b. Polyacryl amide Gel Electrophoresis of Submandibular Saliva Protein from IPR-treated Rats

E, submandibular saliva protein; F, fraction G-I; G, fraction G-II; H, fraction G-III. Proteins which decrease upon IPR treatment (a) and increase upon IPR treatment (No. 1—5) are indicated by arrows.

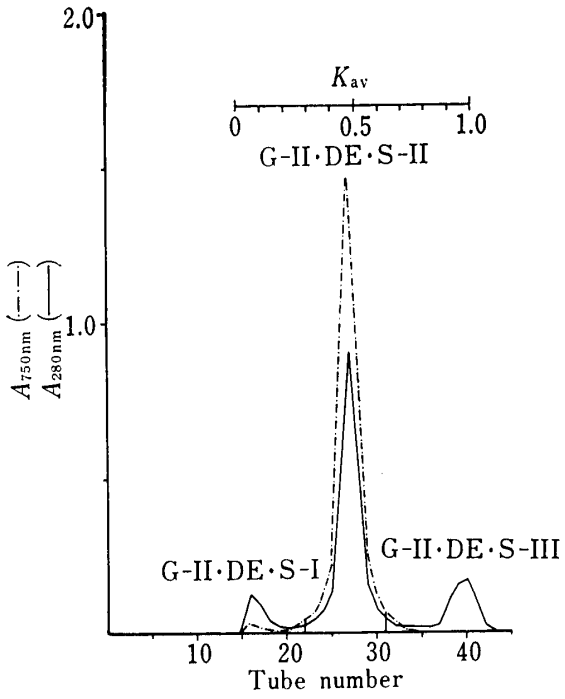

Fig. 3. Rechromatography of partially Purified LM Protein on Sephadex G-75

Fraction G-II. DE (32 mg protein) obtained by preparative polyacryamide gel electrophoresis was treated as described in Fig. 1. Absorbances at 280 nm (——) and 750 nm by Lowry's method (----) were measured.

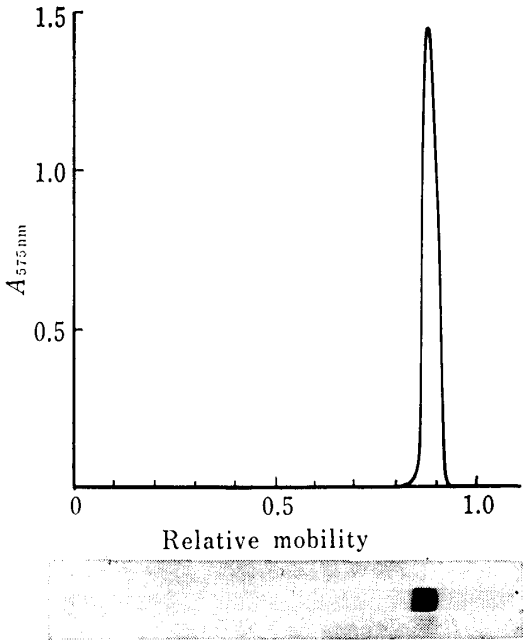

Fig. 4. Densitometric Tracing of Purified LM Protein in a Polyacrylamide Gel Electrophorogram

NII-Electronic Library Service

slices was extracted. Fraction G-II.DE thus obtained gave a single protein band with a relative mobility of 0.92 on analytical polyacrylamide gel electrophoresis. Recovery from the preparative gels was about 80%. In order to remove fine polyacrylamide gel particles in fraction G-II.DE, the fraction was rechromatographed on Sephadex G-75 (Fig. 3). The LM protein eluted at K_{av} 0.49, showed a single protein band as revealed by densitometric tracing (Fig. 4). About 28 mg of LM protein was obtained from 200 mg of the saliva protein in the experimental group.

Double Radial Immunodiffusion

To see whether LM protein is present in the submandibular glands of both groups and also in the parotid saliva, a double radial immunodiffusion method was used. The rabbit anti-LM protein antibody (I) produced a single precipitin line with LM protein (A), the saliva of experimental group (B) and the soluble protein fraction of submandibular glands of experimental group (C). However, no precipitin line was formed with serum (D) or the parotid saliva (E) in the experimental group, or with the saliva (F), the soluble protein fraction of submandibular glands (G) or serum (H) in the control group (Fig. 5).

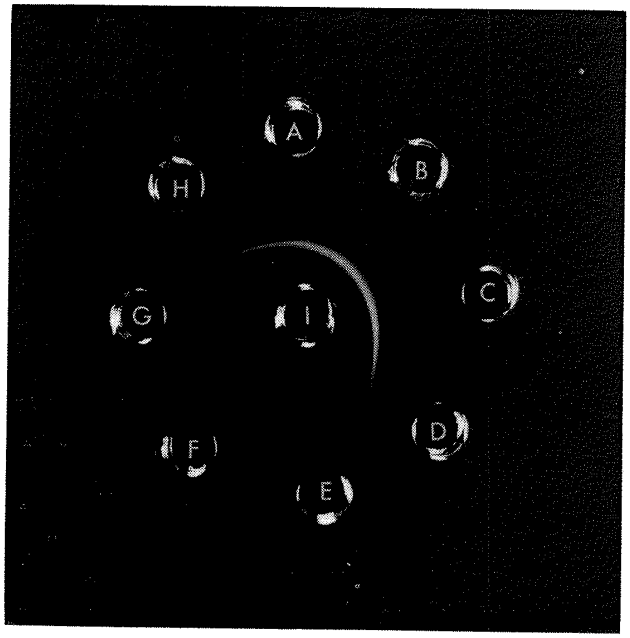

Fig. 5. Double Radial Immunodiffusion

A, purified LM protein; B, submandibular saliva (IPR-treated); C, soluble protein fraction obtained from submandibular glands (IPR-treated); D, serum (IPR-treated); E, parotid saliva (IPR-treated); F, submandibular saliva (control); G, soluble protein fraction obtained from submandibular glands (control); H, serum (control); I, rabbit anti-LM protein. The following amounts of protein (μg) were added to each well: A, 5; B, 15; C, 200; D, 500; E, 800; F, 800; G, 200; H, 500.

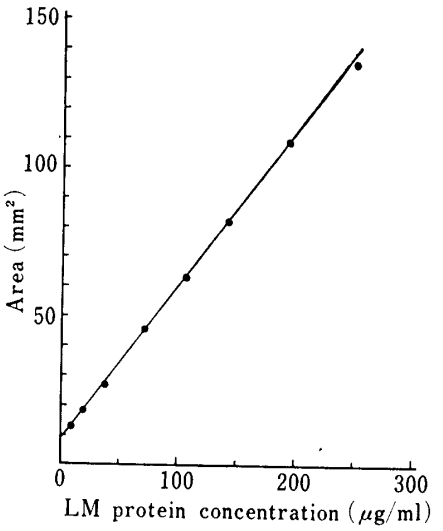

Fig. 6. Single Radial Immunodiffusion

Relation between the concentration of the LM protein and the area of halos. Antigen wells (3 mm diameter) were punched on each antibody-agarose plate (antibody 1/74 dilution; agarose 1%) and filled with 10 μl of antigen solution. The plates were incubated in a humid atmoshphere at room temperature and the area of precipitin rings formed on the plates was measured accurately.

Dose-response Curve

As shown in Fig. 6, the areas of precipitin rings formed by the single radial immunodiffusion method were proportional to the concentration of LM protein in a range from 5 to 250 μg/ml in the standard assay system. The relationship between the dose of IPR and the increase in LM protein was then determined. The rats were given IPR in doses from 0.1 mg/kg to 100 mg/kg for three days. On the fourth day after the initial IPR injection, the LM protein in the soluble protein fraction of the submandibular glands was estimated. The LM protein was not detectable at 0.3 mg/kg but was detectable from 0.5 mg/kg of IPR (6 μg/100 mg of the submandibular glands) (Fig. 7). The amount of the protein increased dramatically from 10 mg/kg and the most effective dose of IPR was found to be 40 mg/kg. The amount of LM

NII-Electronic Library Service

protein decreased significantly at100 mg/kg. The wet weight of submandibular glands also increased as a function of the dose, reaching a maximum at 40 mg/kg of IPR. However, the changes in the amount of LM protein and the wet weight of submandibular glands were not parallel (Fig. 7).

Secretory mechanism of the LM protein

In an attempt to analyze the secretory mechanism of the LM protein, the rats were injected with IPR for three days at a dose of 20 mg/kg/day. On the fourth day after the initial IPR injection, the submandibular saliva was collected by methoxamine stimulation or IPR stimulation (Table I). In the saline-injected control groups, saliva collected by IPR stimulation contained over 8-fold more protein than the saliva collected by methoxamine stimulation, but the flow rate was reduced by 40%. The wet weight of submandibular glands per body weight were similar irrespective of the stimulant used. Consequently, the total protein secreted per hr per submandibular glands was 5-fold higher in saliva collected by IPR stimulation than in saliva collected by methoxamine stimulation. Similarly in

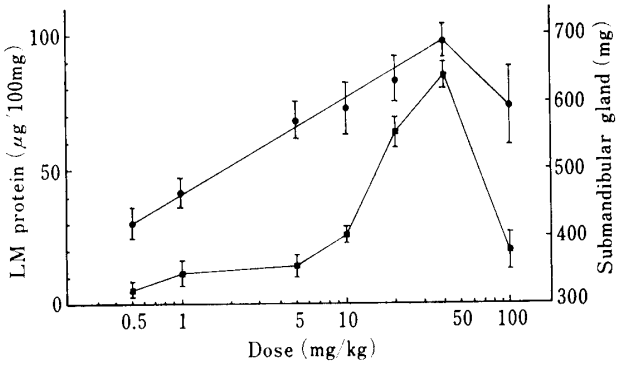

Fig. 7. Dose-response Relationship between IPR and LM Protein or Wet Weight of Submandibular Glands

Various doses were injected intraperitoneally once daily for three days. Rats were killed one day after the last injection and the weight of gland (●—●) was determined. The glands were then homogenized in 10 volumes of 0.01 M phosphate buffer (pH 7.2) in a glass-Teflon homogenizer. LM protein concentration (■—■) in the supernatant fluid (100000×*g*) was determined by a single radial immunodiffusion method. Means±S.E. are given ($n=6$).

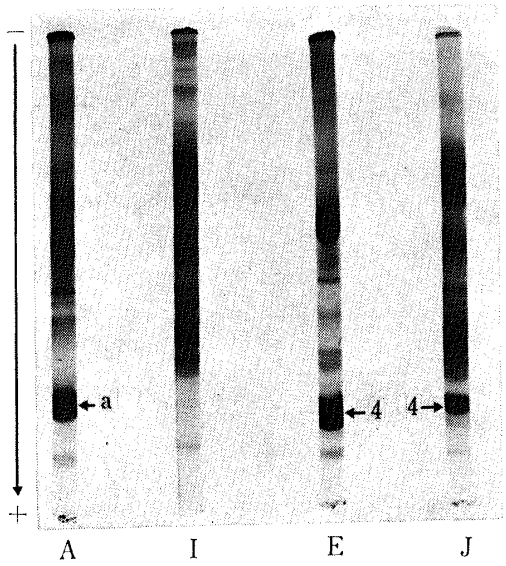

Fig. 8. Polyacrylamide Gel Electrophoresis

Submandibular saliva protein obtained from control rats (A and I), and IPR-treated rats (E and J). A and E: saliva collected by IPR stimulation (20 mg/kg). I and J: saliva collected by methoxamine stimulation (20 mg/kg.) The rats were injected with IPR (20 mg/kg) once daily for three days.

TABLE I. Effects of IPR Treatment and Stimulants on Submandibular Salivary Secretion

Rats were injected with saline or 20 mg of IPR once daily for three days. Saliva was collected one day after the last injection using methoxamine or IPR as a secretory stimulant (20 mg/kg of body weight). The rats were killed after saliva collection and the glands were excised and weighed.

Stimulants	Control groups		Experimental groups	
	Methoxamine Mean±S.E. ($n=7$)	IPR Mean±S.E. ($n=8$)	Methoxamine Mean±S.E. ($n=7$)	IPR Mean±S.E. ($n=8$)
Protein concentration (mg%)	452±110	3810±237	609±105	4105±319
Flow rate (μl/100 mg/hr)	108± 32	64± 10	112± 29	143± 28
Protein secreted (mg/100 mg/hr)	0.49±0.06	2.44±0.07	0.69±0.07	5.88±0.24
$\frac{\text{Submandibular gland}}{\text{Body weight}}$ (mg/100 g)	125± 15	121± 17	171± 21	173± 19

NII-Electronic Library Service

IPR-injected experimental groups, the total protein secreted per hr per submandibular glands was over 8-fold more in saliva collected by IPR stimulation than in saliva collected by methoxamine stimulation.

Proteins of submandibular saliva collected by IPR stimulation (A, E) or methoxamine stimulation (I, J) from control (A, I) and chronically IPR-treated rats (E, J) were analyzed by polyacrylamide gel electrophoresis (Fig. 8). Protein patterns in these four cases were significantly different from each other. The major differences were found in protein (a) with a relative mobility of 0.86 and LM protein with a relative mobility of 0.92. Protein (a) was secreted on IPR stimulation but not on methoxamine stimulation (compare A and I). A large amount of LM protein was secreted on IPR stimulation (E) and a much smaller amount on methoxamine stimulation (J) only in the saliva from chronically IPR-treated rats; the protein was not detectable in the control rats. Another feature is that several protein bands with relative mobilities from 0.54 to 0.79 were detectable in the methoxamine-stimulated saliva.

TABLE II. Effect of Stimulants on the Amount of Soluble Protein in IPR-treated Submandibular Glands

Rats were injected with 20 mg of IPR once daily for three days. The rats were killed before or after saliva collection by stimulation with IPR or methoxamine (20 mg/kg body weight) and the glands were excised and weighed. The soluble protein fraction of the glands was prepared as described in "Materials and Methods". Total protein/submandibular gland and LM protein/submandibular gland were determined.

Stimulants	IPR		Methoxamine	
	Pre-saliva collection Mean±S.E. ($n=7$)	Post-saliva collection Mean±S.E. ($n=8$)	Pre-saliva collection Mean±S.E. ($n=7$)	Post-saliva collection Mean±S.E. ($n=8$)
$\frac{\text{Total protein}}{\text{Submandibular grand}}$ (mg/100 mg)	5.9±0.2	5.3±0.2	5.8±0.2	6.2±0.2
$\frac{\text{LM protein}}{\text{Submandibular gland}}$ (μg/100 mg)	650±59	174±37	682±50	628±58
$\frac{\text{Submandibular gland}}{\text{Body weight}}$ (mg/100 g)	204±29	173±19	199±21	184±18

The presence of LM protein in a larger amount in IPR-stimulated saliva (E and J in Fig. 8) might be a consequence of a difference in the effects of these stimulants on the secretory process from submandibular glands. To test this possibility, the LM protein in the submandibular glands was estimated before and after saliva collection by IPR or methoxamine stimulation (Table II). The LM protein in submandibular glands decreased to 27% of the original value after saliva collection by IPR stimulation whereas the amount of LM protein changed little before and after salive collection by methoxamine stimulation. Total protein/submandibular gland and submandibular gland/body weight changed little under these conditions. These observations are consistent with the appearance of a large amount of LM protein in saliva collected by IPR stimulation (E in Fig. 8).

Discussion

Catecholamines have various effects on salivary glands. Chronic administration of IPR, a β-adrenergic drug, leads to hyperplastic and hypertrophic enlargement of acinar cells of the parotid and submandibular glands with no effect on the sublingual glands.[2-15,29] Wet weight of rat salivary glands (parotid, submandibular and sublingual glands) was proportional to the dose of IPR and the most effective dose of IPR was 50 mg/kg.[29] Similar results were

NII-Electronic Library Service

obtained in the present experiments. Besides the wet weight of salivary glands, increases in total protein, calcium and potassium have been reported in the salivas of rats treated with IPR.[10-12] The increase in the amount of a large mobile (LM) protein in submandibular glands of IPR-treated rats, which was first reported by Menaker *et al.*[13,14] was confirmed by Abe *et al.*[12] This protein was purified to homogeneity and found to be different from the proline-rich protein reported by Fernandez-Soresen and Carlson,[15] as described in the accompanying paper. Various saliva proteins are known to be secreted from submandibular glands through different secretory mechanisms; amylase in acinar cells is secreted by β-agonists,[16-18] whereas epidermal growth factor and nerve growth factor localized in tubular cells are released through the action of α-adrenergic receptors.[19-21] The LM protein which accumulated in the glands upon chronic administration of IPR was secreted by IPR stimulation whereas methoxamine stimulation induced the secretion of only a small amount of this protein (Fig. 8 and Table II). These results suggest that the biosynthesis of LM protein and other saliva proteins is increased in the enlarged submandibular glands by chronic administration of IPR, and that these proteins are secreted from acinar cells through the action of β-adrenergic receptors. The protein (a) secreted upon IPR stimulation in normal rats, was not secreted upon methoxamine stimulation, and the amount of this protein tended to decrease upon chronic administration of IPR. Menaker *et al.*[13] did not observe the presence of this protein in pilocarpine-stimulated whole saliva collected from normal rats.

Acknowledgement I wish to thank Prof. Harumi Okuyama, the Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya, for helpful discussions during the preparation of the manuscript.

References and Notes

1) Location: *7-28-1, Matsufuri-dori, Ichinomiya, 491, Japan.*
2) H. Selye, R. Veilleux and M. Cantin, *Science*, **133**, 44 (1961).
3) K. Brown-Glant, *Nature* (London), **191**, 1076 (1961).
4) T. Barka, *Exp. Cell Res.*, **37**, 662 (1965).
5) R. Baserga, *Life Sci.*, **5**, 2033 (1966).
6) R. Srinivasan, W.W.L. Chang, H. Van Der Noen, and T. Barka, *Anat. Rec.*, **177**, 243 (1973).
7) W.W.L. Chang and T. Barka, *Anat. Rec.*, **178**, 203 (1973).
8) R.S. Bressler, *Am. J. Anat.*, **138**, 431 (1973).
9) J.A.V. Simson, S.S. Spicer, and B.J. Hall, *J. Ultrastruct. Res.*, **48**, 465 (1974).
10) A.L. Wilk and C.T.G. King, *J. Dent. Res.*, **44**, 1374 (1965).
11) A.N. Zengo, I.D. Mandel, A. Solomon, and P. Block, *J. Oral Therap. Pharmacol.*, **4**, 359 (1968).
12) K. Abe, R. Fujita, Y. Yokota, and C. Dawes, *Jap. J. Oral Biol.*, **20**, 521 (1978).
13) L. Menaker, J.H. Sheetz, C.M. Cobb, and J.M. Navia, *Lab. Invest.*, **30**, 341 (1974).
14) L. Menaker, C.M. Cobb, and R.E. Taylor, *Ala. J. Med. Sci.*, **11**, 356 (1974).
15) A. Fernandez-Soresen and D.M. Carlson, *Biochem. Biophys. Res. Commun.*, **60**, 249 (1974).
16) A. Bodlach and M. Schramm, *Biochem. Biophys. Res. Commun.*, **18**, 452 (1965).
17) S. Batzri, Z. Selinger, and M. Schramm, *Science*, **174**, 1029 (1971).
18) J.A. Mangos, N.R. McSherry, T. Barber, S.N. Arvantitakis, and V. Wagner, *Am. J. Physiol.*, **229**, 560 (1975).
19) R.W. Turkington, J.L. Males, and S. Cohen, *Cancer Res.*, **31**, 252 (1971).
20) L.J. Wallace and L.M. Partlow, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 4210 (1976).
21) M.L. Roberts, *Biochim. Biophys. Acta*, **540**, 246 (1978).
22) Y. Yoshida, R.L. Sprecher, C.A. Schneyer, and L.H. Schneyer, *Proc. Soc. Exp. Biol. Med.*, **126**, 912 (1967).
23) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randal, *J. Biol. Chem.*, **193**, 265 (1951).
24) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
25) T.S. Meyer and B.L. Lanberts, *Archs Oral Biol.*, **13**, 839 (1968).
26) A. Mizutani, T. Kitamura, N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.*, **22**, 1955 (1974).
27) Ö. Ouchterlony, *Acta Path. Microbiol. Scand.*, **25**, 186 (1948).
28) G. Mancini, A.O. Carbonara, and J.F. Heremans, *Immunochemistry*, **2**, 235 (1965).
29) P. Pohto and M.K. Paasonen, *Acta Pharmacol. et Toxicol.*, **21**, 45 (1964).

NII-Electronic Library Service