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Isoprenaline-induced changes in rat parotid and submandibular glands are age- and dosage-dependent

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Neonatal rats treated with chronic injections of isoprenaline (isoproterenol) for 10 days revealed differential induction of proline-rich proteins and glycoprotein synthesis between the parotid and submandibular glands. Biosynthesis of proline-rich proteins (M_r 17000–35000) and a M_r-220000 glycoprotein were detectable by solubilization in 10%-trichloroacetic acid extracts from parotid glands 14 days after birth. The enzyme lactose synthase (UDP-galactose:2-acetamido-2-deoxy-D-glucosamine 4β-galactosyltransferase) (EC 2.4.1.22) is also induced 4–7-fold in specific activity compared with control neonatal rats, but again only after 14 days *post partum*, with isoprenaline treatment. This is in accord with the ability of the parotid gland to respond to β-receptor stimulation and subsequent increases in intracellular cyclic AMP necessary for induction of protein synthesis [Grand, Chong & Ryan (1975) *Am. J. Physiol.* **228**, 608–612]. Induction of the proline-rich proteins and a M_r-190000 glycoprotein in the soluble fraction from the submandibular gland were not detected until 49 days after birth under identical conditions in the same animal. Cyclic AMP in the submandibular gland undergoes increases on β-receptor stimulation similar to those achieved in the adult animal, 1 day after birth (Grand *et al.*, 1975). This same differential induction between parotid and submandibular gland was obtained with a range of isoprenaline dosages in adult animals. Trichloroacetic acid-soluble proline-rich proteins were isolated from parotid glands at a dosage of 4.0mg of isoprenaline/kg body wt., but 7.0mg/kg was required to induce also biosynthesis of these proteins in the submandibular gland. Gland hypertrophy showed the same differential dosage kinetics, based on gland weight, between the two glands; however, hypertrophy could be accomplished at a lower dosage of isoprenaline than that used to induce proline-rich-protein biosynthesis.

Administration of the catecholamine isoprenaline (isoproterenol) causes a variety of morphological and cytochemical changes in the parotid and submandibular glands in rats. Initially, isoprenaline causes glandular secretion through interaction with the cell-surface β-receptors, which result in raising the concentrations of cyclic AMP (Schneyer & Hall, 1969; Ekfors *et al.*, 1972; Schramm & Selinger, 1974). Single injections of isoprenaline result in increased synthesis of DNA, RNA and protein (Schneyer, 1972). Continued treatment of rats with isoprenaline results in hypertrophy and hyperplasia of both the parotid and the submandibular glands (Selye *et al.*, 1961; Brown-Grant, 1961; Schneyer, 1962). Biochemical studies on the soluble fraction of cell lysates from both glands after treatment with isoprenaline have shown the induced synthesis of a unique series of basic (pI > 11) proteins rich in the amino acid proline and which are soluble in 10% trichloroacetic acid (Muenzer *et al.*, 1979*a*,*b*; Mehancho & Carlson, 1983). The parotid gland normally produces a single glycoprotein of M_r 200000, but upon chronic injection of isoprenaline a second glycoprotein, of M_r 220000, is induced (Humphreys-Beher *et al.*, 1982). The submandibular gland normally produces two glycoproteins, of M_r 180000 and 200000 (Humphreys-Beher *et al.*, 1982). After injection of isoprenaline, the submandibular gland produces a new glycoprotein, of M_r 158000 (Mehancho & Carlson, 1983). The proline-rich proteins charac-

Abbreviations used: SDS, sodium dodecyl sulphate; Mes, 4-morpholine-ethanesulphonic acid.

teristically have roughly 60% of their amino acid composition as proline, glycine and glutamic acid (Muenzer *et al.*, 1979*a*,*b*). The parotid gland additionally shows a 6–10-fold increase in the Golgi enzyme marker lactose synthase (UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase, EC 2.4.1.22) after chronic treatment with isoprenaline (Zinn *et al.*, 1972; Humphreys-Beher, 1983).

In the neonatal rat, the salivary glands are not fully differentiated and undergo maturation during the first 4–5 weeks *post partum* (Schneyer & Hall, 1969; Redman & Sreebney, 1970; Bressler, 1972). Treatment with isoprenaline at this stage of development leads to precocious morphological development of the parotid and submandibular glands (Barka & van der Noen, 1976; Schneyer, 1978; Sheetz & Menaker, 1979). The ability of the parotid gland to undergo complete morphological and biochemical maturation in response to isoprenaline occurs only after day 14 *post partum* (Grand *et al.*, 1975; Grand & Schay, 1978). However, the ability of the submandibular gland to respond to isoprenaline stimulation by advanced biochemical and morphological development is the same 1 day after birth as it is in the adult (Bressler, 1972; Grand *et al.*, 1975). This effect has been observed not only in terms of accelerated morphological development, but in overall protein composition and response of adenylate cyclase in increasing cyclic AMP (Ekfors *et al.*, 1972; Bressler, 1972; Grand *et al.*, 1975; Grand & Schay, 1978; Lumford & Talamos, 1980). Although the synthesis of the proline-rich proteins is induced in adult animals on treatment with isoprenaline, they are not present at any time during development *post partum* in either the parotid or the submandibular gland (Humphreys-Beher *et al.*, 1982). This suggests these proteins are not under developmental control, although there has been no investigation of the synthesis of these proteins *in utero*.

In the present paper, evidence is presented for the differential induction of the proline-rich proteins and glycoproteins synthesized by the parotid and submandibular glands in response to chronic isoprenaline treatment as a consequence of age. Induced changes in the proline-rich proteins in the parotid gland demonstrate the same kinetics of induction as that of cyclic AMP in response to stimulation of the cell-surface β-receptor. However, the submandibular gland shows no such similarity in the increases in cyclic AMP generated by isoprenaline treatment and the ability to induce proline-rich proteins or glycoproteins. Synthesis of these proteins did not occur until 49 days after birth in the submandibular gland, but they were inducible by day 14 in the parotid gland. Furthermore, the effect of isoprenaline on rat salivary glands is shown to be dosage-dependent in the adult animal, with the parotid gland again showing more sensitivity to treatment than the submandibular gland at lower dosages of the drug.

Materials and methods

Chemicals

(±)-Isoprenaline and trichloroacetic acid were purchased from Sigma. All reagents for polyacrylamide-gel electrophoresis were of ultra-pure quality, obtained from Bio-Rad. UDP-[1-^{14}C]galactose (sp. radioactivity 300 Ci/mmol) was purchased from New England Nuclear. All other chemicals were of analytical-grade quality and obtained from commercial sources.

Isoprenaline treatment

Male Wistar rats (7–35 days old) received 0.025 mg of isoprenaline/g body wt. for 10 days. Adult male rats weighing 200–250 g received intraperitoneal injections of 5 mg of isoprenaline daily and were fed *ad libitum* unless otherwise stated in the Results and discussion section.

Tissue preparation

Parotid and submandibular glands were identified by gross morphology. The glands were removed from animals that had been anaesthetized with sodium pentobarbital and killed by exsanguination, and soluble and insoluble membrane fractions were prepared by homogenization in 10 mM-Tris/HCl buffer, pH 8.0, with a Dounce apparatus. The slurry was then centrifuged at 100000*g* for 1 h. Protein assays were performed by a modification of the Lowry method with bovine serum albumin as standard (Schacterle & Pollack, 1973). For purification of the salivary-gland glycoproteins and proline-rich proteins, the soluble fraction was diluted with an equal volume of 20% trichloroacetic acid. The proline-rich proteins and parotid and submandibular glycoproteins remain soluble in 10% trichloroacetic acid (Muenzer *et al.*, 1979*a*,*b*). Insoluble proteins were removed by centrifugation at 15000*g* for 15 min, and trichloroacetic acid was removed by repeated extraction with diethyl ether.

Polyacrylamide-gel electrophoresis and protein staining

Protein samples were electrophoresed in an 8%- or 12.5%-polyacrylamide gel by using a modification of the Laemmli Tris/glycine system (Pugsley & Schnaitman, 1979). All gels were fixed and stained by a modification of the method of Fairbanks *et al.* (1971) for staining total protein with Coomassie Blue R-250, or by staining with periodic acid/Schiff reagent (Fairbanks *et al.*, 1971) for identifica-

tion of glycoproteins. In staining polyacrylamide gels containing proline-rich proteins, staining time was shortened to 30min with Fairbanks' staining solution no. 1 only and destaining was performed in several changes of 10% acetic acid over 24h. Samples for gels were made up to 1mg of protein/ml of sample buffer. Lane 3 of Figs. 2(*a*) and 3(*a*) has 40μl of sample added to enhance the background proteins present before induction of proline-rich proteins by isoprenaline.

Galactosyltransferase assay

The activities of UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase and UDP-galactose:*N*-acetylgalactosaminyl-protein 3β-galactosyltransferase were measured by a modification of the assay used by Carlson *et al.* (1973). Membrane fractions were obtained as described above and resuspended in 10mM-Tris/HCl buffer, pH8.0, to give a final protein concentration of 20mg/ml. The assay mixture (total volume 50μl) contained 0.1M-Mes, pH6.3, 25mM-$MnCl_2$, 0.5% Triton X-100, 1mM-UDP-[1-^{14}C]galactose (6×10^6c.p.m./μmol) and either 10mM-*N*-acetylglucosamine or asialo-(sheep submaxillary mucin) and 0–0.5mg of the enzyme preparation. The asialo-mucin was sheep submaxillary mucin from which sialic acid residues had been removed by sialidase treatment or by acid hydrolysis (Carlson *et al.*, 1973).

After incubation at 37°C for 40min, the reaction was stopped by adding 10μl of 10mM-EDTA in 7.5% (w/v) sodium tetraborate. High-voltage paper electrophoresis was performed on Whatman 3MM paper for 45min at 1700V as described by Carlson *et al.* (1973).

Protein purification

Trichloroacetic acid-soluble glycoproteins were isolated from the proline-rich proteins by chromatography on a Sephadex G-100 column (1cm × 90cm). The protein was eluted with 10mM-Tris/HCl buffer, pH7.5, and 2ml fractions were collected. Protein elution was monitored by A_{230} (Mehancho & Carlson, 1983). Since the parotid-gland glycoproteins after isoprenaline treatment could not be separated by this method, the two proteins were co-purified for subsequent analysis.

Amino acid analysis

Purified glycoproteins were subsequently dialysed to remove Tris buffer and freeze-dried. A 0.2mg sample of protein was analysed for amino acid composition after hydrolysis in 6M-HCl for 18h, as described by Muenzer *et al.* (1979*b*). Amino acid analysis was performed on a Beckman 119 analyser, by using single-column methodology.

Results and discussion

Beginning at 7 days after birth, neonatal animals were given daily injections of 0.025mg of (±)-isoprenaline/g body wt. for 10 days. After this time the parotid and submandibular glands had undergone gross morphological changes in appearance, resembling those glands isolated from mature animals, as well as showing increased hypertrophy. The soluble fraction isolated from cell lysates also gave patterns on SDS/polyacrylamide-gel electrophoresis resembling those of the proteins produced by adult animals (Fig. 1). Although not fully differentiated, the protein pattern at 7 days *post partum* (treated animal) bears a similarity to the protein profile for the adult (Fig. 1, lanes 2 and 5 respectively). Only in the 14-day-old animal can the changes in parotid-gland glycoprotein (M_r 220000) be seen, whereas these changes do not occur in the parotid gland of the 7-day newborn (control or isoprenaline-treated) or the adult (Fig. 1). Treatment of neonatal-rat submandibular gland in the same manner resulted in precocious changes in the soluble cell proteins typical of the gel profile found

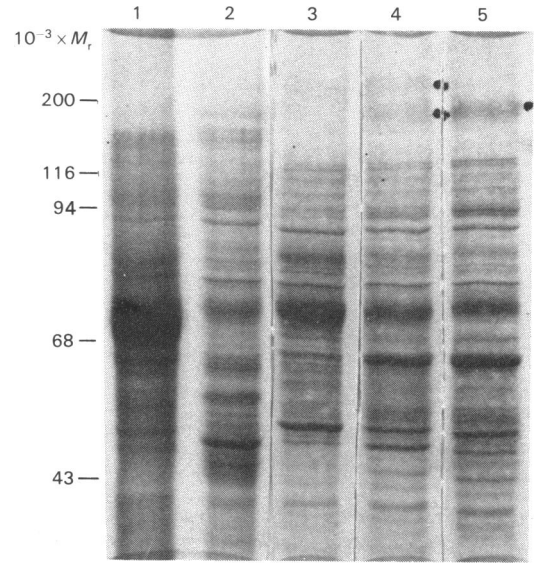

Fig. 1. *Electrophoresis (8%-polyacrylamide gel) of the 100000g soluble fraction of parotid-gland cells, stained for total protein by Coomassie Brilliant Blue*

Lane 1, 7-day newborn, untreated; lane 2, 7-day newborn, treated for 10 days with isoprenaline; lane 3, 14-day newborn, untreated; lane 4, 14-day gland treated with isoprenaline; lane 5, adult untreated soluble fraction. Dots indicate the M_r-200000 and -220000 glycoproteins. M_r markers were myosin (200000), β-galactosidase (116000), phosphorylase (94000), serum albumin (68000) and ovalbumin (43000).

in the adult control gland (results not shown). After this analysis, the soluble protein fraction of the parotid and submandibular gland was extracted with trichloroacetic acid to detect better the induced expression of proline-rich proteins and glycoproteins. As shown in Figs. 2(*a*) and 3(*a*), the soluble cell extracts from parotid glands of neonatal rats were able to respond to chronic isoprenaline treatment with the induction of synthesis of proline-rich proteins and the M_r-220000 glycoprotein after day 14 after birth (Fig. 2, lane 5; Fig. 3, lane 4). In comparison, lanes 8 and 9 (Figs. 2*a*) refer to parotid glands from control and isoprenaline-treated adult animals respectively. Lanes 9 and 10 of Fig. 3(*a*) show the periodate–Schiff-stained glycoproteins isolated from control and isoprenaline-treated parotid glands. The neonatal animals beyond 14 days of age are able to respond to treatment in the same manner as the adult animals in terms of the proteins induced.

The UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase activity could be stimulated from 0.27 (untreated rat newborns) to 1.38–1.87 nmol/min per mg of protein after 14 days from birth on treatment with isoprenaline (Table 1). This was not the case for enzyme activity assayed in animals 7, 9 or 11 days *post partum* which received injections of isoprenaline for 10 days (Table 1). Enzyme activity for UDP-galactose:*N*-acetylgalactosamine 3β-galactosyltransferase (no EC number) remained unchanged for both treated and untreated animals over the developmental period studied (results not shown).

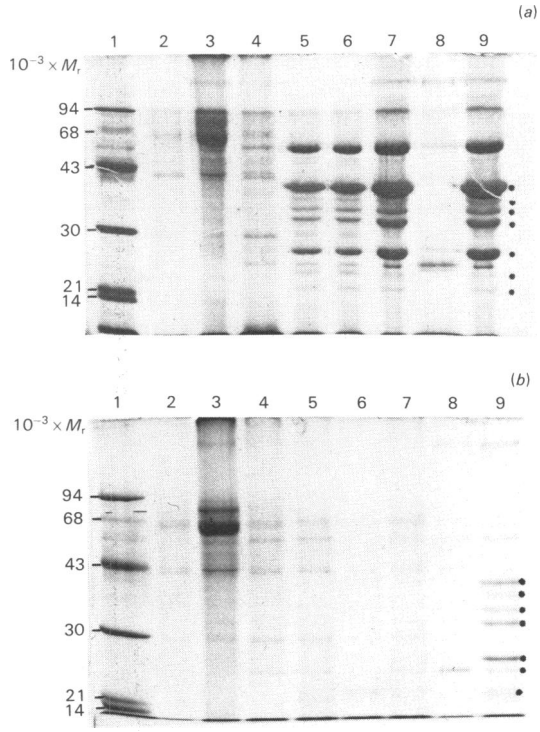

Fig. 2. *Electrophoresis (12.5%-polyacrylamide gel) of 10%-trichloroacetic acid-soluble proteins isolated from (a) parotid glands and (b) submandibular glands of rats treated for 10 days with isoprenaline*

The gels are stained with Coomassie Brilliant Blue R-250 for total protein present. Lanes in both parts: 1, M_r standards; 2, 7-day neonatal rat; 3, 9-day neonatal rat; 4, 11-day neonatal rat; 5, 14-day neonatal rat; 6, 28-day neonatal rat; 7, 35-day neonatal rat; 8, adult control; 9, adult rat injected for 10 days with isoprenaline. Dots represent migration of proline-rich proteins. Each lane contains 20 μg of total protein, except lane 3, which was run with 40 μg to show differences in the acid-soluble proteins between induced and non-induced states.

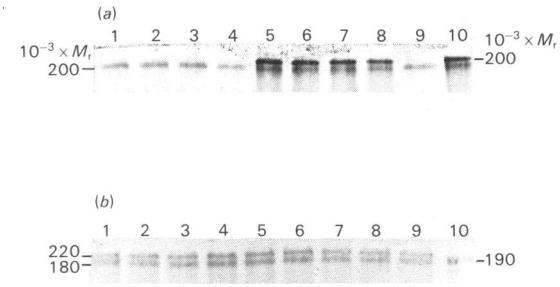

Fig. 3. *Electrophoresis (8%-polyacrylamide gels) of trichloroacetic acid-soluble proteins stained with periodic acid/Schiff reagent for glycoproteins from the parotid (a) and submandibular (b) glands of rats treated with isoprenaline*

Lane 1, untreated 7-day newborn. Lanes 2–8, 7-, 9-, 11-, 14-, 21-, 28- and 35-day neonatal rats treated with isoprenaline. Lane 9, 49-day-old untreated adult. Lane 10, adult animal (49 days), chronic isoprenaline treatment.

Table 1. *Effects of isoprenaline on the activity of 4β-galactosyltransferase in neonatal-rat parotid glands*

Results are averages of three experiments for both control and isoprenaline-treated animals.

	Enzyme activity (nmol of galactose/min per mg of protein)	
Age (days)	Control	Isoprenaline-treated
7	0.27	0.31
9	0.31	0.41
11	0.31	0.28
14	0.39	1.79
21	0.35	1.38
28	0.28	1.87
35	0.31	1.79
49	0.34	1.81

When the submandibular glands from the same animals were examined for the induction of the trichloroacetic acid-soluble proteins, no evidence was found for their synthesis before 49 days after birth (Figs. 2*b* and 3*b*). The control submandibular gland from adult and neonatal animals has been previously reported (Humphreys-Beher *et al.*, 1982) to produce two glycoproteins, of M_r 180000 and 200000 (Fig. 3*b*), which appear to be present in trichloroacetic acid extracts from neonatal rats treated with isoprenaline. The acid-soluble glycoproteins from a 7-day newborn (untreated control) are shown in Fig. 3(*b*), lane 1. The M_r-190000 glycoprotein is unique to the submandibular-gland trichloroacetic acid extracts of adults (Fig. 3*b*, lane 10) and could be the M_r-158000 protein reported by Mehancho & Carlson (1983), but demonstrating a slightly higher M_r owing to the differences in gel systems used.

To test the relatedness of M_r-190000 to the M_r-158000 glycoprotein (gp 158), these proteins were purified by trichloroacetic acid extraction and subsequent chromatography on a Sephadex G-100 column eluted with 10mM-Tris/HCl buffer, pH7.5 (results not shown). The glycoprotein isolated from the isoprenaline-treated submandibular gland was analysed for a comparison of its amino acid composition with that of the composition of gp158 (Mehancho & Carlson, 1983). The overall amino acid composition suggests that gp158 and the M_r-190000 glycoprotein are different proteins of the proline-rich type.

In an attempt to understand further this phenomenon of differential induction of biosynthesis of glycoprotein and proline-rich proteins, adult rats were given various doses of isoprenaline for 10 days. Syntheses of the proline-rich proteins were induced with as little as 1mg of isoprenaline/200g animal in the parotid gland, whereas a greater dosage (1.75mg/animal) was necessary to induce synthesis fully in the submandibular gland (Figs 4*a* and 4*b*). The biosynthesis of the M_r-190000 submandibular glycoprotein occurs at a concentration of 1.5mg/animal (Fig. 5*b*). Because of the gel concentration (12.5%), the induction of the M_r-220000 glycoprotein from the parotid is not evident in Fig. 4(*a*), but it is indeed induced at 1.0mg of isoprenaline/animal (results not shown). Finally, gland hypertrophy was examined after the 10 days with various dosages of isoprenaline. This response was found to be more sensitive to isoprenaline treatment than was induction of proline-rich-

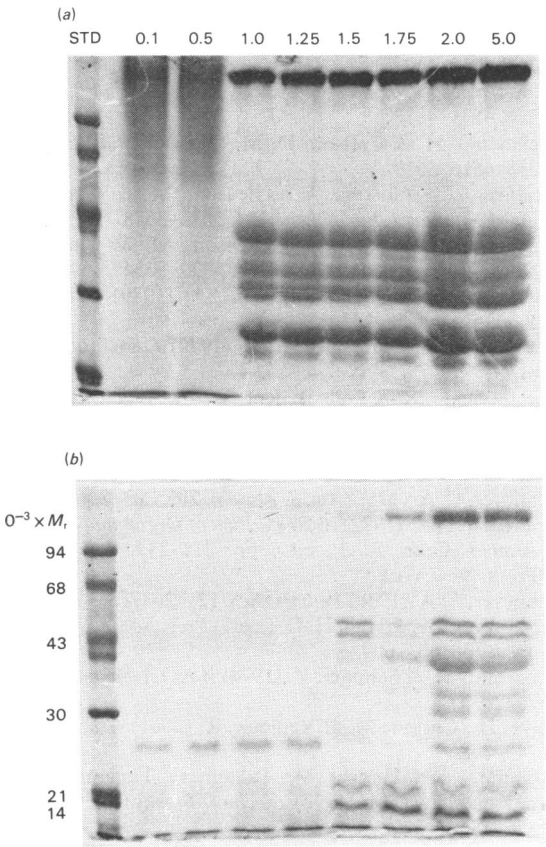

Fig. 4. *Electrophoresis (12.5%-polyacrylamide gels) of trichloroacetic acid-soluble proteins isolated from adult rat parotid (a) and submandibular (b) glands*

The concentrations of isoprenaline shown at the tops of the gels were given each day for 10 days to 200g animals. The gels are stained with Coomassie Brilliant Blue. STD, M_r markers given in (*b*): phosphorylase *b* (94000); bovine serum albumin (68000); ovalbumin (43000); carbonic anhydrase (30000); soya-bean trypsin inhibitor (21000); lysozyme (14000).

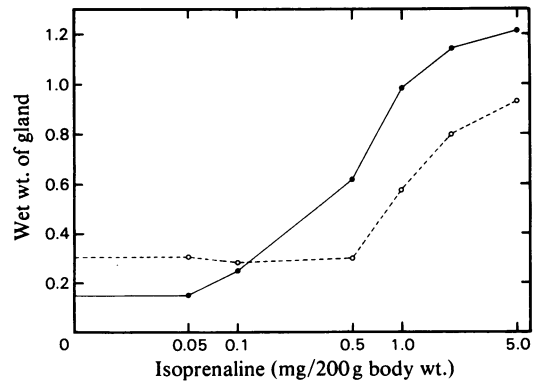

Fig. 5. *Gland hypertrophy after 10 days treatment with various concentrations of isoprenaline*

●, Parotid gland; ○, submandibular gland.

protein biosynthesis. As shown in Fig. 5, at a concentration of 0.1 mg of isoprenaline/animal the parotid gland has increased in wet weight. However, the submandibular gland again requires a 10-fold higher concentration of isoprenaline before hypertrophy becomes evident, and requires an even greater dosage of isoprenaline to induce subsequent synthesis of the proline-rich proteins.

Isoprenaline stimulates parotid and submandibular-gland activity by interaction with the cell-surface β-receptors. The interactions leads to a rise in cyclic AMP through activation of adenylate cyclase. In the parotid gland the ability of isoprenaline to cause an increase in cyclic AMP is not evident until 14 days *post partum* (Grand *et al.*, 1975; Grand & Schay, 1978; Lumford & Talamos, 1980) and has been suggested to result from a low content of guanine-nucleotide-binding regulatory protein, required for adenylate cyclase activity (Lumford & Talamos, 1983). However, this does not explain the inability of the submandibular gland to synthesize the proline-rich proteins and M_r-190000 glycoprotein in the neonatal animal or at corresponding doses of isoprenaline in the adult animal. It may be that induction of these proteins in the submandibular glands requires the participation of other factors induced by isoprenaline in other glands, such as hormones, or it may be that the submandibular gland is more refractory to isoprenaline stimulation, owing to its lower content of cell-surface β-receptors compared with the parotid (Grand *et al.*, 1975). Injections of both thyroxine and cortisol have been shown to cause precocious development of neonatal-mouse parotid glands (Kumigawa *et al.*, 1977; Takuma *et al.*, 1978; Imai *et al.*, 1982). Clearly, further investigation is required to determine the exact nature of this difference in isoprenaline sensitivity.

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