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Comparative developmental analysis of the parotid, submandibular and sublingual glands in the neonatal rat

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Analysis of the soluble protein fractions from the rat parotid, submandibular and sublingual glands by polyacrylamide-gel electrophoresis reveals similarities in overall patterns of protein synthesis at birth. Tissue-specific changes in protein and glycoprotein synthesis occur shortly after birth and again at the time of weaning, 21–28 days later. Incorporation of [^{3}H]thymidine into DNA was at its highest after birth and gradually decreased in both the parotid and submandibular gland, whereas [^{3}H]thymidine incorporation in the sublingual gland was low throughout the time of neonatal development. [^{14}C]Leucine incorporation into total protein increased in all glands with age after birth, showing an accelerated rate 21–28 days later. Trichloroacetic acid/phosphotungstic acid-precipitable [^{3}H]fucose in glycoproteins declined over the time of neonatal development in the parotid and submandibular gland, but its incorporation remained higher in the sublingual gland. α-Amylase (EC 3.2.1.1) in the salivary glands increased at the time of weaning, as judged by detectability in sodium dodecyl sulphate/polyacrylamide gels and by immune precipitation. Two membrane-bound enzymes, UDP-galactose:2-acetamido-2-deoxy-D-glucosamine 4β-galactosyltransferase (EC 2.4.1.22) and UDP-galactose:2-acetamido-2-deoxy-D-galactosaminyl-protein 3β-galactosyltransferase (no EC number), undergo tissue-specific changes rather than changes induced by physiological stimulation of the salivary glands.

The salivary-gland system of the rat, including the parotid, submandibular and sublingual glands, has been studied extensively as a developmental model for tissue maturation (for review, see Schneyer, 1972). Whereas most work has centred on studying development through electron microscopy (Yamashina & Mizuhira, 1976; Denny & Cope, 1977), only more recently have attempts been made at understanding development at the molecular level.

Histological observations have established that the neonatal parotid and submandibular glands are immature with regard to terminally differentiated acinar cells at the time of birth (Ball, 1974). Innervation of these glands by sympathetic and parasympathetic processes occurs after birth, along with cytodifferentiation of the gland cell types. The importance of neuronal stimulation to the process of cytodifferentiation has been studied by Schneyer & Hall (1969). Parasympathectomy of neonatal rats results in retarded gland growth as well as decreased mitosis. In contrast with the immaturity of function and structure observed in the parotid and submandibular glands, the sublingual gland at the time of birth is functionally and structurally similar in organization to the adult gland (Redman & Ball, 1978).

Biochemical analysis of the neonatal development of the salivary glands of the rat had not been extensive. Several secretory enzymes, including α-amylase (EC 3.2.1.1) and RNAase (EC 3.1.27.5), were studied by Ball (1974) and Ball & Nelson (1978). Increases in α-amylase and RNAase in the parotid gland were observed shortly after birth, but only transient increases were found at birth for these enzymes in the submandibular gland. The sublingual gland, owing to its function as the primary producer of salivary mucins, was devoid of these enzyme activities. The catecholamine isoprenaline (isoproterenol) has been used to study morphological changes in the parotid and submaxillary glands because of its ability to induce precocious development in the neonatal rat (Schneyer, 1972). Among the biochemical changes induced in the

Abbreviations used: RNAase, ribonuclease; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholine-propanesulphonic acid.

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parotid gland of adult male rats by chronic isoprenaline injection have been increases in the enzyme UDP-galactose:2-acetamido-2-deoxy-D-glucosamine 4β-galactosyltransferase (Zinn *et al.*, 1972) and the appearance of a series of proline-rich proteins and glycoproteins (Fernandez-Sorenson & Carlson, 1974; Muenzer *et al.*, 1979*a*,*b*).

In studies reported here, radiolabelled compounds were employed to follow changes in developing salivary glands. Alterations in total protein and glycoprotein biosynthesis are analysed by SDS/polyacrylamide-gel electrophoresis. Emphasis is placed on determining whether the observed changes induced in adult rats with isoprenaline were the result of renewed transcription and translation of developmental genes expressed in the neonatal rat parotid gland. UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase (EC 2.4.1.22) and UDP-galactose:*N*-acetylgalactosaminyl-protein 3β-galactosyltransferase (no EC number) were assayed for changes with development in neonatal rats. α-Amylase (EC 3.2.1.1) was used as a control marker of gland maturation.

Materials and methods

Materials

All reagents used for polyacrylamide-gel electrophoresis were purchased from Bio-Rad. [^{14}C]-Leucine (300mCi/nmol), [^{3}H]fucose (40Ci/mmol) and [^{3}H]thymidine (100Ci/mmol) (for incorporation *in vivo*) were obtained from Amersham. All other chemicals were of analytical-grade quality and obtained through commercial sources.

Animals

Sprague–Dawley rats were used in all experiments. The ages of neonatal animals were the same in all experiments; 1–7-day neonatal animals and rats obtained every 7 days thereafter were selected for developmental analysis. Rats 7 weeks of age and weighing 200–225g were considered to be adults.

Tissue preparation

Salivary glands of neonatal rats were identified by gross morphology. The glands were removed, and soluble and insoluble membrane fractions were prepared either by homogenization in 10mM-Tris/HCl buffer, pH7.2, with a Dounce apparatus, or by a rapid freeze–thaw in an acetone/solid-CO_2 bath followed by sonic disruption. The slurry was then centrifuged at 100000**g** for 1h. Protein assays were performed by a modification of the Lowry method, with bovine serum albumin as standard (Schacterle & Pollack, 1973).

Polyacrylamide-gel electrophoresis and protein staining

Protein samples were subjected to electrophoresis in an 8%-polyacrylamide gel by using the Tris/glycine system of Laemmli (1970). All gels were fixed and stained by 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 the identification of glycoproteins. For fluorography of radiolabelled proteins the gel was further fixed in the presence of the scintillator En^{3}Hance (New England Nuclear Corp.). The gels were dried on filter-paper backing and exposed to X-ray film for 24h at −70°C.

Radiolabelling of cellular material

Synthesis of DNA, protein and glycoprotein was studied in animals by injecting them intraperitoneally with [^{3}H]thymidine, or with a combination of [^{3}H]fucose and [^{14}C]leucine at a final concentration of 1μCi/g body wt. After 3.5h the animals were anaesthetized with sodium pentobarbital and the glands of interest were surgically removed. The parotid, submandibular and sublingual glands were placed into 1ml of cold 10mM-Hepes buffer, pH7.2, and subsequently frozen in a solid-CO_2/acetone bath. After thawing at room temperature, the glands were further disrupted by sonication at 4°C. A mixture of cold 10% (w/v) trichloroacetic acid and 1% phosphotungstic acid (8ml) was added and the precipitate was collected by filtering on Whatman GF/A glass filters. The filters were dried and radiolabel incorporation was measured by liquid-scintillation counting with Beckman non-aqueous (NA) 'cocktail'.

Glycoprotein labelling *in vitro* was performed by the method of Gahmberg & Hakomori (1963). Galactose residues were oxidized by the addition of 25 units of galactose oxidase (Sigma Chemical Co.)/mg of protein. The glycoprotein/enzyme mixture was incubated for 2h at room temperature, and 250μCi of $NaB^{3}H_4$/mg of protein in 0.1M-NaOH was added at a final concentration of 5mM.

Galactosyltransferase assay

The activities of UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase (enzyme I) and UDP-galactose:*N*-acetylgalactosaminyl-protein 3β-galactosyltransferase (enzyme II) 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, to give a final protein concentration of 20mg/ml. The assay mixture (total volume 50μl) contained 0.1M-Mes, pH5.7, 25mM-$MnCl_2$, 0.5% Triton X-100, 1mM-UDP[1-^{14}C]galactose, (10^{6}c.p.m./μmol) and either 10mM-*N*-acetylglucos-

amine or asialo-(sheep submaxillary mucin), and 0–0.5mg of the enzyme preparation. The asialomucin is 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 7.5% sodium tetraborate/0.20M-EDTA. Electrophoresis was performed on Whatman 3M paper for 45min at 1700V as described by Carlson *et al.* (1973).

α-Amylase assay

α-Amylase was assayed as described by Bernfeld (1955). Values are expressed as nmol of maltose equivalents formed/min per mg of protein.

Results and discussion

Reports from other laboratories (Schneyer, 1972; Schneyer & Hall, 1969; Yamashina & Mizuhira, 1976) on the development of rat salivary glands have been confirmed and extended by observing changes in DNA, protein and glycoprotein compositions at various developmental stages and in the adult rat. Table 1 shows that the incorporation of [^{3}H]-thymidine into DNA, highest at birth, decreases with age. [^{3}H]Thymidine incorporation into sublingual glands is essentially constant after day 3, which concurs with the morphological studies of Leeson & Booth (1961), who reported that the sublingual glands are almost completely developed at birth. Most major changes take place in sublingual glands 18 days *post conceptio* (Redman & Ball, 1978). On the basis of the data presented in Table 1, the parotid glands develop slowly after birth, and the submandibular glands are essentially fully developed by day 28. Cellular proliferation and differentiation of the major salivary glands progress most rapidly during the first 3 weeks after birth (Schneyer & Schneyer, 1961; Schneyer & Hall, 1969; Schneyer, 1972). Relative changes in the biosynthesis of proteins and of fucose-containing glycoproteins were followed by dual labelling with [^{3}H]fucose and [^{14}C]leucine (Table 2). Incorporation of [^{3}H]fucose, presumably into glycoproteins, decreases with age in all three glands. However, the decrease is most dramatic in the parotid glands. The high ratio of [^{3}H]fucose/[^{14}C]leucine incorporation for the sublingual glands is consistent with the known function of these glands as mucus-secreting glands. Protein synthesis, as determined by [^{14}C]leucine incorporation, is constant after day 5 in the sublingual glands, but increases dramatically at about day 28 for both the parotid and submandibular glands.

The major glycoproteins synthesized by all three glands are of similar molecular weights (180000 and 200000; Fig. 1), and these are most prevalent during the first week after birth. Both of these high-molecular-weight glycoproteins are present in the submandibular and in the sublingual glands of the adult rat (Fig. 1*b*), except that the relative amounts seem to change in the submandibular glands. In the parotid glands, only the 200000-mol.wt. glycoprotein remains after day 28 (Fig. 1*a*). Materials heavily stained with periodic acid–Schiff reagent (Fig. 1*b*, lane 4), which just enters the separation gel, are probably mucus glycoproteins. These high-molecular-weight substances are generally excluded from 8%-polyacrylamide gels (Holden *et al.*, 1971*a*,*b*). It appears that only the sublingual glands,

Table 1. [^{3}*H*]*Thymidine incorporation into parotid, submandibular and sublingual glands during neonatal development*
Values are expressed in c.p.m./mg of protein from a 20mg tissue slice and are set relative to activity in the adult rat. Samples were prepared by taking 15mg of trimmed tissue frozen in 1ml of 10mM-Hepes buffer, pH 7.2. This mixture was thawed and sonicated to disrupt cells. Samples were diluted in 10% trichloroacetic acid/1% phosphotungstic acid and filtered through glass filters, dried and counted for radioactivity. Results are averages for two experiments, with two animals per experiment.

Age (days)	Gland ...	Incorporation		
		Parotid	Submandibular	Sublingual
49 (adult)		1.0 (679 c.p.m./mg)	1.0 (1057 c.p.m./mg)	1.0 (329 c.p.m./mg)
1		4.10	6.03	2.34
3		4.68	3.07	1.28
5		3.99	2.79	1.20
7		3.65	2.81	1.16
14		3.49	1.73	1.13
21		2.69	1.63	1.10
28		2.44	1.12	1.05
35		2.54	1.11	0.98

Table 2. *Incorporation of [^{14}C]leucine and [^{3}H]fucose into trichloroacetic acid/phosphotungstic acid-insoluble protein in neonatal-rat salivary glands*

Results are averages for four animals per time point per two experiments.

Time after birth (days)	10^{-3} × Incorporation (c.p.m./h per mg of gland protein)								
	Parotid			Submandibular			Sublingual		
	[^{3}H]Fucose	[^{14}C]Leucine	^{3}H/^{14}C	[^{3}H]Fucose	[^{14}C]Leucine	^{3}H/^{14}C	[^{3}H]Fucose	[^{14}C]Leucine	^{3}H/^{14}C
1	4.2 (±0.82)	0.69 (±0.25)	6.08	4.18 (±0.35)	1.5 (±0.48)	2.79	4.75 (±0.12)	1.6 (±0.32)	2.97
3	6.20	1.16	5.34	3.16	2.50	1.26	4.44	1.70	2.61
5	2.60	1.15	2.26	2.50	2.50	1.01	3.95	1.40	2.83
7	2.00	1.21	1.66	2.26	2.30	0.98	2.67	3.12	0.85
14	1.76	1.84	0.94	2.86	2.40	1.19	3.18	3.80	0.84
21	1.88	2.41	0.78	2.48	3.78	0.65	3.20	3.78	0.85
28	1.90	5.84	0.32	2.14	7.28	0.28	2.70	3.80	0.71
35	1.58	7.25	0.21	1.94	6.46	0.30	2.80	3.35	0.83
49 (adult)	1.84	8.37	0.22	1.85	6.38	0.28	3.30	3.33	0.99

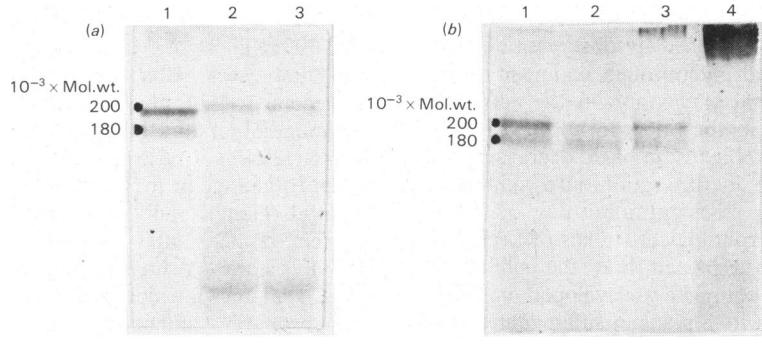

Fig. 1. *Identification of salivary-gland glycoproteins from total soluble protein*

(*a*) SDS/8%-polyacrylamide gel of the soluble fractions of the parotid gland stained for carbohydrate-containing proteins with periodate/Schiff reagent, and (*b*) SDS/polyacrylamide gel of the soluble fraction of the neonatal submandibular gland and sublingual gland stained with periodate/Schiff reagent. (*a*) Lane 1, 1-day neonatal gland; lane 2, 21-day neonatal gland; lane 3, adult parotid gland. The molecular weights correspond to 200000 and 180000 respectively. (*b*) Lane 1, 7-day submandibular gland; lane 2, adult submandibular gland; lane 3, 7-day sublingual gland; lane 4, adult sublingual gland.

as expected, contain high amounts of mucus glycoproteins.

Soluble fractions of parotid, submandibular and sublingual glands were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 2). There appeared to be a two-step change in protein synthesis by the parotid glands (Figs. 2*a* and 2*b*); between day 3 and day 4 *post partum* (lanes 5 and 6, Fig. 2*a*), synthesis of a protein of apparent mol.wt. 180000 stopped. This observation is consistent with the data presented in Fig. 1. The 200000-mol.wt. glycoprotein is the major glycoprotein of adult-rat parotid-gland secretion, as determined by analysis of secretions collected by cannulating the parotid-gland duct.

Other major changes in proteins appear at 21–28 days after birth. α-Amylase (58000 mol.wt.) is detected during this developmental period, as is a new protein band of apparent mol.wt. 25000 (Fig. 2*b*, lanes 6 and 7). The 58000-mol.wt. component was immunoprecipitated by α-amylase antibody, confirming its identity (results not shown). A major protein of about 60000 mol.wt. decreases in amount. Protein profiles for extracts of submandibular and sublingual glands (Figs. 3*a* and 3*b*) were similar to those exhibited for parotid glands, but additional changes appearing to be tissue-specific differences were noted during development. Electrophoresis patterns of the submandibular and sublingual glands

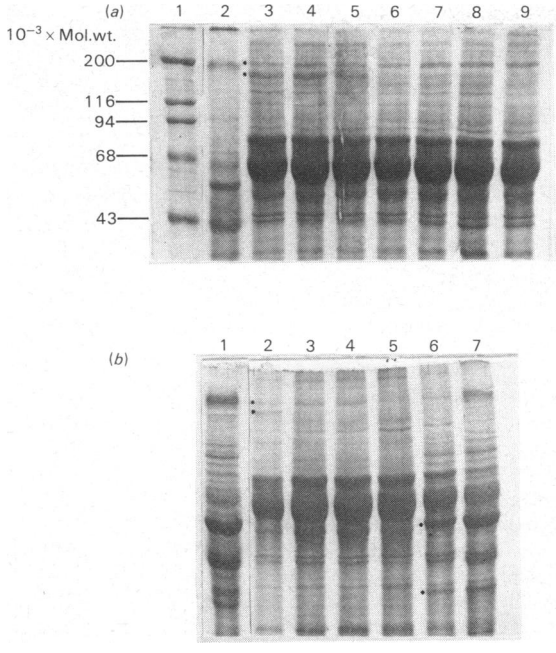

Fig. 2. *SDS/8%-polyacrylamide-gel electrophoresis of parotid-gland 100000 g soluble fraction stained for total protein by the method of Fairbanks et al. (1971)*

(*a*) Lane 1, molecular-weight standards: myosin (200000); β-galactosidase (116000); phosphorylase *a* (94000); bovine serum albumin (68000); ovalbumin (48000). Lane 2, adult male rat; lane 3, 1-day neonatal; lane 4, 2-day; lane 5, 3-day; lane 6, 4-day; lane 7, 5-day; lane 8, 6-day; lane 9, 7-day parotid gland. The bold dots represent the 200000- and 180000-mol.wt. glycoproteins respectively. (*b*) Lane 1, adult; lane 2, 1-day; lane 3, 7-day; lane 4, 14-day; lane 5, 21-day; lane 6, 28-day; lane 7, 35-day parotid gland. The lower bold dots represent amylase (mol.wt. 58000) and the appearance of a minor protein with mol.wt. 25000.

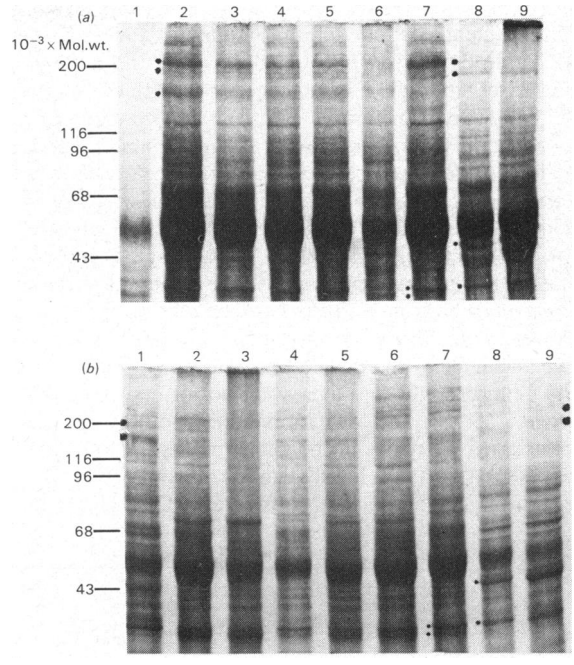

Fig. 3. *SDS/8%-polyacrylamide-gel electrophoresis of (a) the soluble proteins from the neonatal-rat submandibular gland and (b) the soluble proteins from the neonatal-rat sublingual gland*

(*a*) Lane 1, adult-rat submandibular gland; lane 2, 1-day neonatal submandibular gland; lane 3, 4-day; lane 4, 5-day; lane 5, 7-day; lane 6, 14-day; lane 7, 21-day; lane 8, 28-day; lane 9, 35-day submandibular gland. (*b*) Lanes 1–9 correspond to the developmental ages (days *post partum*) for the submandibular gland in (*a*). The bold dots represent 200000-mol.wt. and 180000-mol.wt. glycoproteins, and the 150000-mol.wt. protein of the submandibular gland. The small dots represent the 58000-mol.wt. and 25000-mol.wt. doublets and single protein respectively on the right side of gels (*a*) and (*b*).

show only a single protein band of about 25000 mol.wt. after day 28 (Figs. 3*a* and 3*b*, lanes 7 and 8), whereas parotid gland had two proteins of close to 25000 mol.wt. A protein of about 150000 mol.wt. was prominent at day 1 (Fig. 3*a*, lane 2), but this material gradually decreased in amount and was not detected at day 28, or in the adult.

Several studies have shown changes in various enzyme activities associated with development and differentiation of the salivary glands, pancreas and other tissues. α-Amylase and ribonuclease in neonatal rats show specific and divergent patterns of activities for the parotid, submandibular and sublingual glands (Ball, 1974; Ball & Nelson, 1978). A dramatic increase of over 6-fold in the specific activity of UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase in parotid glands of isoprenaline-treated rats was reported by Zinn *et al.* (1972), but the specific activity of a companion galactosyltransferase, UDP-galactose:*N*-acetylgalactosaminyl-protein 3β-galactosyltransferase, did not change. Removal of isoprenaline treatment resulted in a rapid return of transferase activity to the normal value. Changes in these same two galactosyltransferases have been followed with development of the pancreas in the rat from 13 days *post conceptio* to birth (Carlson *et al.*, 1973), and during embryonic development in chick pectoral muscle (Ullrich *et al.*, 1981). We report here on the relative specific activities of these two galactosyltransferases in

development *post partum* in rat salivary glands (Fig. 4; Table 3). In the parotid glands there is a rapid decrease in UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase with a concomitant increase in the other transferase, until about equal activities are observed at about day 14. A relatively constant increase in both transferases was noted with submandibular glands. There was little change for the transferase activities in sublingual glands, except in the first few days of the neonatal period. Data presented in Table 3 compare the activities of the two transferases from salivary glands, pancreas, gut and liver of the developing rat. When studies on the pancreas, gut and liver were performed (Carlson *et al.*, 1973), the developmental profiles on the transferase activities in the gut after day 16 were not reliable. After day 16, nucleotide pyrophosphatase, which hydrolyses the substrate UDP-galactose, increased dramatically. Lau & Carlson (1981) report a procedure for inhibiting this nucleotide pyrophosphatase activity.

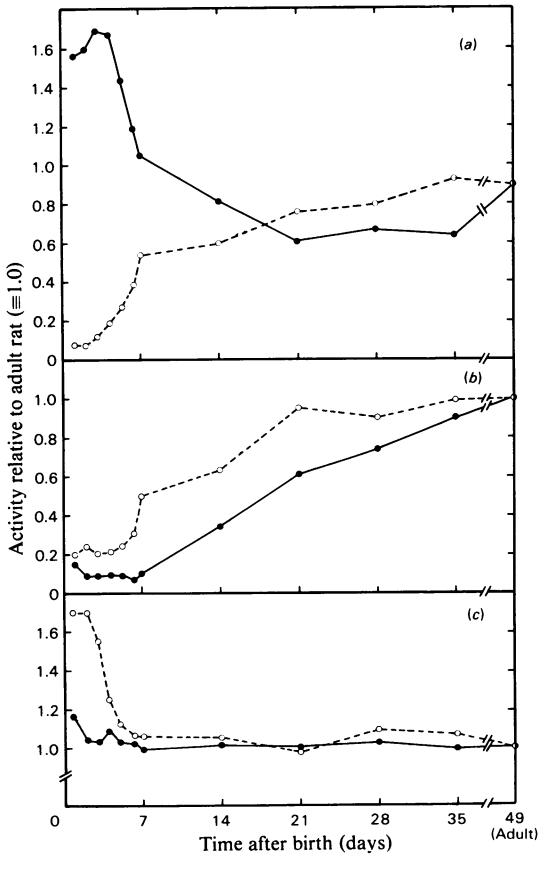

Fig. 4. *Activity of UDP-galactosyltransferase enzymes I* (●) *and II* (○) *relative to adult rat parotid, submandibular and sublingual gland development*
(*a*) Parotid gland; (*b*) submandibular gland; (*c*) sublingual gland.

Table 3. *Galactosyltransferase activity in developing rat salivary gland and other tissues*

Acceptor species ...	Activity (mmol/min per mg of protein)	
Gland and age	*N*-Acetyl-glucosamine	*N*-Acetyl-galactos-aminyl-protein
Adult, parotid	20	180
1-Day neonatal, parotid	52	15
Adult, submaxillary	180	240
1-Day neonatal, submaxillary	62	15
Adult, sublingual	22	29
1-Day neonatal, sublingual	21	89
Adult, pancreas	2	102
Adult, liver	32	80
Pancreas, *in utero* (16-day)	47*	210*
Gut, *in utero* (16-day)	48*	21*
Liver, *in utero* (16-day)	73*	129*
Newborn		
Pancreas	3*	129*
Liver	51*	90*

* Values from Carlson *et al.* (1973).

The biological significance of the changes in glycosyltransferase activities with development is as yet not known. The UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase is in soluble form in the embryonic-chick brain (Den *et al.*, 1970), but as the chick develops (day 14) the enzyme is found only in the membrane fraction. The same enzyme in the rat pancreas increases several-fold from 13 days *in utero* to birth (Carlson *et al.*, 1973), but the enzyme activity falls to near zero shortly after birth. The increase in specific activity of UDP-galactose:*N*-acetylglucosamine 4β-galactosyltransferase in parotid glands of isoprenaline-treated rats (Zinn *et al.*, 1972) parallels the synthesis of a new glycoprotein in the parotid glands, which has mol.wt. 220000 (Humphreys-Beher *et al.*, 1981).

α-Amylase activity of the three salivary glands was assayed as a tissue marker for developing rats. Results were consistent with those reported by Ball (1974). α-Amylase activity increased in parotid glands with age, the submandibular gland showed an intermediate activity and the sublingual glands had very low activity; specific activities for α-amylase at day 35 were 550, 130 and 10 mmol for the respective glands. The high amount of mucus glycoprotein

(Fig. 1*b*, lanes 3 and 4) is also a marker for the sublingual glands (Shackleford, 1963).

In summary, tissue-specific changes in protein and glycoprotein biosynthesis are observed during neonatal development of the major rat salivary glands. Dramatic changes in protein synthesis are known to occur in the parotid glands of rats treated with isoprenaline in addition to the induced hypertrophy and hyperplasia (Muenzer *et al.*, 1979*a*,*b*). A series of proteins high in proline (over 43% proline) comprise over 50% of the total soluble protein of the parotid glands after treatment. In the rat liver it has been shown for the isoenzymes of glucose:ATP phosphotransferase, aldolase and pyruvate kinase, foetal isoenzymes no longer expressed in the adult, are the predominant form of these enzymes in rat hepatomas (for review, see Weinhouse, 1973). Analysis of neonatal parotid-gland soluble fractions failed to detect the presence of the proline-rich proteins during parotid development. However, the expression of these proteins may still take place during the development of the salivary gland *in utero*.

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