

Morphogenesis and functional differentiation of the rat parotid gland *in vivo* and *in vitro*

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SUMMARY

In comparison with the submandibular and the sublingual glands, the parotid develops slowly in the rat. The foetal rudiment appears a day later than that of the submandibular gland and the formation of adenomeres is slower, leading to a more diffusely branched structure. Cytodifferentiation, in the form of traces of mucopolysaccharide in the tubules and terminal buds, begins at, or just before, birth. There is a transitory increase in mucopolysaccharide production for a few days after birth until the presumptive acinar cells become pyramidal in shape with basal nucleus and granular cytoplasm. Amylase activity of the gland begins to rise between the second and third day after birth and reaches the adult level at weaning. That of the submandibular gland remains at the foetal level.

Parotid rudiments were cultivated on a film of agar over a medium of fowl plasma and chick embryo extract. The oxygen in the gas phase of air and 5 % CO₂ was increased to 50 % after the first 9 days *in vitro*. Under these conditions the mass of the rudiments increased tenfold during 18 days cultivation and the initially unbranched rudiment formed adenomeres in which the cytodifferentiation followed the same course as *in vivo*. The rise in amylase activity of the explants was only slightly delayed compared with that *in vivo*, suggesting that systemic or environmental factors are not obligatory in the early postnatal development of the rat parotid.

INTRODUCTION

The parotid gland in the majority of rodents is a serous gland characterized by the secretion of amylase and other enzymes in the saliva (Siuda & Szymańska, 1961; Junqueira & De Moraes, 1965; Junqueira, 1967). Since the parotid gland of the rat, unlike the submandibular, has a well-defined differentiation product in the enzyme amylase, it should be a suitable system for studying the factors that influence the functional development of salivary gland epithelium, thus complementing existing information from the mouse submandibular gland on salivary gland morphogenesis (Borghese, 1950*a, b*; Grobstein, 1953*a–c*; Grobstein & Cohen, 1965; Kallman & Grobstein, 1965, 1966).

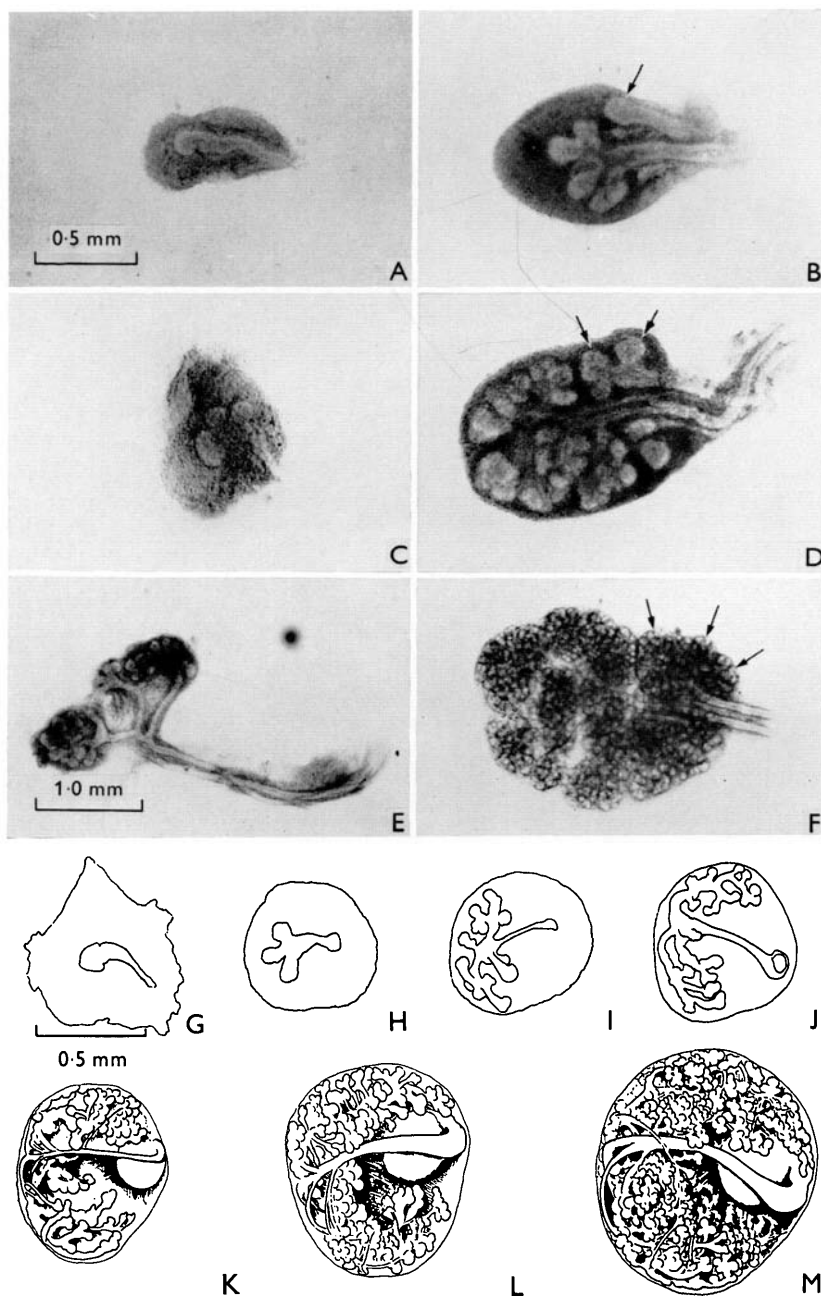
Although the structure and function of the adult gland have been extensively studied (see for example two recent symposia edited by Sreebney & Meyer (1964) and by Schneyer & Schneyer (1967)), its early development appears to have been neglected. This paper describes the foetal and early postnatal development of the rat parotid gland and an organ culture system which allows its prolonged development.

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MATERIALS AND METHODS

Albino rats were used in which parturition regularly occurred on the 23rd day after mating, counting the morning on which sperm were found in the vagina as day 1.

The parotid rudiment was most conveniently removed from 16- to 19-day



foetuses by decapitating above the shoulders and removing the skull and palate by cutting from the angle of the mouth through the ears. The lower jaw was then dissected further under the microscope. In 20-day foetuses and in young rats the ventral skin of the neck was reflected, the transparent parotid glands with surrounding tissue were removed and trimmed free of adhering muscle and lymph nodes under the microscope. Young rats were immobilized by cold anaesthesia and the parotid glands were removed and rapidly freed of extraneous tissue in ice-cold saline. This method gave negligible loss of amylase activity compared with cervical section and subsequent dissection in saline.

Tissue culture. The watch-glass method (Fell & Robison, 1929) was used. The medium consisted of two parts fresh fowl plasma mixed with one part chick-embryo extract (one part pulp from 9-day-old embryos + two parts Tyrode's solution). The explant was placed either in direct contact with the clot, or separated from it by a piece of Millipore filter (25 μ thick, 45 m μ pore diameter), or by a thin film of agar. The latter was prepared by dipping strips of cellulose acetate net (*ca.* 7 \times 5 mm) in warm 0.5 % agar in Tyrode's solution. For optimum viewing of the living cultures it was more convenient to cut a rectangular hole in the rayon strip and allow the agar to set across it as a transparent window. Alternatively, the explants were grown in direct contact with a medium consisting of two parts 1 % agar in Tyrode's solution, two parts cockerel serum and one part embryo extract (modified from Wolff & Haffen, 1952). The medium was changed every third day.

The culture dishes were incubated at 37 °C in mechanically sealed containers in which the gas phase was a humidified, commercially prepared mixture of either (a) air and 5 % CO₂, (b) 50% O₂, 45 % N₂ and 5 % CO₂, or (c) 95 % O₂ and 5 % CO₂.

Histological techniques. Tissues were fixed in Helly's fluid and embedded in paraffin wax. Sections were stained with Mayer's haemalum and eosin, or by the PAS technique in combination with alcian blue.

FIGURE 1

(A–F) Early morphogenesis of the rat parotid gland compared with that of the submandibular and sublingual glands.

(A) Parotid rudiment from a 16-day foetus. Scale line applies to (A)–(D).

(B) Submandibular and sublingual (arrow) rudiments from the same foetus as in (A).

(C) Parotid rudiment from a 17-day foetus.

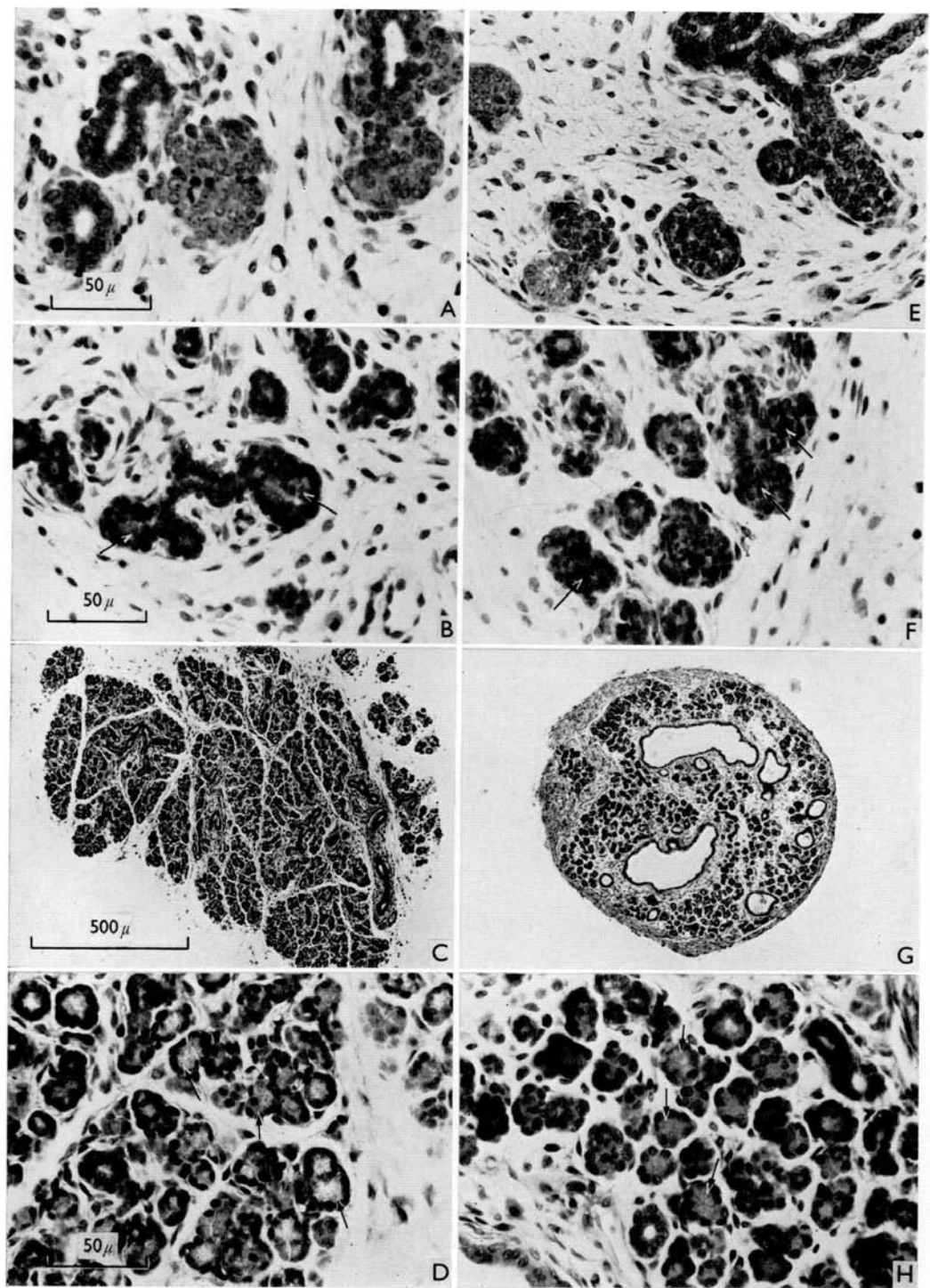
(D) Submandibular and sublingual (arrows) glands from the same foetus as in (C).

(E) Parotid rudiment from a 19-day foetus. Note slower and less compact epithelial budding compared with the sublingual (F). Scale line applies to (E) and (F).

(F) Submandibular and sublingual (arrows) glands from the same foetus as in (E).

(G–M) Morphogenesis of a 16½-day parotid rudiment in culture. Line drawings made from photographs. Scale line applies to (G)–(M).

(G) *ex utero*, (H) 1 day *in vitro*, (I) 2 days *in vitro*, (J) 3 days *in vitro*, (K) 5 days *in vitro*, (L) 7 days *in vitro*, (M) 9 days *in vitro*.



Amylase estimation. Tissues were rinsed in 0.9 % NaCl, homogenized in distilled water and the homogenate diluted to 1 or 1.5 ml. Amylase activity was estimated on 0.5 ml of the homogenate using Bernfeld's reducing sugar method (Colowick & Kaplan, 1955) and a glycogen substrate at pH 6.8. Results with the glycogen substrate were more reproducible than with a starch substrate and had the additional advantage of negligible blank values. The unit of amylase activity was arbitrarily defined as resulting in the production of reducing groups equivalent to 0.19 mg glucose in 1 min at 30 °C, based on a standard curve prepared from an homogenate of adult rat parotid gland. Enzyme activity was expressed as units/mg total organic solids, the latter being measured on 0.4 ml of the homogenate (Johnson, 1949).

During preliminary experiments with the assay system, it was found that there was up to 80 % irreversible loss of amylase activity in dilute homogenates containing less than 40 µg total organic solids/ml. The enzyme solution was stable if diluted in the presence of the boiled homogenate, bovine serum albumin or substrate. It was not stabilized by the addition of Ca²⁺, 0.9 % NaCl, the ashed component of the homogenate, sorbitol or polyvinylpyrrolidone. Care was therefore taken to ensure that the homogenate was sufficiently concentrated. Extracts of foetal and young rat parotid glands, at stages before appreciable amylase activity can be found, were tested on adult parotid gland for the presence of inhibitors to amylase activity. In addition, adult parotid extract was boiled for 1 min and included in the assay of amylase activity of extracts from foetal parotids to test for the presence of a heat stable activator present in the adult gland, but absent from the foetus.

FIGURE 2

Rat parotid histogenesis *in vivo* (A–D) and *in vitro* (E–H).

(A) Parotid from 20-day foetus. Stage I.

(B) Parotid from 2-day-old rat. Stage III. Arrows indicate PAS-positive material.

(C) Low power view of parotid from 8-day-old rat showing young acini.

(D) High power view of (C) showing young acini with granular cytoplasm (arrows). Stage IV.

(E) Parotid from 17-day foetus cultivated *in vitro* for 6 days. Stage I. Magnification as in (A).

(F) As in (E) but cultivated for 12 days. Stage III. Arrows indicate PAS-positive material in the terminal buds. Magnification as in (A).

(G) Low power view of 17-day parotid cultivated for 18 days. Magnification as in (C).

(H) High power view of (G) to show acini with basal nuclei and granular cytoplasm (arrows). Stage IV. Magnification as in (A).

RESULTS

Normal development in vivo

Morphogenesis in the foetus. The parotid rudiment appears in the 16-day rat foetus as a rod of epithelium (Fig. 1A) extending dorsally from the lateral part of the oral cavity. By the 17th day the future collecting duct has lengthened, reflexed postero-ventrally under the external ear towards the mid-line, and the distal knob has expanded into three swellings from which further budding subsequently occurs (Fig. 1C). The epithelium is surrounded by mesenchyme which, unlike that of the submandibular gland, does not form a well-defined capsule. The reflexion of the future main collecting duct ensures that the parotid gland continues to grow between the external ear and the proximal part of the submandibular gland. The branching pattern of the terminal buds is more diffuse and develops at a slower rate than in the submandibular and sublingual glands (Fig. 1A-F).

Histogenesis. For convenience in reference the development of the parotid gland in the embryo and young suckler has been divided into four stages based on the cytogenesis of the terminal buds:

Stage I. The terminal buds of the diffusely branching epithelial tree consist of cells with large central nuclei and basophilic cytoplasm (Fig. 2A). This stage lasts from the appearance of the initial unbranched rudiment until term.

Stage II. Patches of densely PAS-positive material can be found in a few cells of the terminal buds in some foetuses at term, in newborn and 1-day-old rats.

Stage III. Patches of PAS-positive material are now larger and more abundant (Fig. 2B). A few patches of alcian blue-staining material can also be found. The nucleus occupies a more basal position in the cell, the apical portion of cytoplasm is eosinophilic. This stage is mainly found in 2- and 3-day-old rats.

Stage IV. The young acinar cell of 4-day and older rats is obviously polarized with a basal nucleus and prominent, eosinophilic apical cytoplasm which has a granular appearance; the granules are PAS positive (Fig. 2C, D). Dense deposits of PAS-positive material characteristic of stage III are only occasionally found.

The surrounding diffuse mesenchyme at all stages is characterized by strands of intercellular material which stain faintly with alcian blue.

Amylase activity. Amylase activity in the parotid gland remains at a very low level until after birth (Fig. 3). Activity increases significantly between the second and third day *post partum* and continues to rise until the adult level is reached at the time of weaning.

There was no effect of an extract of foetal parotid glands on the amylase activity of adult parotid gland nor of boiled adult extract on the amylase activity of foetal parotid glands.

Development *in vitro*

The effect of substrate. Morphogenesis, i.e. branching and budding, of the epithelium of 17-day foetal parotid glands occurred equally well for the first 6 days *in vitro* on all the media and substrates tested. Neither the solid agar substrate, direct contact between explant and plasma clot, nor the Millipore filter, were suitable for longer culture periods; in the first situation because the explant rounded into a sphere and the centre became necrotic, and in the second

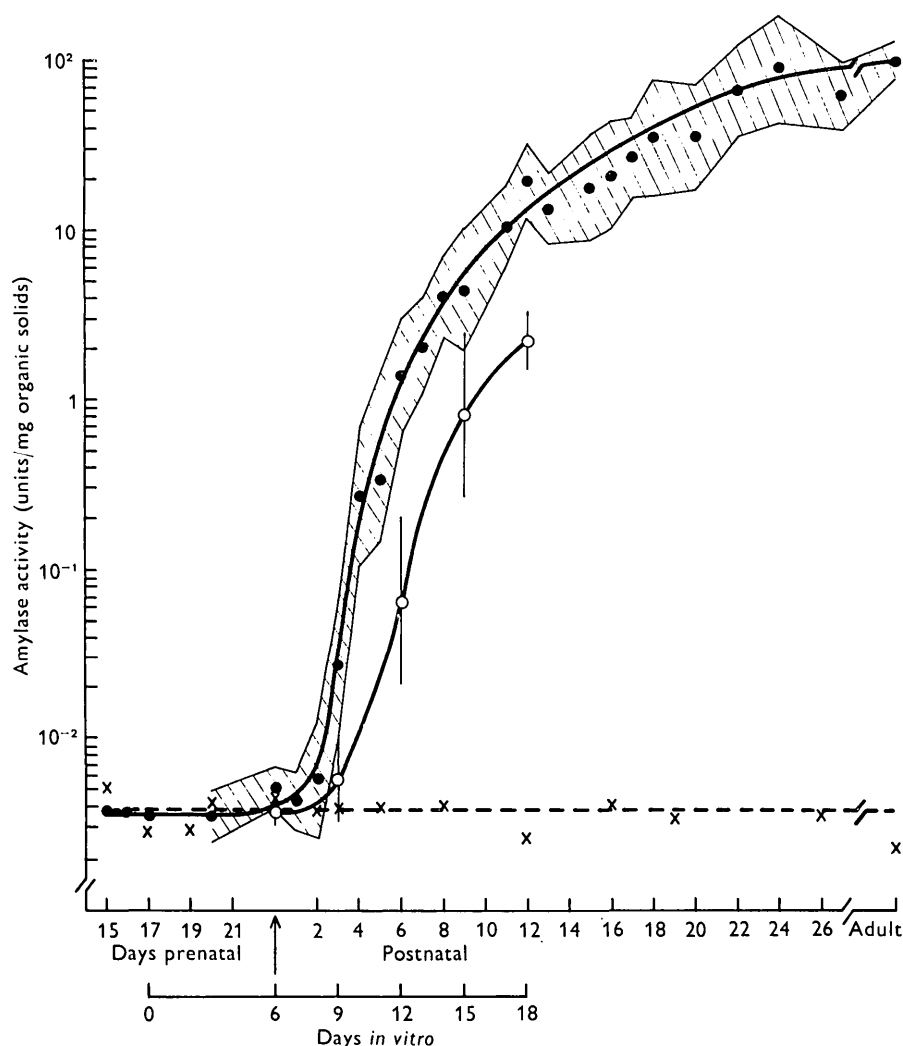


Fig. 3. Amylase activity of the developing rat parotid (●) and submandibular (×) glands, and of the cultivated parotid from 17-day foetuses (○). 95 % confidence limits are represented by the hatched area and by the vertical lines for the parotid *ex vivo* and *in vitro* respectively. The values for 15–17-day foetuses were derived from one or two samples only, each containing about 25 rudiments.

and third because the mesenchyme migrated away from the epithelium which then became disorganized. In contrast, the explants separated from the plasma clot by a thin film of agar remained disc-shaped and the epithelial tree continued to develop (Fig. 1 G-M). This last-mentioned system was therefore adopted.

The effect of oxygen. During the first 9 days *in vitro* organ cultures of 17-day parotid rudiments remained healthy in an atmosphere of air and 5 % CO₂, but

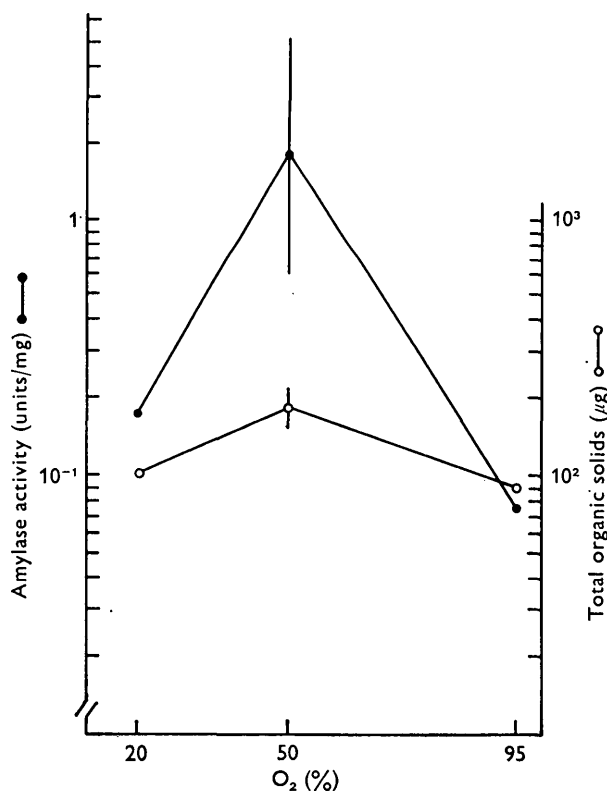


Fig. 4. Effect of different oxygen concentrations applied during the 9th to 18th day *in vitro*, on the final size (○) and amylase activity (●) of parotid rudiments from 17-day foetuses. Vertical lines represent 95 % confidence limits for comparison between means derived from the analysis of variance for each type of estimation.

thereafter progressive necrosis, beginning at the centre towards the base of the explant, became apparent. Raising the initial oxygen concentration to 50 or 95 % was toxic within 2 days, the effect being more rapid in the higher concentration. When the explants were transferred to higher oxygen levels after 9 days cultivation the effect of 95 % oxygen was less drastic. Histological examination after 18 days cultivation revealed well differentiated columnar ducts but the acini, particularly peripherally, had many pycnotic nuclei, were poorly differentiated, and the amount of diffuse mesenchyme appeared to be reduced. No mitoses were found. In contrast glands cultivated from the 9th to the 18th

day in 50 % oxygen were better differentiated compared with those in air and there was no necrosis (Fig. 2G). Mitoses were still present, particularly in the epithelium, after 18 days cultivation. The final size was greater by 80 % and amylase activity tenfold higher (Fig. 4).

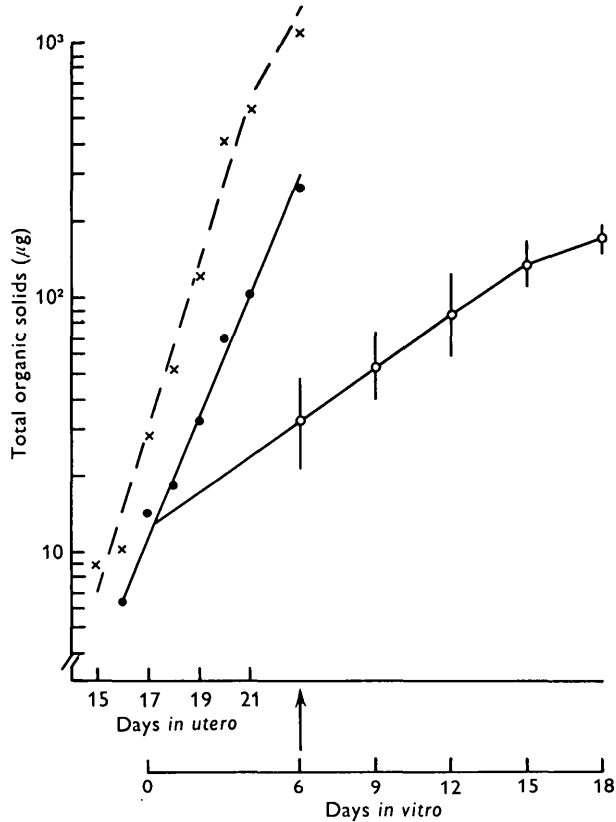


Fig. 5. Growth of the 17-day foetal parotid gland *in vitro* (○) in terms of total organic solids, in comparison with the growth of the parotid gland (●) and of the submandibular gland (x) *in utero*. Vertical lines represent 95 % confidence limits.

Further description of material *in vitro* concerns only explants cultivated for the first 9 days in air and 5 % CO₂, and thereafter in 50 % O₂, 45 % N₂ and 5 % CO₂.

Growth. The total organic solids content of rudiments from 17-day foetuses increased throughout the culture period, with a tenfold increase within 18 days (Fig. 5).

Histogenesis. The parotid rudiment passed through the same histogenetic stages *in vitro* as *in vivo*. After 6 days cultivation rudiments from 17-day foetuses were still at stage I (Fig. 2E). At 9 days traces of PAS-positive material, indicating stage II, could be found. At 12 days there was more abundant PAS-

positive material (stage III, Fig. 2F). Stage IV, showing acinar cells with granular cytoplasm, was reached in 15–18 days (Fig. 2H).

Amylase activity. Amylase activity of 17-day foetal rudiments remained at a low level until after the ninth day of culture. There was significant increase in activity by the 12th day and by 18 days the specific activity had increased more than 500-fold from the initial level (Fig. 3).

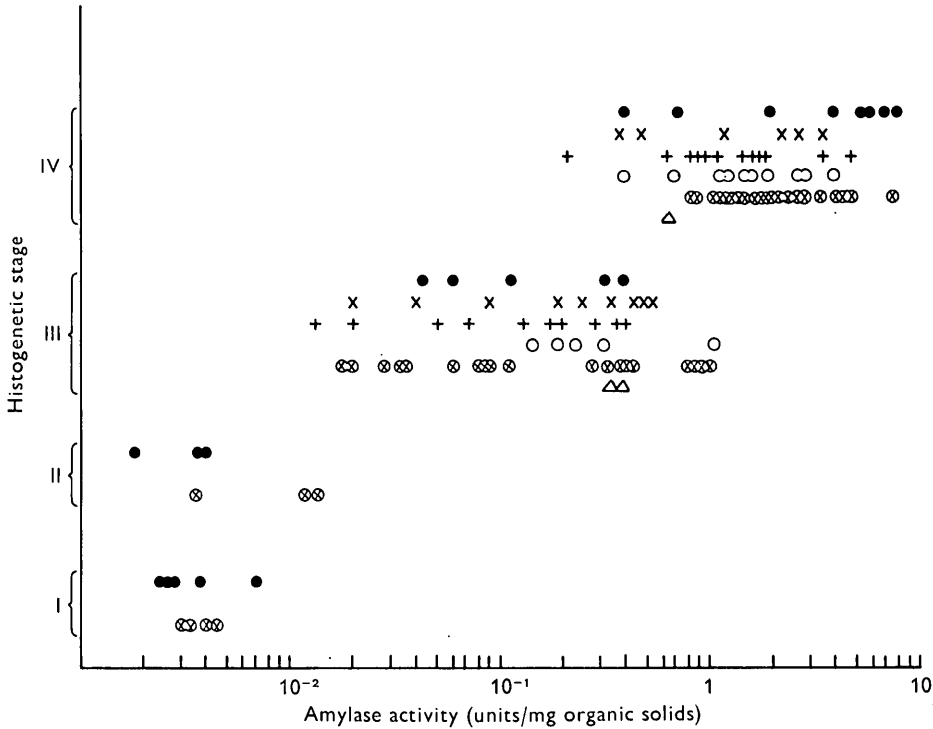


Fig. 6. Comparison of histogenetic stage with amylase activity in parotids *ex vivo* (●) and in cultivated parotids from 20-day (x), 19-day (+), 18-day (O), 17-day (⊗) and 16-day (Δ) foetuses.

Correlation of histogenesis and amylase activity in vivo and in vitro

Although the parotid gland not only grew much faster (Fig. 5) but also differentiated somewhat faster *in vivo* than *in vitro*, amylase activity was associated with defined histogenetic stages both *in vivo* and in glands taken from embryos of different ages and cultivated for varying lengths of time (Fig. 6). When possible these comparisons were made on paired glands; when several glands were required for amylase assay, determination of histogenetic stage was made on material from a litter-mate.

DISCUSSION

The foetal growth and development of the parotid gland in the rat is slower than that of the other salivary glands. Although its epithelial rudiment is identifiable at the same time as that of the sublingual gland, the formation of adenomeres is slower in the parotid gland and leads to a treelike structure in comparison with the shrubby appearance of the sublingual and associated submandibular glands. Both sublingual and submandibular glands show secretory activity in the foetus (Szymańska, 1962, 1963; Gerstner, Flon & Butcher, 1962, 1963; Di Giovine Vecchione, 1967; Plasmeyer, 1969), but histochemical indications of mucopolysaccharide synthesis in the terminal buds of the parotid gland were only found in foetuses at term. In contrast to the present work Shubnikova & Chunaeva (1967) reported functional signs in the foetal parotid gland after the 18th day of gestation.

The immaturity of the postnatal parotid gland has been previously emphasized (Schneyer & Schneyer, 1961; Schneyer & Shackleford, 1963; Schneyer & Hall, 1969). From histological observations Schneyer & Shackleford (1963) reported that even at 16 days most of the cells are still undifferentiated; however, this statement is not supported by the increase in amylase activity which begins about 3 days after birth, nor by the polarization of the acinar cells which becomes increasingly prominent after the fourth day. This polar organization, which is present in parotids from 4- and 5-day-old rats but is not found in foetal or newborn material, is characterized by a basal nucleus and endoplasmic reticulum and apical zymogen granules (J. Bluemink, personal communication).

The transitory appearance of patches of mucopolysaccharide which stain heavily with PAS and sporadically with alcian blue has been previously recorded in the unweaned rat and other mammals by Bignardi (1961) and in foetal and young unweaned rats by Shubnikova & Chunaeva (1967). The significance of the phenomenon, which precedes the rise in amylase activity of the whole gland, and occurs both *in vivo* and *in vitro*, is not known. From the present work it is not possible to say whether the cells which at stage III have prominent PAS-positive patches also later produce zymogen granules and presumably abundant amylase.

The absence of any effect of foetal parotid extract on the amylase activity of adult parotid glands or of boiled adult extract on the amylase activity of foetal parotid glands suggests that the rise in amylase activity which begins in the parotid gland 2–3 days after birth is more likely to be due to synthesis of the enzyme, rather than to loss of an inhibitor or development of a heat-stable activator. That neither a suckling stimulus, nor innervation of the gland, nor an altered hormonal balance, play a major role in initiating this rise is indicated by the comparable development which was achieved *in vitro*. Whether the difference in amylase activity between older cultures and glands of the same age *in vivo* is inherent in the culture technique or due to the absence of autonomic

stimulation (Schneyer & Hall, 1967) or other factors thought to be important for the postnatal function of the gland, is open to experimental investigation. The relative difference between the amylase activity of the adult rat parotid gland and that of the submandibular gland, which remains at the foetal level, is within the range of that previously recorded for unstimulated glands (Schneyer & Schneyer, 1960).

That the necrosis which appears in the central and basal portions of the explants after 9 days *in vitro* is due to anoxia is indicated by its relief with 50 % oxygen. An atmosphere of 95 % oxygen is frequently used for adult tissues (Moscona, Trowell & Willmer, 1965) and allows short-term survival *in vitro* of parotid glands from young rats (Jones, 1966; Lucas, 1969), but was toxic for the immature tissue studied in the present work. Even 50 % oxygen was deleterious for the freshly isolated rudiment. The basis of the toxicity is not known; the areas most affected are the adenomeres, but it is not clear whether the effect acts directly on the dividing cells, on some other morphogenetic mechanism within the epithelium, or indirectly via the diffuse mesenchyme which is also affected under abnormally high oxygen concentrations.

RÉSUMÉ

Morphogénèse et différenciation fonctionnelle de la glande parotide du rat, in vivo et in vitro

Comparée aux glandes submandibulaires et sublinguales, la parotide se développe lentement chez le rat. L'ébauche foetale apparaît un jour après celle de la glande submandibulaire et la formation des adénomères est plus lente, conduisant à une structure ramifiée, plus diffuse. La cytodifférenciation se traduisant par la présence de traces de mucopolysaccharides dans les tubules et les bourgeons terminaux débute à la naissance ou juste avant. On observe un accroissement transitoire de la production de mucopolysaccharides pendant les quelques jours qui suivent la naissance jusqu'au moment où les cellules acinaires présumptives deviennent pyramidales, avec un noyau basal et un cytoplasme granulaire. L'activité de l'amylase dans la glande commence à augmenter entre le deuxième et le troisième jour après la naissance et atteint le niveau adulte au sevrage. Celle de la glande submandibulaire demeure au niveau foetal.

Des rudiments parotidiens ont été cultivés sur un film d'agar et dans un milieu contenant du plasma aviaire et de l'extrait d'embryon de poulet. L'oxygène dans la phase gazeuse: air et 5 % de CO₂ est augmenté jusqu'à 50 % après les neuf premiers jours *in vitro*. Dans ces conditions, la masse des rudiments augmente de 10 fois pendant 18 jours de culture, et le rudiment, non ramifié initialement, forme des adénomères où la cytodifférenciation suit le même cours qu'*in vivo*. L'accroissement de l'activité amylasique des explants n'est que peu retardé par rapport à celui qui s'observe *in vivo*, suggérant que des facteurs systémiques ou d'environnement ne sont pas requis pour le développement postnatal précoce de la parotide du rat.

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