# PROLIFERATIVE BEHAVIOR OF DIFFERENTIATING CELLS IN THE DEVELOPING RAT PAROTID GLAND

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ABSTRACT

Parotid glands of litters of rats at age intervals from 20 days *in utero* to 100 days were assayed for DNA content and examined by light- and electron-microscopy. The age differences in total DNA and DNA concentration indicated that there was a rapid rate of proliferation of parenchymal cells until 25 postnatal days, after which the rate declined rapidly, and that there was a rapid increase in cell size between 18 and 25 days. These findings were substantiated by histologic observations, such as the presence of numerous mitotic figures until 25 days of age, and the rapid maturation of the acinar cells between 18 and 25 days. These data suggest that the acinar cells of the rat parotid gland comprise an expanding cell population. Light- and electron-microscopic observations consistently indicated that cells with mitotic figures were about as well differentiated as other parenchymal cells at all stages of gland development, including mature acinar cells observed at ages 23 and 25 days. These observations support the view that the division of cells in advanced stages of differentiation may be important in the growth of certain organs and tissues.

## INTRODUCTION

It is a widely accepted theory that as cells differentiate they lose the ability to divide. An elaboration of this theory has been presented by Rusch (13) in his proposal that increasing differentiation of cell populations is accompanied by decreasing rates of cell division until the ability to divide is lost. Recently, Leblond (5) presented evidence for a classification of cell populations based in part on their proliferative behavior. In *static* populations, e.g. neurons, the cells cease to divide and then mature during the perinatal period. In *renewing* populations, e.g. stratified squamous epithelium, relatively undifferentiated stem cells divide continuously throughout life; some of their progeny cease dividing, differentiate, and are destroyed or shed at a rate that, in the adult, maintains the population at approximately the same number of cells. Cells of both of these classes thus seem to fit or support the proposal of Rusch. However, cells of the third class, or *expanding* populations, continue to divide after achieving a considerable degree of differentiation, and to maintain the ability to divide for some time after the animal attains adulthood.

In the examples of expanding populations cited by Leblond (kidney, skeletal muscle, pancreas, etc., of the rat), there were three lines of evidence for his conclusions: (*a*) Total DNA per organ increased substantially after the organs or tissues had achieved mature morphologic characteristics. (*b*) Mitotic figures were seen in moderately well-differentiated parenchymal cells. (*c*) Radio-

autographs indicated uptake of thymidine-$^3$H by the nuclei of apparently mature parenchymal cells. Though this evidence is strong, the fact that the morphologic observations were made by light microscopy leaves room for doubts about the actual location of the mitotic figures and labeled nuclei within differentiated cells.

The purpose of the present article is to present evidence which (*a*) permits the acinar cells of the rat parotid gland to be categorized as an expanding population, (*b*) suggests that in the later stages of development acinar cells undergoing division have attained essentially the same advanced degree of differentiation as all of the acinar cells, and (*c*) thus supports the views of Leblond.

## METHODS

Litters of 8–12 Sprague-Dawley rats were sacrificed at the following ages: 20 days *in utero*, newborn, 1, 3, 5, 9, 12, 15, 18, 21, 23, 25, 30, 35, 40, and 100 days (adult). Parotid glands to be assayed biochemically were removed, trimmed of nonparenchymal tissues, frozen on "dry ice", lyophilized, weighed, pooled by litter, diced, and stored in sealed vials at −20°C until assayed. Preliminary tests indicated that no loss of DNA occurred in parotid tissues so treated, even after a year or more of storage. Samples of the tissues were homogenized in 10% trichloroacetic acid (17), extracted according to the method of Schneider (14), and assayed for DNA by the method of Burton (2). One or more determinations were made from each of six litters at each of the designated ages except for litters under 5 days of age, where pooling of tissues from up to 10 litters was necessary for a minimum of three determinations. Two glands from each litter were fixed in Zenker's-acetic acid fluid and processed for routine histologic examination. One gland from each of two animals from at least two litters of every age was taken fresh from the rat and fixed for electron microscopy by a method developed by Buckland (1). The tissues were diced to pieces 1–2 mm on a side in a drop of the primary fixative (6.25% glutaraldehyde and 0.5% acrolein in 0.067 M Sörensen's phosphate buffer, pH 7.4), and placed in a vial of this fixative for 2 hr at room temperature.

After an overnight wash in the buffer with 0.14 M sucrose, the tissues were postfixed for 1 hr at room temperature in 1.0% osmium tetroxide in Caulfield's buffer (0.057 M Veronal acetate and 0.14 M sucrose, pH 7.4). The tissues were then processed and embedded in araldite, following the recommendations of Luft (6). Sections approximately 1 micron thick were cut for light microscopy with a Porter-Blum ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.) and stained by the method of Richardson et al. (10). Sections for electron microscopy were cut 400–600 A thick (silver to gray by reflected light) on a Huxley ultramicrotome (Cambridge Instrument Co., New York), placed on carbon-coated grids (20), and stained with uranyl acetate and lead hydroxide, after the methods of Watson (19) and Millonig (7), respectively. The grids were viewed and photographed in an RCA EMU-3G electron microscope.

## OBSERVATIONS

The amounts of DNA per gland and per milligram of lyophilized tissue are shown in Fig. 1. DNA per gland increased rapidly until about 25 days, except for a brief pause from 12 to 15 days. The rate of increase diminished rapidly thereafter, with the DNA per gland at 35 days being 92% of the adult (100 day) value. DNA per unit of tissue appeared to increase slightly or remain constant from birth to 18 days, decreased between 18 and about 35 days, and remained fairly constant or decreased slowly thereafter. Since it has been shown that the amount of DNA per cell remains virtually constant in most tissues and organs of the developing rat (4), changes in DNA per gland are an index of changes in cell numbers, and changes in DNA per unit of tissue are an inverse index of changes in cell size. Thus the results of the DNA assays can be interpreted as follows. Cellular proliferation proceeds at a rapid rate until 25 days, then tapers off. Cell size changes very little during most of the period before 18 days, increases rapidly between 18 and 25 days, and continues to increase slowly thereafter.

Histologic observations tended to confirm these interpretations. Acinar cells became larger and there was a decrease in the interparenchymal connective tissue space. Numerous mitotic figures were seen in the samples of parotid glands of rats through 25 days, and they became less frequent with increasing age. At 100 days they were rarely seen. Similar rates of mitotic activity have been reported in the developing rat parotid gland by Schneyer and Hall (15).

The increases in acinar cell size were accompanied by increases in the amounts of rough endoplasmic reticulum (r.e.r.), Golgi apparatus, and secretory granules which they contained (9). Since these organelles are the hallmarks of the mature parotid acinar cell (11), it is apparent that differentiation and proliferation proceed simultaneously in the gland as a whole, and that high rates of proliferation occur during the period 18–35 days, after the acinar cells have achieved a very advanced stage of differentiation.

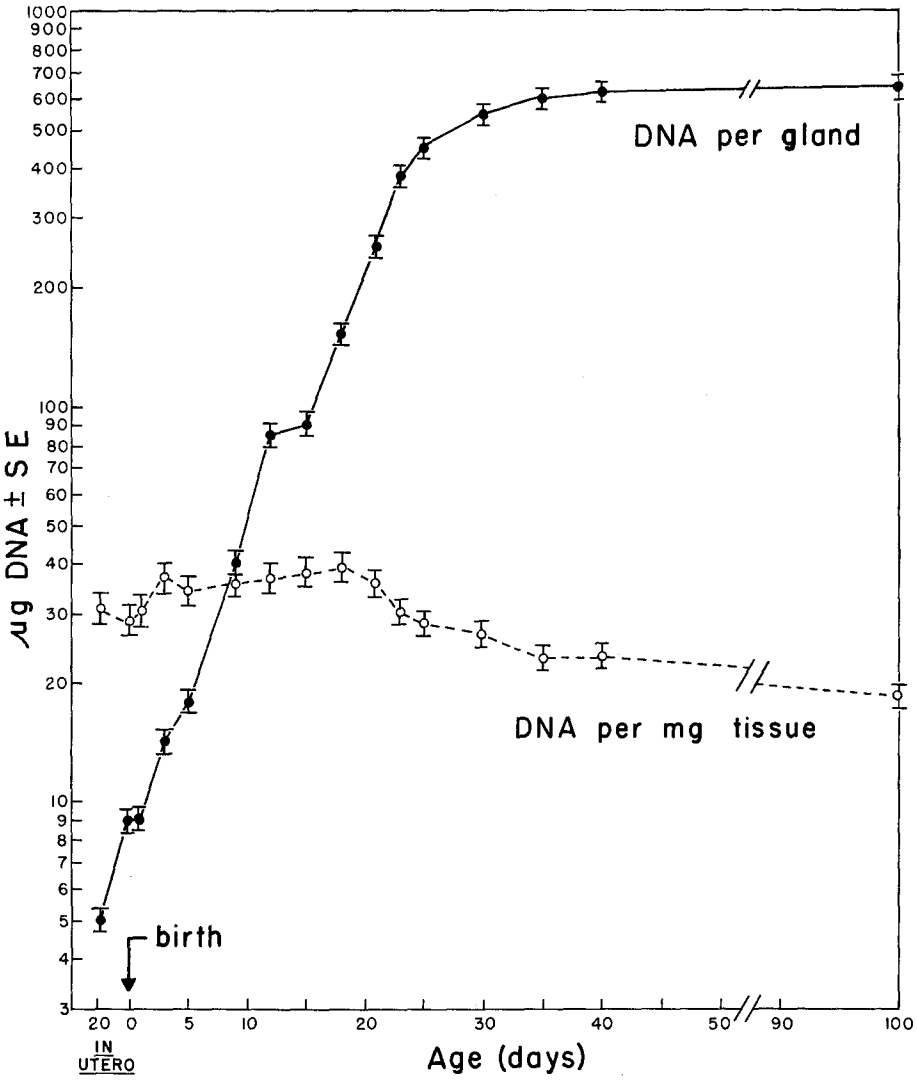



FIGURE 1 Total DNA and DNA per milligram of lyophilized tissue ± standard error of the mean, in parotid glands of rats at selected ages.

There are at least two ways in which the foregoing could take place. (*a*) If cell proliferation and differentiation are mutually exclusive, then division takes place only in groups of relatively undifferentiated cells, while other groups of cells cease to divide and then differentiate into mature, functioning units. (*b*) Most or all of the cells of the gland, including those engaged in proliferative activity, tend to differentiate at the same pace, i.e., in a synchronous fashion.

Figs. 2–7 are electron micrographs representative of dividing cells of the rat parotid gland between 20 days *in utero* and 25 days after birth. The cells containing mitotic figures showed progressive differentiation as the ages of the rats increased. Among the more than 30 cells with evidence of mitotic activity that were observed in samples from rats between 5 and 25 days of age, not one appeared to be markedly less differentiated than its neighboring cells. In fact, cells with mitotic figures appeared to contain nearly the same amounts of rough e.r., Golgi apparatus, and secretory granules as other cells in the same gland.

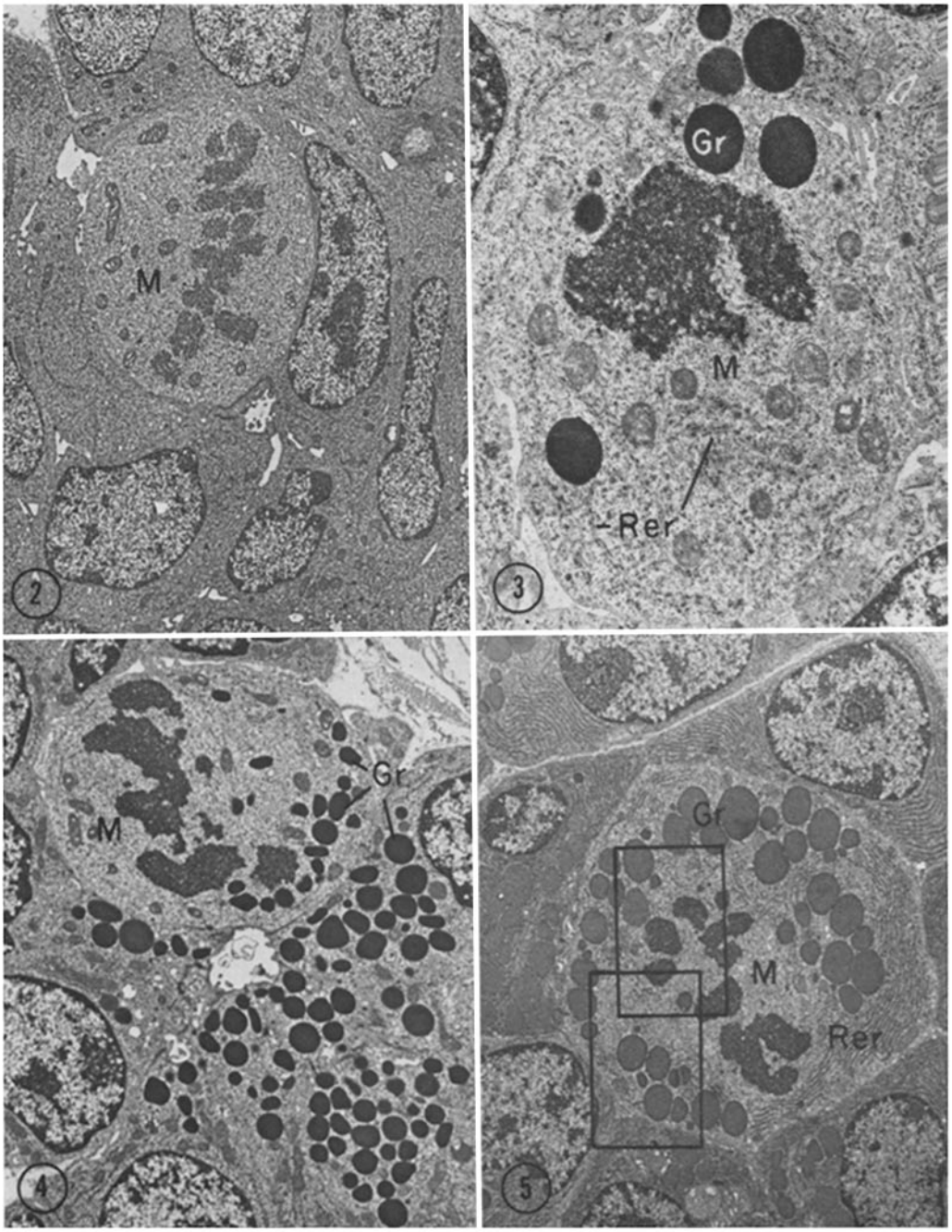
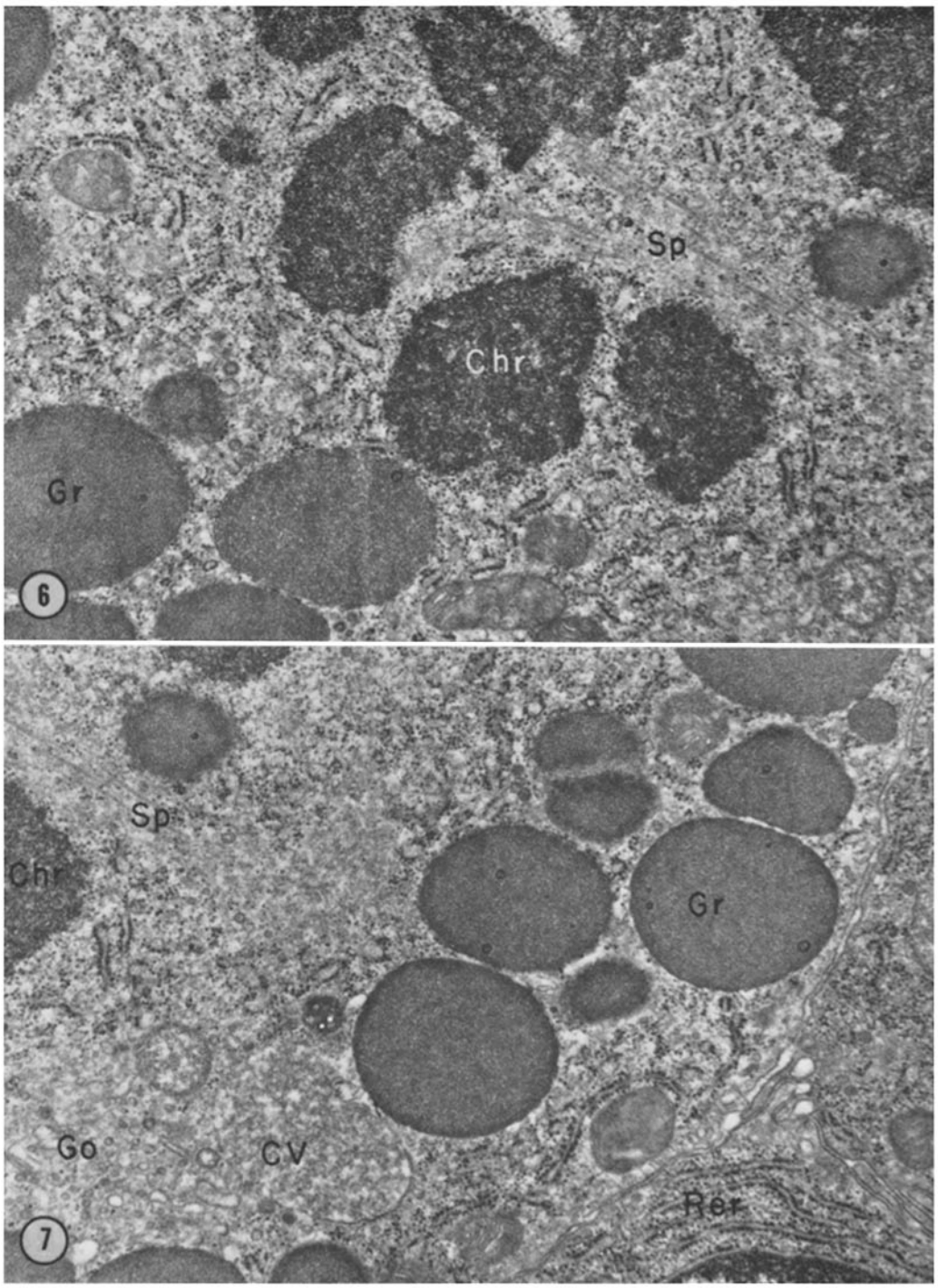



FIGURE 2 Terminal cluster of parenchymal cells of parotid gland from a rat at age 20 days *in utero*. Cell with mitotic figure (*M*) is similar to its neighbors in having scanty amounts of rough e.r. and no discernible secretory granules. *Circa* × 2800.

FIGURE 3 Parotid gland of 5 day postnatal rat. Cell with mitotic figure (*M*) contains several large secretory granules (*Gr*) and a modest amount of rough e.r. (*Rer*). *Circa* × 10,000.

FIGURE 4 Acinus of parotid gland of 12 day old rat. Cell with mitotic figure (*M*) contains as many secretory granules (*Gr*) and as much rough e.r. as most of its neighbor cells. *Circa* × 3500.

FIGURE 5 Parotid acinar cells of 23 day old rat. Cell with mitotic figure (*M*) contains large amounts of secretory granules (*Gr*) and rough e.r. (*Rer*). *Circa* × 3000.




FIGURES 6 and 7 Higher magnification of portions of the cell cited in Fig. 5. (Fig. 6 is of area designated by upper rectangle; Fig. 7, by lower rectangle). Chromatin (*Chr*), microtubules of the mitotic spindle (*Sp*), rough e.r. (*Rer*), Golgi apparatus (*Go*), and secretory granules (*Gr*) are all in the same cell. In Fig. 7 is a granule of low density, presumably a condensing vacuole (*CV*), or secretory granule in a formative stage. Both electron micrographs are *circa* × 25,000.

## DISCUSSION

These observations indicate that the growth of the rat parotid gland involves increases in both cell number and cell size until the rat has attained adulthood. The greatly diminished numbers of mitotic figures in the gland after 25 days indicate that, at least after that age, there is not a high rate of turnover of cells, i.e., of cell renewal. As more than three-fourths of the cells of the adult gland are acinar cells (11), it would appear, therefore, that the acinar cells of the rat parotid gland can be classified as an *expanding* population of cells, according to Leblond's definition. In order to completely rule out the alternative that they comprise a *renewing* cell population, additional evidence would be needed, e.g., a negligible loss of cells pulse-labeled with thymidine-$^3$H over a period of time. Since this kind of evidence has been demonstrated in other exocrine organs of the rat, including the submandibular and sublingual glands (5), it would be surprising if the acinar cells of the rat parotid gland should exhibit a significant difference in their proliferative behavior.

The resolution of cell boundaries in the present series of electron micrographs is such that, beyond question, the cited cells contain both mitotic figures and the organelles associated with acinar differentiation. This constitutes evidence in support of Leblond's concept that as the cells in an expanding population undergo progressive differentiation, they continue to divide even in an apparently fully mature state until the organ reaches full size.

Further support can be drawn from recently published electron micrographs illustrating mitotic activity in developing cardiac muscle cells (12), mast cells (3), and Paneth and goblet cells (18). The last-named are examples of the division of differentiating cells in *renewing* cell populations. On the other hand, Wessells (21) has shown that the acinar cells of the pancreas in the embryonic mouse and rat cease to divide before proceeding to advanced stages of differentiation. Furthermore, cell division, as measured by the uptake of thymidine-$^3$H, ceased entirely shortly before birth, and all of the acinar cells appeared to be fully mature at birth. The implication was made that in these cells more than a moderate degree of differentiation and the ability to divide were mutually exclusive. Yet, other evidence seems to contradict these conclusions. In postnatal life the pancreases of the rat and mouse show a great deal of growth (16). Evidence that this growth involves the proliferation of well-differentiated acinar cells was cited earlier (5). Preliminary findings from our own laboratories support the suggestion that well-differentiated cells in the pancreas of the postnatal rat indeed do undergo mitotic division. The available evidence thus seems to point toward a remarkable difference between the embryonic and postnatal modes of growth in the pancreas of the mouse or rat.

Prescott (8) has suggested that the preparation for cell division and the synthesis of specific cell products must be mutually exclusive or competitive on several levels. Thus, the synthesis of RNA must come to a temporary halt while DNA replication is underway, and the synthesis of the proteins for the mitotic apparatus and new cell structures must compete with the synthesis of the cell products for amino acids and energy. It should be interesting to find out to what extent, if any, this sort of competition results in the slowing of the synthesis of secretory enzymes in mature acinar cells of the pancreas and parotid gland. Although this would be difficult to demonstrate by morphologic data alone, the structure presumed to be a condensing vacuole in the acinar cell in Fig. 7 suggests that synthesis and intracellular transport of secretory enzymes may have been taking place at the same time that the cell was undergoing division.

The observations presented here are part of a growing body of evidence which indicates that division of well-differentiated cells occurs more commonly than has been heretofore supposed. Further work is needed to establish the importance of this phenomenon in the over-all pattern of growth and development in organs such as the rat parotid gland and pancreas, and to clarify the conditions under which it occurs.

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