

An Autoradiographic and Morphological Investigation of the Postnatal Development of the Pineal Body

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ABSTRACT Cell proliferation in the pineal body of the hooded rat was studied with thymidine- H^3 autoradiography. Animals were either injected at birth and allowed to survive for variable periods or were injected at different ages and allowed to survive for a fixed period. Cell proliferation was high in the neonate (one and six hours of age) and continued at a decreasing rate into adulthood. The final development of the pineal body was believed to be due to cellular hyperplasia in the young animal and cellular hypertrophy in the adult. The morphological evaluation of the autoradiograms indicated that the principal proliferating components were parenchymal cells.

Earlier autoradiographic studies revealed the presence of labeled cells in many regions of the brain in rats after injection of thymidine- H^3 during infancy (Altman and Das, '65, '66). These postnatally forming cells were interpreted to be precursors of either neurons with short axons or of neuroglia cells and other supporting elements. In these studies it was observed that a large proportion of cells in endocrine glands associated with the brain, namely the pituitary and pineal, were labeled. This paper is concerned with an evaluation of cell proliferation in the pineal body combined with identification of the cell types formed.

Studies dealing with development of the pineal body have shown that in the mouse, mitotic activity drops from a neonatal maximum to a low level by two weeks (Dill and Walker, '66); data obtained by counting mitotic figures in the rat, showed that cell proliferation is rapid up to two weeks of age and then drops to a low frequency that continues into the adult period (Quay and Levine, '57). After two weeks of age the size of the pineal body increases up to about ten weeks; this change has been attributed primarily to parenchymal cell hypertrophy (Izawa, '25; Quay and Levine, '57).

Autoradiographic material obtained from earlier studies seemed to indicate that proliferation in the rat pineal body continued for a longer time and at a higher rate than had been suggested by prior investigations. Accordingly this study was carried out in an attempt to resolve this

question concerning cell proliferation in the pineal body using the technique of fine-resolution thymidine- H^3 autoradiography.

MATERIALS AND METHODS

In this study Long-Evans rats were used. The animals were given intraperitoneal injections of thymidine- H^3 , dissolved in isotonic saline at a dose of $10 \mu\text{c}/\text{gm}$ body weight, ($1.0 \text{ mc}/\text{ml}$; specific activity $6.7 \text{ C}/\text{mM}$). The younger animals were sacrificed by decapitation with immediate immersion of the heads in 10% neutral formalin; the older animals were sacrificed by cardiac perfusion with formalin. The brains were fixed in formalin for a minimum of one week and then embedded in Paraplast. Serial sections were cut at 6μ and three consecutive sections (or set of sections) were preserved out of every 10 to 30. Of the preserved sections, two sets were stained with Einarson's modification of gallocyanin-chromalum; one of these was coated in the dark with Kodak NTB-3 nuclear emulsion by the dipping technique, exposed at 5°C for 91 days and then developed. The procedures are described in more detail elsewhere (Altman, '64). In addition to the gallocyanin-chromalum stained control sections, sagittal sections were obtained from several adult animals and were stained with cresylecht-violet.

Quantitative autoradiographic studies using thymidine- H^3 and aimed at estimat-

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ing rates of cell proliferation are predicated on the assumption that thymidine- H^3 injected in a single dose remains available for a limited period and that the cells that are in the S phase at the time of injection are the only ones that will utilize and retain the radiochemical (Hughes et al., '58; Cronkite et al., '59; Messier and Leblond, '60). The other assumption (Hughes, '59) is that DNA is metabolically stable and, therefore, thymidine- H^3 is lost only when the cell multiplies and half of its DNA is transferred to its daughter cells. Hence, continued multiplication of already labeled cells will lead to an increase in number of labeled cells combined with dilution of the radiochemical and reduction in the number of silver grains over cell nuclei. The various possible relationships between number of labeled cells and concentration of label within the cells in populations with different rates and time course of multiplication have been indicated in an earlier study dealing with the general topic (Altman and Das, '56).

A summary of the design of the experiment with respect to age of the animals and survival time after injection is presented in table 1. The variability in proportions of labeled cells in the replicated pineals is between 5 and 8%. The animals were maintained on an 8:00 AM to 8:00 PM photoperiod with the injections administered, with the exception of the six hour group where injection was determined by the time of birth, at approximately the midway point in the cycle. There are

TABLE 1

Plan of experiments showing age at injection of thymidine- H^3 and subsequent interval before termination of the experiment

Age at injection	Interval between injection of thymidine- H^3 and death of rat						
	1 Hour	6 Hours	1 Day	3 Days	6 Days	60 Days	120 Days
6 hours	2	2	2	2	2	2	2
2 days	2	2					2
6 days	2	2					2
		2	2				2
30 days							2
120 days							2

several advantages to such a design; by studying slides coated with photographic emulsion (autoradiograms) from animals with a one or six hour survival after injection, it is possible to obtain information on regional rates of cell proliferation at different ages. Studying autoradiograms from animals with longer survival after injection provides information about the time course of cell proliferation and the extent of postnatal development of the pineal body. In this study both autoradiographic and control sections containing the pineal body were utilized. In two cases, however, the quality of the pineal sections was inadequate for quantitative analysis.

Evaluation of cell multiplication.

From serial sections of the pineal body, the one with the largest cross-section was taken for quantitative evaluation. For qualitative analysis, serial sections were used. Homologous sections and homologous regions within sections were scanned for all animals compared.

Cross-sectional measurements of the pineal body were taken by using a Leitz Aristophot with a tracing attachment. A $50 \times$ enlargement of the pineal body was projected onto a sheet of paper and the enlarged projection was traced. Planimetric measures were then taken of the tracings.

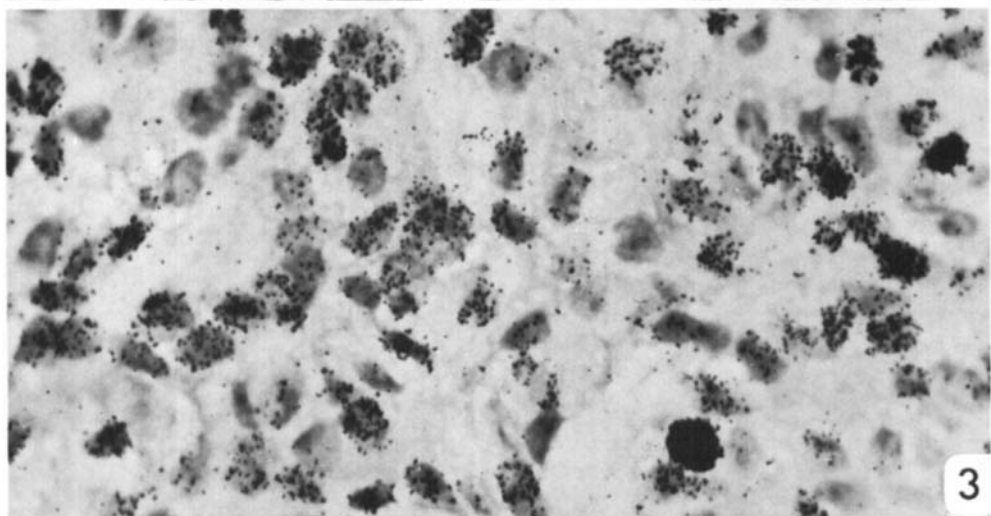
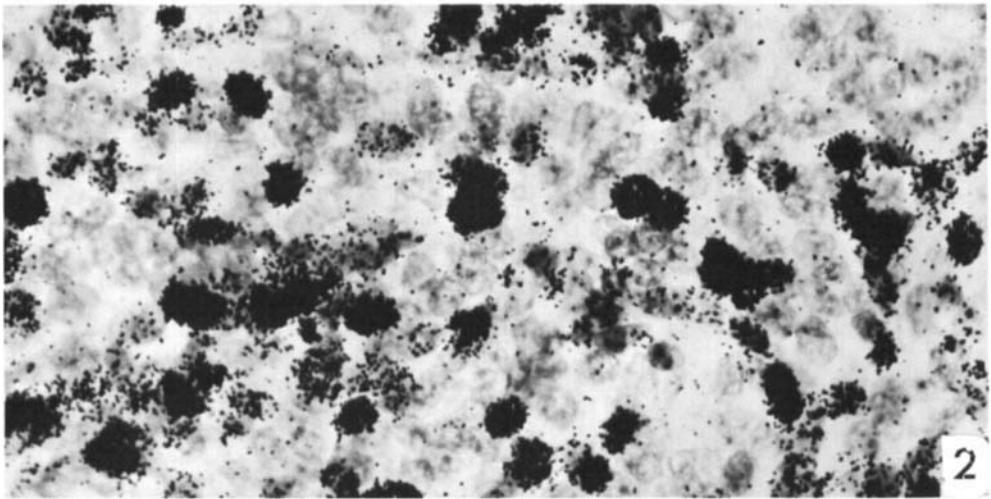
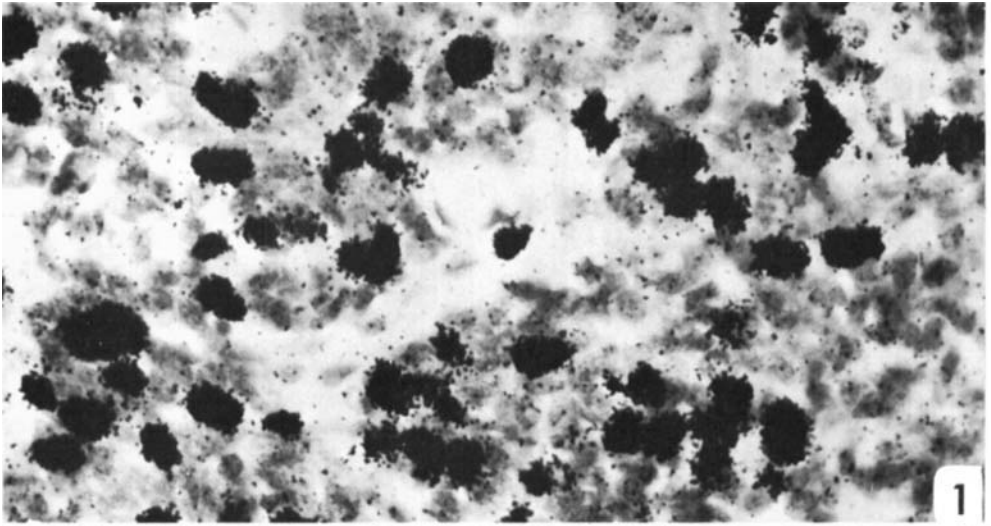
Cell packing densities, the number of cells present, were determined microscopically at $500 \times$ magnification in a diagonal series of five squares 182μ on a side.

The stained autoradiograms were examined at $500 \times$ magnification and the proportion of labeled to unlabeled cells in a set of squares 182μ on a side determined. In each section a minimum of 1000 cells were counted and classified. Cells were classified as either labeled or unlabeled but an attempt was also made to determine label concentration over the cell nuclei, to estimate degree of remulti-

Fig. 1 Photomicrograph of an autoradiogram from an animal injected at six hours and surviving for six hours.

Fig. 2 Photomicrograph of an autoradiogram from an animal injected at six hours and surviving for three days.

Fig. 3 Photomicrograph of an autoradiogram from an animal injected at six hours and surviving for 60 days. A progressive dilution of label concentration over cells can be noted. Galloxyanin-chromalum. $\times 640$.



Figures 1-3

plication of labeled cells by classifying the labeled cells into four categories; solid black cells were those most intensely labeled over which the blackened grains formed a continuous opaque surface. Predominantly black cells were those less intensely labeled in which grain-free areas were present to some extent, but the blackened grains predominated. Predominantly light cells were those over which there were larger patches of grain-free than grain-filled areas. Finally, very light cells were those over which only a few blackened grains were visible, about two to three times above the background level in comparable cell-free areas of the section. For purposes of analysis, however, these were combined into two categories, namely, darkly labeled and lightly labeled cells.

RESULTS

Figures 1, 2 and 3 illustrate the progressive dilution of label within cells as a function of repeated multiplications.

Autoradiographic evaluation. The proportion of labeled cells in the pineal body of animals injected at different ages with a one- or six-hour survival after injection is plotted in figure 4. It indicates a gradually declining rate of proliferation from birth to 13 days and also an increasing accumulation of labeled cells with a six-hour survival (except at 13-days) as compared to one hour of survival after injection. Although the relative proportions of lightly and darkly labeled cells are not indicated,

they are essentially the same for both survival periods.

The labeling of cells in the pineal body and progressive dilution of label concentration over cell nuclei as a function of repeated multiplication with increasing survival after injection is illustrated in figure 5. The data indicate that after the initial accumulation of lightly and darkly labeled cells up to one day, there is a phase of rapid decline in darkly labeled cells by the third day and a concomitant increase in the proportion of lightly labeled cells. These facts indicate a rapid rate of proliferation during this time period. By six to 13 days of survival, however, proliferation is slowing down as indicated by the gradual decrease in number of darkly labeled cells and the slowly increasing number of lightly labeled cells. This suggests that proliferation is continuing but at a lower rate than in the neonate. If the rate of proliferation remained high, the curve showing the number of lightly labeled cells would reach an asymptote and then decline while the curve for darkly labeled cells would drop to zero. If on the other hand proliferation were to stop, the curve of lightly labeled cells would stabilize at an asymptote and the curve of darkly labeled cells would not decline.

In the animals with 120 days of survival at least 60% of the cells of the pineal body are still labeled. Since it is possible that, due to the high rate of proliferation in the first week of life, label dilution beyond the point of detectability might have taken

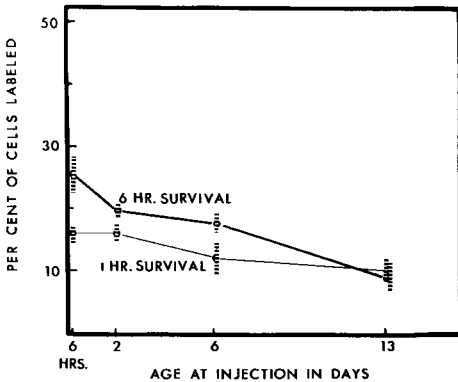


Fig. 4 Percentage of labeled cells in the pineal body in animals injected at different ages and surviving for one or six hours. The vertical broken lines represent the crude ranges of the data.

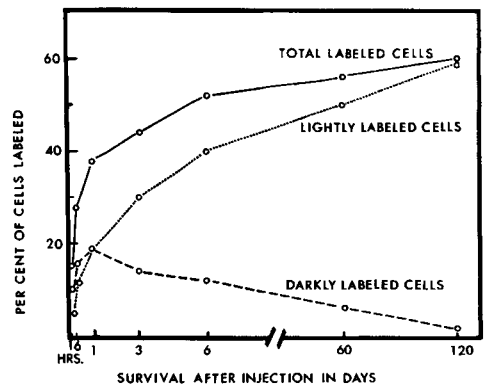


Fig. 5 Percentage of labeled cells in the pineal body in animals injected as neonates and surviving for different periods after injection.

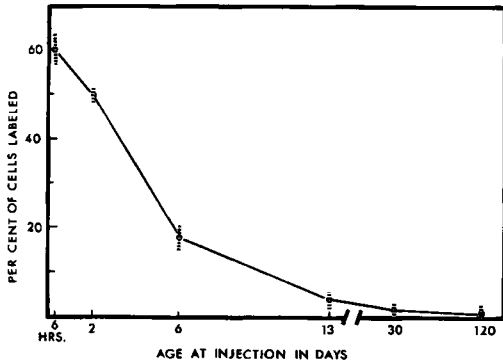


Fig. 6 Percentage of labeled cells in the pineal body in animals injected at different ages and surviving for 120 days. The vertical broken lines represent the crude ranges of the data.

place, these curves express conservative estimates of pineal cell multiplication; the actual proportions of postnatally formed cells could be much higher.

Animals injected at different ages and surviving for 120 days (fig. 6) provide information as to the proportions of cells contributed at different ages to the final development of the pineal body. Most of pineal body development, at least in relation to cell production, occurs during the first two weeks of life. Since our long survival material indicates that most cells of the pineal body are formed during the first two weeks of life, we have considered proliferation as being high during this time.

If in fact proliferation is high during the first days of life and becomes gradually more sluggish as the animal matures, then by comparing animals injected early in life and surviving for either one hour, six hours or 120 days, the last survival period should show a high percentage of labeled cells (figs. 4, 6). This is indeed the case for very early injection ages but at 13 days of age at injection, 120 day survival shows a lower value.

Figure 7 shows that, as determined by cross-sectional measurements, the pineal body grows in size up to at least 60 days; at the same time the packing density decreases steadily. Previous reports have indicated that increase in size of the pineal body is primarily attributable to hypertrophy of parenchymal cells rather than to a growing number of cells (Quay and Levine, '57). The data would seem to substantiate this observation since the

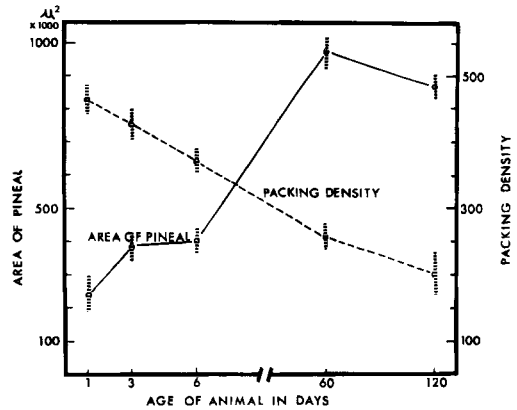


Fig. 7 Area of the pineal body cross-section and packing density (number of cells present in a diagonal series of five squares 182μ on a side) in animals that survived for different periods after injection. The vertical broken lines represent the crude ranges of the data.

pineal body increases in size up to 60 days whereas cellular proliferation drops to moderate levels by the end of the second week of life. It is possible, however, that essentially two processes contribute to the final development of the pineal body, hyperplasia in the neonate and this merging into cellular hypertrophy as the animal matures.

Morphological evaluation. Although autoradiographic data provide information concerning the rates and time course of proliferation, the procedure in this instance does not provide detailed information on the types of cells labeled due to the concentration of the opaque label over the nucleus. Accordingly control sections, processed according to standard histological procedures, were evaluated.

Cells of the pineal body are classified into the following categories: parenchymal or chief cells, spongioblasts, pia cells, endothelial cells, and ependymal cells (Aristila and Hopsu, '64; Aristila, '66; Gardner, '49; Hungerford and Pomerat, '62). Parenchymal cells have large pale staining nuclei with darkly staining nucleoli; in their differentiated state they invariably have a large amount of cytoplasm; according to Del-Rio-Hortega ('32) these cells have long processes which end in bulb-like swellings. The spongioblasts or neuroglial cells are characterized by a small, oval, intensely stained nucleus with little or no cytoplasm;

it is claimed that the majority of glial cells are astrocytes (Wartenberg, '68) but the staining technique was inadequate to make this determination. Endothelial cells are characterized by long thin deeply staining

nuclei and generally surround blood vessels. Figures 8-9 illustrate the different cell types determined. Using these criteria all of the control material as well as a few nonradioactive adult animals were scanned

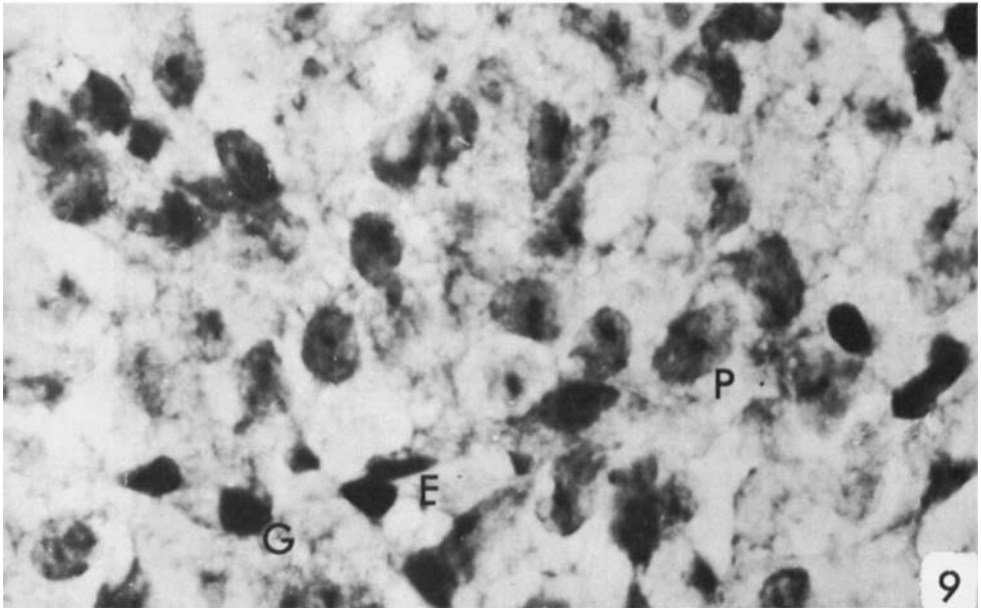
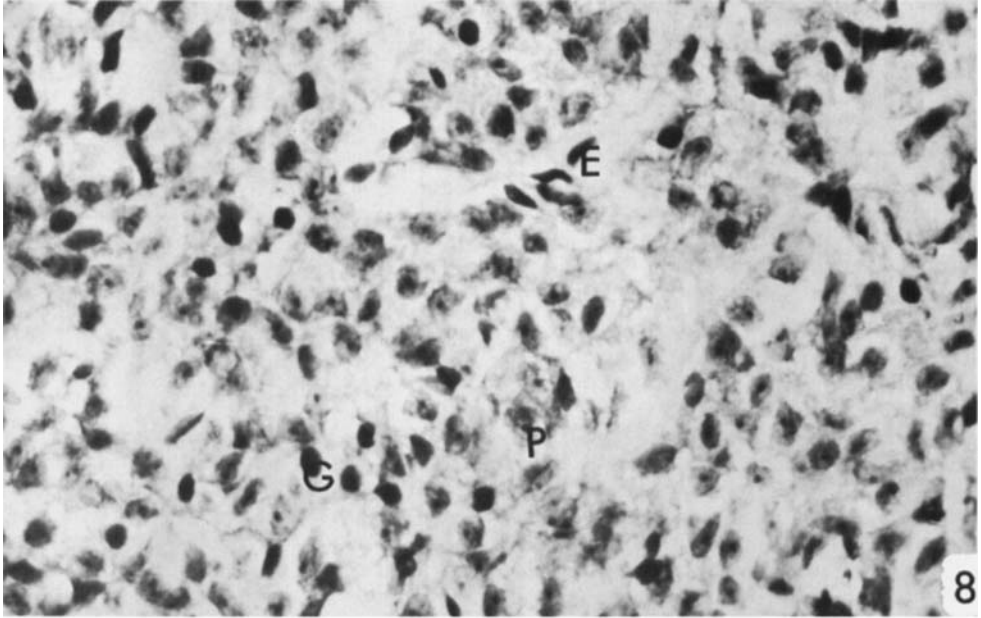


Fig. 8 Photomicrograph of rat pineal showing parenchymal cells (P), glial cells (G), and ependymal cells (E). Gallocyanin-chromalum. $\times 512$.

Fig. 9 Photomicrograph of rat pineal showing parenchymal cells (P), glial cells (G), and ependymal cells (E). Gallocyanin-chromalum. The various cell types found in the rat pineal are illustrated here. $\times 1024$.

and the proportions of different cell types present counted. In the adult animal about 82% of the cells scanned were parenchymal, 12% were neuroglial, 5% endothelial and 2% unidentified. In very young animals the different cell types could not be readily differentiated; parenchymal cells could first be clearly identified at about two weeks of age. As has been mentioned, the nature of the autoradiographic procedure precludes a detailed morphological evaluation; in a few cases, however, some labeled parenchymal cells could be seen.

DISCUSSION

The first anlage of the pineal body appears as an evagination of the roof of the diencephalon. In embryos between 14 and 14.5 days, the first anlage of the body can be seen in the albino rat (Ariens Kappers, '60); according to Gardner ('49) pineal body development in the hooded rat starts at about the twelfth day and following this, pineal body development proceeds rapidly. The pineal anlage at the fourteenth day is differentiated into ependymal, mantle and marginal layers; the five cell types mentioned above can be distinguished on the eighteenth day (Gardner, '49).

Postnatal proliferation of cells in the pineal body of the rat demonstrated with thymidine- H^3 autoradiography, substantiates the histological evidence regarding growth of the pineal body. The curve for pineal cell proliferation (fig. 5) is characteristic of a cell population with rapid initial multiplication that slows down over time as the animal matures (Altman and Das, '66). Supporting this inference is the work of Fujita ('62) who indicated that the stage of development might affect the rate of DNA synthesis. This contrasts with earlier studies which indicated that postnatal mitotic activity in the rat pineal body drops from a neonatal maximum to a low or trace levels by two weeks (Quay and Levine, '57). The value of 60% labeled cells in the pineal body obtained here in rats injected as neonates and with 120 days of survival is an underestimate due to two factors. First, there was loss of label as a result of repeated multiplications and, secondly, since only one injection was given to these animals, not all cells were in the S phase when the radiochemical was available.

The long survival material does provide information concerning the prolonged time course of cellular proliferation in the rat pineal body. It is apparent, however, that growth of the pineal body as evidenced by figure 7 cannot be principally attributed to cell multiplication since the pineal body continues to increase in size beyond the point at which the rate of cell proliferation has dropped off. By the end of the second week of life there is a merging of cellular hyperplasia and hypertrophy with the latter primarily contributing to growth of the structure. This fact is supported by the decrease in packing density with increase in age and agrees with earlier observations concerning size and weight increase in the pineal body (Quay and Levine, '57; Roth, '65). Possible explanations for the lower number of labeled cells following 120 days of survival with 13 days of age at injection could lie in the following factors. By 13 days cells other than parenchymal might have begun to multiply, and hypertrophy of parenchymal cells could alter the distribution of labeled cells. This may, therefore, represent a sampling artifact. Another possibility could be the degeneration of a certain proportion of parenchymal cells between days 3 and 21 as noted by Quay and Levine ('57).

The data concerning rate of proliferation during the first two weeks of life together with the total number of labeled cells strongly indicates that a large part of the pineal body is formed postnatally. In animals receiving multiple injections at birth and surviving for four months, about 80% of the cells of the pineal body are labeled. The fact that 82% of the cells identified in the adult animal are parenchymal and the fact that at least 60% of the total pineal body section shows label in the long survival material, clearly demonstrates that a significant proportion of the proliferating components are parenchymal. It is possible, however, that the different cell types classified in the pineal body might have differing developmental histories; due to problems in clearly identifying cellular morphology in the autoradiograms, this issue cannot be resolved. These observations suggest that, although in young animals development of the pineal body is fairly uniform, as the animals grow older and lobules begin to develop in the pineal

body, proliferating cells are restricted to the periphery of these lobules.

It has been maintained for some time that thymidine- H^3 labeling of multiplying cells was a pulse labeling process and that by one hour after injection most of the radiochemical was metabolized and passed out of the body (Amano, Messier, and Leblond, '59; Rubini et al., '60). The data, however, show that more cells are found after six hours than after one hour of survival (fig. 4). There are at least two possible explanations for this. If in the six-hour interval between injection and sacrifice, labeled cells would have the opportunity to remultiply then this would account for the noted increase. Suggestive confirmation of this is seen in the paper by Hommes and Leblond ('67) where they showed the number of labeled mitotic figures increasing up to five hours by day or eight hours by night following a single injection of thymidine- H^3 . Such factors as the route of administration of the radiochemical (Skougaard and Stewart, '59) and a variable duration for the S phase in different tissue systems also can affect such a count (Quastler and Sherman, '59). Alternatively there exists the possibility that labeling with thymidine- H^3 is not a pulse process and that radiochemical is available for longer periods of time than has been earlier believed; thus prolonged availability of thymidine- H^3 would label cells that entered the synthetic or S phase at different times during the survival interval.

ACKNOWLEDGMENT

This work was supported by the United States Atomic Energy Commission.

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