SCHWANN CELL PROLIFERATION IN DEVELOPING MOUSE SCIATIC NERVE

A Radioautographic Study

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ABSTRACT

Proliferation of Schwann cells in neonatal mouse sciatic nerve was studied radioautographically in 1-μ glycol methacrylate sections. 28 mice were injected with thymidine-^{3}H, 4 μc/g, 48 hr after birth, and were killed serially over the next 4 days. For the cell cycle following injection, the generation time was approximately 24 hr as determined by grain-count halving data; the duration of synthesis phase was 8 hr as determined from a curve constructed from the per cent of mitotic figures containing label; and the labeling index was 9% at 2 hr after injection. With these estimates, the per cent of Schwann cells proliferating was calculated to be 27%. In addition, roughly 25% of dividing cells appeared to cease division during the cell cycle under study. The relationship of these findings to other events during maturation of nerve is discussed.

INTRODUCTION

Schwann cells take their positions along peripheral nerve fascicles during embryonic life. Although the sources of Schwann cells and their routes of migration into nerve are still in doubt (10, 17), it is clear from casual observations of radioautograms that, after Schwann cells join with nerve, they proliferate in situ at all levels along the spinal roots and nerves. This process begins on or before the 12th embryonic day in the mouse and continues well into postnatal life. We have seen mitoses of Schwann cells in the 2nd postnatal week, a finding in accord with that of Diner who studied rat sciatic nerve (7). Peters and Muir, on the basis of quantitative light microscopic studies coupled with electron microscopy, concluded that the increase in numbers of Schwann cells they observed in the late embryonic period resulted mainly from proliferation of Schwann cells that were already within nerve, rather than from accretion of postmitotic cells by migration (11).

In recent years, elaboration of radioautographic methods has allowed a more precise analysis of cell population kinetics. The methods have been applied, for the most part, to cell systems chosen because all cells are of one type and are engaged equally and randomly in proliferative activity. Schwann cells were chosen for this study because of their key role in the growth, function, and pathophysiology of the peripheral nervous system, not because they are an ideal model of cellular kinetics. The present report deals with Schwann cell proliferation in the 2-day-old mouse sciatic nerve.

METHODS

C3H/HeJ mice from several litters, born at various hours of the day, were injected subcutaneously with tritiated thymidine, 4 μc/g, (New England Nuclear Corporation; spec. activ. 1.7–3.0 c/mmole), 48 hr after birth, and killed 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,

12, 13, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 60, 72, and 96 hr later, and this gave a total of 28 time points from the 2nd to the 6th day postnatally. Mice were decapitated, skinned, and fixed by immersion in 10% acrolein in 0.9% saline overnight at 4°C. The intact hind limb and brain were dehydrated over 24 hr in three changes of methanol-methyl cellosolve (1:1, v/v). The sciatic nerves were

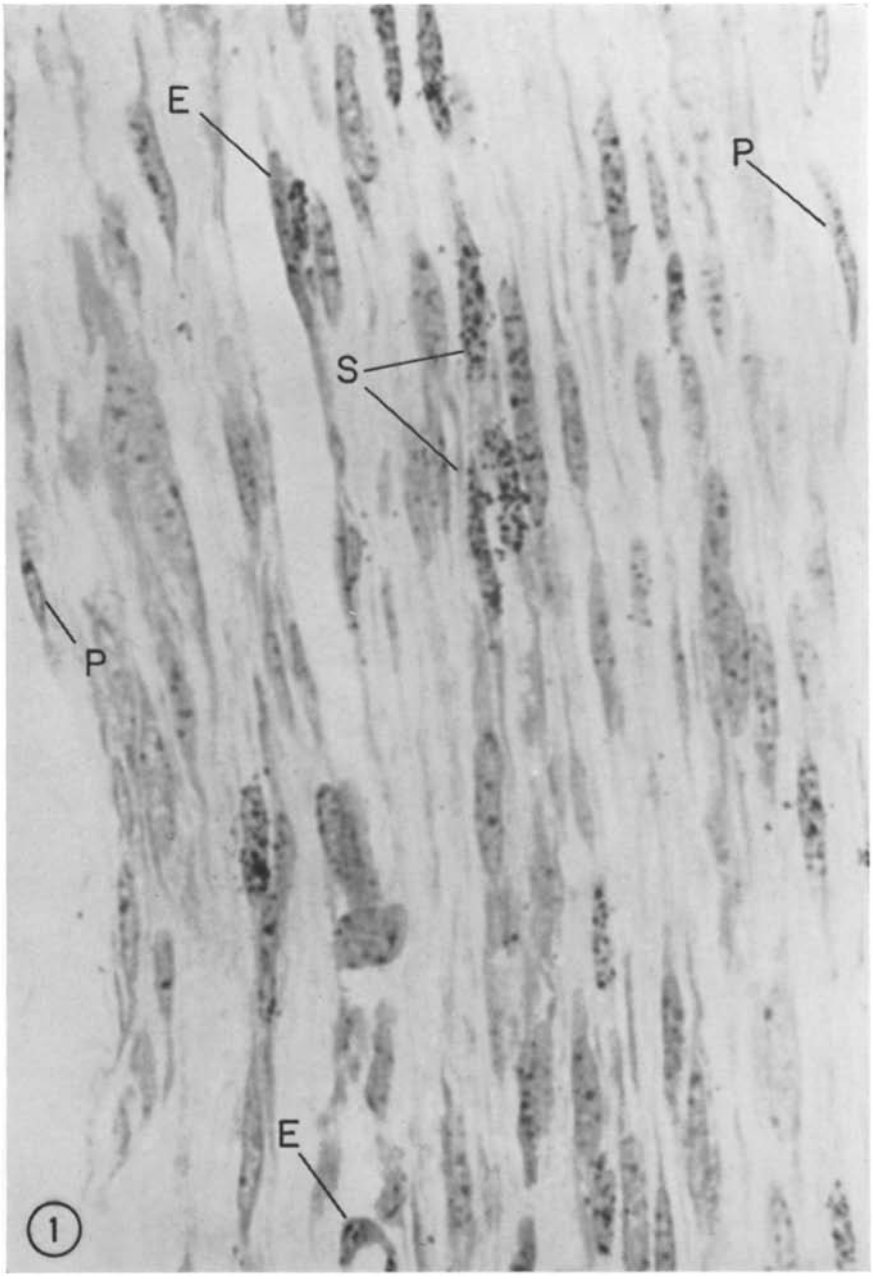

FIGURE 1 Longitudinal section of neonatal mouse sciatic nerve at 28 hr after injection of thymidine-^{3}H. *S*, Schwann cell nuclei; *P*, perineurial cell nuclei; *E*, endothelial cell nuclei. Most labeled Schwann cell nuclei have few grains overlying them by 28 hr, but some heavily labeled nuclei persist. Toluidine blue stain. × 760.

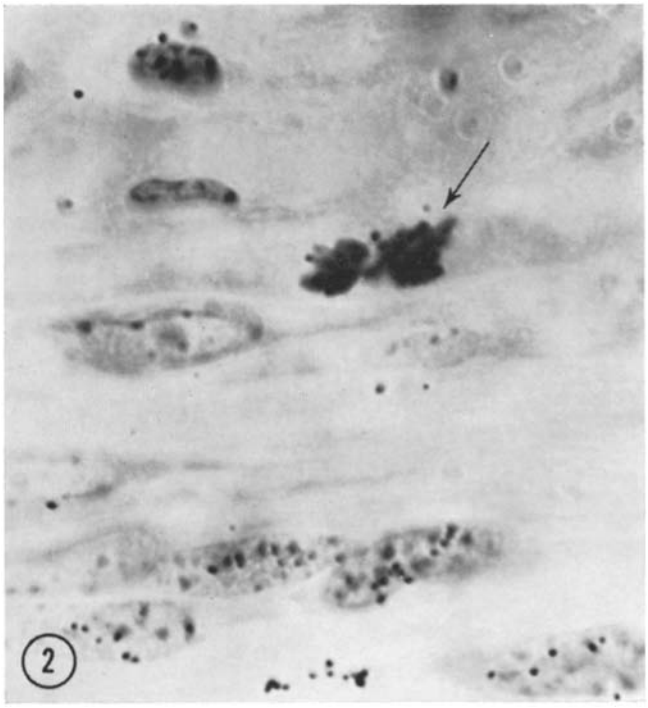

FIGURE 2 Labeled mitotic figure (arrow) in sciatic nerve of neonatal mouse. Note also labeled interphase Schwann cell nuclei at bottom. Toluidine blue stain. × 1800.

dissected free with the aid of a dissecting microscope, and infiltrated with glycol methacrylate monomer, which was then polymerized at 60°C for 1–2 days (1). 1-μ longitudinal sections were cut on an MT-1 Porter-Blum microtome with a glass knife, floated on a drop of water on clean glass slides, and allowed to dry in air. We prepared radioautograms in total darkness by dipping the slides into liquid photographic emulsion (Kodak NTB-2) at 40°C, allowing them to drain for 30 min at 28°C, and storing them at −70°C in light-tight boxes for periods from 8 to 90 days. The preparations were developed in Dektol developer (Kodak) for 3 min, fixed, washed, and stained with 0.5% toluidine blue for 5–10 min, rinsed, dried in air, and mounted for microscopy. We dipped, exposed, and developed simultaneously sections used for grain-counting in order to minimize error introduced by the radioautographic method itself.

Quantitative information was obtained with a 100x oil immersion objective as follows: 1) 1000 consecutive Schwann cell nuclei were counted for each of time points 2, 8, 13, 16, 24, 28, 36, and 48 hr after injection of thymidine-^{3}H, and recorded as labeled or unlabeled. 2) The number of grains overlying each of 200 consecutive labeled interphase Schwann cell nuclei was determined for each of time points 2, 13, 24, 36, and 48 hr after injection of thymidine-^{3}H. 2- and 13-hr specimens were selected because they were technically better than the 1- and 12-hr specimens. 3) 100 Schwann cells in mitosis were classified as labeled or unlabeled for each time point from 1 hr through 16 hr after injection of thymidine-^{3}H (see Fig. 2). However, at time points 6, 8, and 9 hr, only 71, 49, and 81 mitotic figures, respectively, were available to be recorded. Early prophases were not included in the counts.

In the nerves, only Schwann cells were counted. Schwann cells were readily identified by their elongated, oval nuclei which were always arranged parallel to the long axis of the nerve, each nucleus lying in relation to a nerve fiber (Fig. 1, *S*). In contrast, endothelial cell nuclei were shorter and usually plumper than Schwann cell nuclei. They did not lie in longitudinal orientation but were aligned in obvious relationship to vessels, and often were curved or indented to conform to the vessel lumen (Fig. 1, *E*). Perineurial cells were identified by their anatomic position, scanty cytoplasm, and narrow nuclei (Fig. 1, *P*). Mast cells were recognized easily by their prominent intracytoplasmic granules in the toluidine blue preparations. Occasional oval nuclei lying adjacent to vessels, but not oriented longitudinally and not in relationship to nerve fibers, were designated as adventitial cell nuclei. The category of endoneurial fibroblast, as used by others, corresponds to our group of adventitial cells. Electron microscopic studies of mouse peripheral nerve have

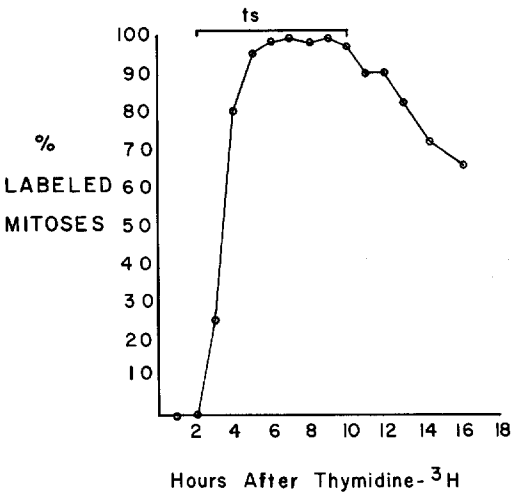

FIGURE 3 Per cent of Schwann cell mitoses which are labeled in 2-day-old mouse sciatic nerve plotted against time after injection of thymidine-^{3}H. The duration of S (t_S) may be estimated from the resulting curve (8 hr). S is measured from the initial appearance of labeled mitoses to the initial disappearance of labeled mitoses, e.g., the first labeled cells to leave S to the last labeled cells to leave S.

indicated that less than 5% of cells in nerve are endoneurial fibroblasts[1] and that at least 85% are Schwann cells (5). This ratio is confirmed by studies on other species, including the human (16). In any event, the endoneurial fibroblast population of electron microscopic studies and the adventitial cell population of our study are too small numerically to influence the data presented here.

There were three advantages of 1-μ, glycol methacrylate sections. 1. Excellent histological detail was obtained, making identification of individual nuclei almost always possible. (See reports by Feder and colleagues for a fuller account of glycol methacrylate as an embedding medium (1, 8)) 2. Localization of silver grains to individual nuclei was precise. Four grains/nucleus was arbitrarily considered threshold for labeling, although background was low enough so that three grains/nucleus might have been sufficient. 3. Closer correlation of grain-counts to isotope content was gained. Caro estimates that ideally 20% of disintegrations from tritium produce silver grains when 1-μ sections are used, whereas only 2% do so when 2-μ sections are used (4).

Grain-counts per nucleus ranged from four, the arbitrary threshold, to over 100, although only an occasional nucleus had more than 80 grains. Accuracy of counts above 40 was lessened because of clustering of grains. Below 40, the counts were accurate.

[1] Not all authors agree (14).

RESULTS

Assumptions in the following analysis are: 1. Schwann cells divide asynchronously, and 2. The generation time for dividing Schwann cells is relatively uniform. No assumptions were necessary concerning the total pool size. Preliminary observations suggested that the majority of Schwann cells were no longer dividing by the end of the 2nd day of life, and that after each successive cycle a progressively smaller percentage of the daughter cells continued to divide.

In Fig. 3, the per cent of mitotic figures which are labeled has been plotted against time after thymidine-^{3}H injection. The ascending limb of the curve is steep, indicating that the G2[2] portion of interphase is constant in the labeled population (12). The average duration of G2 plus ½ M is the time from zero hour to the time of labeling of 50% of mitotic figures, or 3.5 hr. DNA synthesis time is taken as the time between the point at which the curve begins to rise (2 hr after thymidine-^{3}H) and the point at which the curve begins to fall (10 hr after thymidine-^{3}H), which gives a duration for S of 8 hr (2).

This means of determining the duration of S is felt to be more reliable than the usual device of measuring the time-span between 50% levels on the ascending and descending limbs of the curve, since the descending portion beginning at 10 hr is considerably less steep than the ascending one and fails to return to 50%. A probable explanation for the failure of the curve to descend sharply after 10 hr includes the variability, from cell to cell, in the durations of S, M, and, to a minor degree, G2 (15). At times later than 16 hr after thymidine-^{3}H (not shown in Fig. 3), flattening of the curve occurs, making identification of a second peak uncertain. Another possible cause of curve flattening is reutilization of liberated DNA precursors containing tritium which have been shown to reappear after a pulse-dose of thymidine-^{3}H (3, 6, 9, 13).

If, as suspected, a portion of daughter cells ceases dividing after each cell cycle, histograms of grain-count distributions at different times after

[2] S = DNA synthesis phase; G1 = postmitotic, presynthetic phase; G2 = premitotic, postsynthetic phase; M = mitosis; T, (or generation time) = the period from the end of mitosis to the end of the next mitosis.

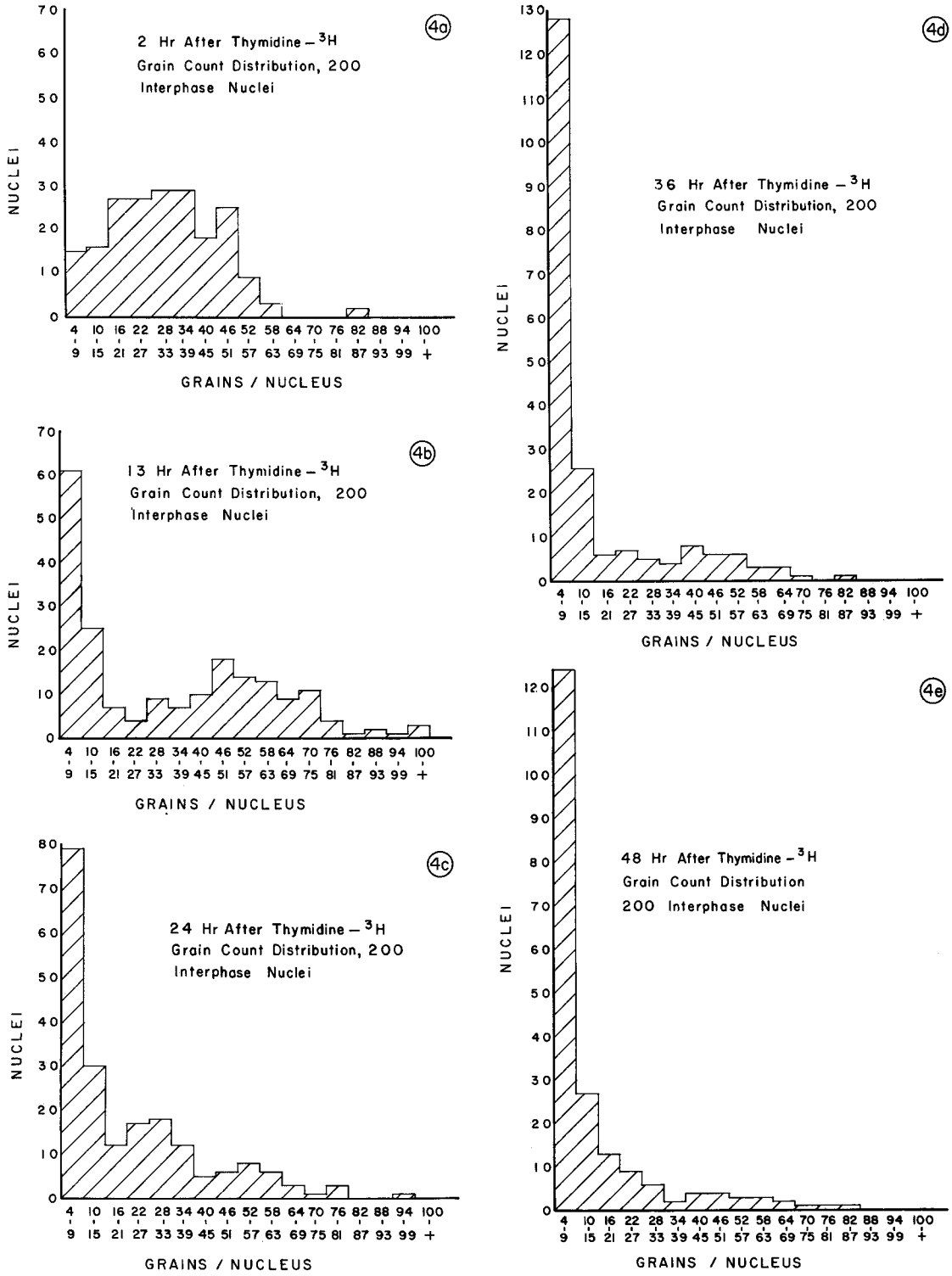

FIGURE 4 *a–e* Distribution of grain-counts/interphase Schwann cell nucleus in the sciatic nerve of neonatal mice killed 2, 13, 24, 36, and 48 hr after a single injection of thymidine-^{3}H. Mean grains/nucleus decrease with time after injection, but a heavily labeled population with 40 or more grains/nucleus persists.

injection of thymidine-^{3}H should show a persisting, heavily labeled, postmitotic population. Such a population is indeed present (Figs. 4 *a–e*). At 2 hr after thymidine-^{3}H, the grain-counts lie predominantly between 15 to 50 per Schwann cell nucleus and are distributed randomly about a median of 32 grains. By 13 hr after thymidine-^{3}H, the counts shift to lower values in the majority of the cells, and by 24 hr, 40% of nuclei have fewer than 10 grains.

A population of heavily labeled cells, approximately 10% of the total, persists at 72 and 96 hr after thymidine-^{3}H, the latest time points examined quantitatively. At 48 hr after thymidine-^{3}H, 19 of the 200 cells counted (9.5%) had 40 or more grains per nucleus (total = 1080 grains; mean = 57 grains), and can be assumed to have divided only once after labeling, or conceivably not at all. Estimation of the extent of the nondividing, labeled population obtained at 48 hr provides a useful correction in calculations given below for the approximate generation time. In addition, Schwann cells of sciatic nerves examined radioautographically in 5-month-old mice that had received a thymidine-^{3}H injection daily for the 1st 10 days after birth show a labeling index[3] approaching 50%, indicating that once the cells stop dividing in the neonatal period they normally do not begin again.

In Fig. 5, mean grain-counts/nucleus are plotted against time after thymidine-^{3}H (open circles). These averages are based upon grain-counts of the labeled, nondividing population as well as the dividing cells, so that the mean grain-counts are falsely high, an error that increases at each succeeding time point. If the 19 nondividing, labeled cells and their associated 1080 grains are subtracted from the total cells and total grains at 48 hr, and the same numerical correction is made at each previous time point (*X*'s in Fig. 5), a curve may be drawn that indicates a grain-count halving time of 24 hr. This value is still only a crude approximation of the generation time, since no correction has been made for systematic error introduced by the arbitrary threshold of four grains/nucleus. 15% of cells at 2 hr after thymidine-^{3}H have fewer than 16 grains. These cells will have been through mitosis twice by 48 hr and grain-counts of their progeny will have been diluted below threshold. Neglect of the "real" grain-counts which are below

[3] Labeling index = % of nuclei labeled, i.e., four or more grains overlying.

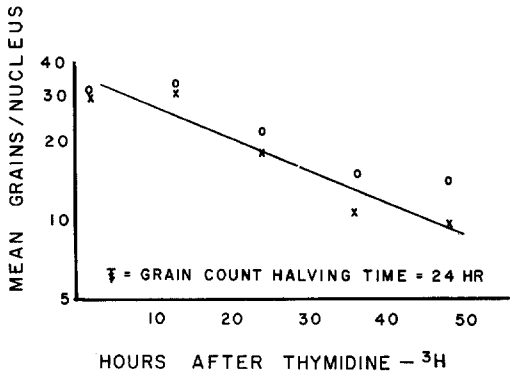

FIGURE 5 Mean grains/interphase Schwann cell nucleus plotted semilogarithmically against time after injection of thymidine-^{3}H in 2-day-old mice. Open circles represent mean grains/nucleus if no correction is made for the persisting heavily labeled population. *X*'s represent mean grains/nucleus if the persisting heavily labeled population at 48 hr (19 nuclei with 1080 grains) is neglected at every time point. Line is drawn to the *X*'s. By this method, the grain-count halving time is 24 hr.

threshold tends to decrease the slope of the line plotted in Fig. 5, thereby making the estimate of generation time too long. Lowering the threshold from four to three grains/nucleus lowers the entire grain-count halving curve, but changes the generation time by only an hour, from 24 to 23 hr. Other possible factors affecting the estimated generation time, such as radiation-induced death of labeled cells and reutilization of labeled DNA precursors derived from dying cells, have an opposite effect, i.e., tend to increase the slope. These points make it plain that the generation time as calculated here is only a rough approximation, but fortunately is accurate enough for some further calculations.

For a cell population of which only a portion is mitotically active, one can determine the relative sizes of the dividing and nondividing groups, at a given time, from the following equation:

$$\frac{\text{Duration of } S}{\text{Generation time}} = \frac{\text{Labeling index}}{\%\text{ of cells proliferating}} \quad (1)$$

The duration of *S* in the dividing Schwann cell population on the 2nd postnatal day is 8 hr, and the generation time is estimated to be about 24 hr. The labeling index, a measure of the total cell population in *S* at the time of thymidine-^{3}H injection, is 9% at 2 hr after thymidine-^{3}H (Fig. 6).

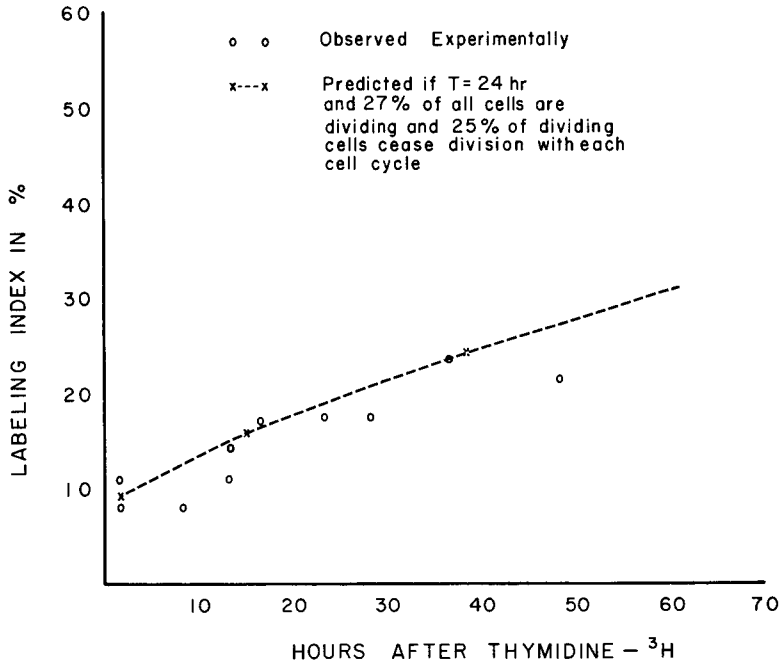

FIGURE 6 Labeling index (open circles) of interphase Schwann cell nuclei in sciatic nerve plotted against time after injection of thymidine-^{3}H in the 2-day-old mouse. X---X is the predicted rise of labeling index based on the information already obtained, i.e., T = 24 hr, 27% of all Schwann cells are dividing, and 25% of dividing cells cease division with each cell cycle (see Table I). Each experimental point represents a count of 1,000 Schwann cells. For the time point, 2 hr after thymidine-^{3}H, two independent counts on different radioautograms were made. The average labeling index at 2 hr was 9%. A similar double count was carried out for time point 13 hr after thymidine-^{3}H. The experimental points approximate the predicted rise.

Knowing the other three numbers of the proportion, we can calculate the per cent of cells proliferating to be 27%; thus, almost three-quarters of the Schwann cells are not proliferating.

Both numerators in the equation are reasonably accurate; less reliable is the generation time, but even gross departure of as much as 10 hr either way from the estimated generation time of 24 hr does not alter the conclusion that cell proliferation in the mouse sciatic nerve at 2 days is carried on by a minority of the Schwann cells.

If the Schwann cell population were completely homogeneous, and all the cells, both labeled and unlabeled, continued to divide, the labeling index should remain constant at the initial value of 9% for some hours, and then gradually fall as the label was diluted below threshold. However, it was found that the proportion of labeled nuclei rose (Fig. 6). This indicates that most of the unlabeled cells were not proliferating, so that the labeled compartment came to occupy a larger and larger part of the total.

Beginning with a labeling index of 9% at 2 hr after injection of thymidine-^{3}H, one can project an expected rise of labeling index with time by using other facts learned in this experiment. This has been done in Table I, and the projected labeling index is plotted (interrupted line) in Fig. 6. Values for generation time, initial labeling index, and percentage of all cells dividing have already been determined, and an arbitrary guess was made, on the basis of the histograms (Figs. 4 *a–e*), that 19 cells ceased division during the 1st cell cycle. These data make it possible to estimate that a starting group of 75 cells at time zero would increase to 200 in 48 hr if 19 cells cease division during the 1st cell cycle and a like proportion in the succeeding one. Since 19 of the original group of 75 cells cease dividing during the 1st cycle, a drop-out rate of 25% may be estimated. Many potential sources of error could be cited in an exercise of this nature; however, these factors are either unimportant here or are mutually canceling,

TABLE I

Predicted Rise in Labeling Index of Schwann Cell Nuclei in Neonatal Mouse Sciatic Nerve

Time after thymidine-^{3}H		Labeled cells/ Total cells	Labeling index
hr			%
1st cycle	2	9/100	9
2nd	14*	18/110	16
3rd	38	32/136	24
	62	52/173	30

* First doubling of labeled nuclei occurs in 12 hr ($S + G_2 + M$).

The observed labeling index at 2 hr after thymidine-^{3}H is 9%. Labeling indices (right hand column) at subsequent times (left hand column) are predicted from information previously obtained: T = 24 hr, and 27% of all Schwann cells are dividing. It was estimated that 25% of dividing cells cease division with each cell cycle (See text for explanation). Labeling indices are plotted in Fig. 6.

since the predicted rise in labeling index approximates the experimental one fairly well.

DISCUSSION

Most developmental studies of the Schwann cell have been directed toward learning its origin and route of migration, and relatively little attention has been paid to Schwann cell proliferation following migration to peripheral nerve. By the 12th embryonic day in the mouse, proliferating cells are aligned longitudinally along spinal roots and nerve fascicles. Schwann cell division continues into the 2nd postnatal week, at least in the sciatic nerve. By the 2nd postnatal day in the mouse, almost three-quarters of the Schwann cells are no longer proliferating, and presumably are maturing because myelin is forming at a rapid rate in the 1st postnatal week. The large size of the nondividing group is determined by comparing the labeling index (the proportion of all cells in *S*) to the ratio of duration of *S* to generation time (Equation 1). Another indication of a large nondividing pool of cells is the rise of labeling index with time (Fig. 6). We interpret this rise to mean that an initially small, actively dividing, labeled population occupies a larger and larger proportion of the total cell number, thus reducing the relative size of the nondividing group. In the cell cycle immediately following injection of thymidine-^{3}H, approximately 25% of the dividing population ceases to divide, and persists as heavily labeled cells. These heavily labeled cells are most likely differentiating, but it is also possible that they are continuing to divide, but too slowly to be noticed in the 4-day span studied. If it is true that a Schwann cell which has begun to lay down myelin no longer divides (11), the present finding that three-quarters of the Schwann cells are not dividing is in accord with the rapid myelin formation which occurs in the mouse at this age.

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