THE REPLICATION TIME AND PATTERN OF THE LIVER CELL IN THE GROWING RAT

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ABSTRACT

Three-week-old male rats of the Wistar strain were given tritiated thymidine, 1 μc/gm body weight, intraperitoneally and were killed at intervals from 0.25 to 72 hours later. Autoradiographs were made from 5 μ sections, stained by the Feulgen method. The replication time and its component intervals were determined from the scoring of the labeling of interphase nuclei as well as of prophase, metaphase, anaphase, and telophase nuclei. Absorption of the intraperitoneally injected label is rapid and is attended by "flash" labeling during interphase. The results show that at any one time about 4 per cent of the liver cells are synthesizing DNA preliminary to cell division. These cells alternate with waves of other cells and it is estimated that about 10 per cent of the liver cell population is engaged in cell duplication. The replication time is about 21.5 hours, and its component intervals occupy the following times: DNA synthesis, 9 hours; post-DNA synthesis gap, 0.50 hour; prophase, 1.3 hours; metaphase, 1.0 hour; anaphase, 0.4 hour; telophase, 0.3 hour; post-mitosis gap, 9.0 hours. A group of liver cells has been recorded in at least 3 successive replication cycles.

INTRODUCTION

Since the formation of new cells represents the end result of many metabolic activities, its study offers a potentially useful technique for the investigation of some of the properties of cells. One phase of this problem is the analysis of the time intervals of the several stages of new cell formation. The introduction of radioactive materials as labels of deoxyribonucleic acid (DNA) has made possible the study of these phases of cell replication, and has been employed in the investigation of many cell lines (1–18, 21–29).

The present study concerns the formation of new liver cells in the growing rat. An attempt has been made to estimate the respective times required for DNA synthesis, mitosis, and new cell formation from autoradiographic studies with tritiated thymidine (H^3TDR). This measurement system has since been employed as an index of change in the liver cell under different experimental conditions.

METHODS

Three-week-old male rats of the Wistar strain were fed Purina checkers and water *ad libitum*. They were given 1 μc/gm body weight of H^3TDR[1] intraperitoneally, and at stated intervals, 0.25 to 72 hours later, groups of 2 to 6 animals were sacrificed. The

[1] The H^3TDR had a specific activity of 0.36 c per millimole and was obtained from Schwarz Bio-research Inc., Orangeburg, New York. It was diluted in distilled water to 50 μc/ml and was administered intraperitoneally.

completeness of the absorption of the injected material was checked by analysis of the peritoneal washings 0.25, 0.5, and 1.0 hour after administration. The livers were fixed in Carnoy's fluid and sections were made at 5 μ. After staining the sections by the Feulgen method, strips of Kodak AR 10 film were applied in the dark and the slides were stored for 30 days in the cold, after which time they were developed.

The sections were examined under oil immersion to determine the percentages of labeled and unlabeled interphase, prophase, metaphase, anaphase, and telophase nuclei. Estimates of interphase labeling and of per cent of mitoses were made from the random examination of about 1500 to 2500 nuclei per slide. The labeling of the mitotic stages was determined separately, by the random scoring of these nuclei. Slides, containing at least 3 sections each, were made from 2 to 6 different areas of every liver. Each section covered an area of about 15 to 20 mm^2. Two observers checked the estimates independently and obtained good agreement.

The determination of whether or not a cell was labeled presented little difficulty, since the number of grains over the labeled nucleus was always many times that over the background. The latter was estimated from the examination of areas equal to the area of the labeled cell. When there was uncertainty about the labeling of a cell, it was not counted. Grain counts were made in stated instances from the random scanning of sections.

Cronkite *et al.* (1) have discussed in detail some of the assumptions and problems involved in the use of radioactive labeling of DNA, combined with autoradiography, in the study of the formation of new cells. The replication time and its component parts may be estimated from the determination of the time phase waxing and waning of mitotic labeling and from interphase labeling (21). Each labeled mitosis is derived from a labeled interphase nucleus. Thus, if the curves of mitotic labeling for each cycle of new cell formation are similar and symmetrical, or reasonably so, the cell replication time may be derived from the estimation of the time interval between any 2 points comparably placed on each of the 2 adjacent curves. As examples, points might be chosen at the beginnings of mitotic labeling, at the peaks of labeling, or at their midpoints (either ascending or descending limbs). The method requires cycle after cycle of cell division, so that the group of initially labeled interphase nuclei remain in phase and continue in the division cycle. Finally, the labeled cells in each succeeding cycle must be derived from the labeled cells in the preceding cycle.

In each slide, the labeling of prophase, metaphase, anaphase, and telophase nuclei was scored separately. The generation time was estimated from the data on prophase labeling in the manner indicated above. This stage of mitosis was selected because of the ease of its recognition and because the number of grains over this stage was usually greater than that found over the other mitotic stages, at the same time after H^3TDR administration. In addition, the labeling curves for the prophase mitoses were the most symmetrical of all of the mitotic classes.

The several parts of the replication cycle were timed in the following manner. The post-DNA synthesis gap (G_2) was determined from the time of injection of H^3TDR to the earliest time of appearance of labeled prophase. Except for telophase, the time for each mitotic stage was estimated by extending the ascending limb of each curve of mitotic labeling to intercept the abscissa. From the observed differences of the respective times of interception, prophase, metaphase, and anaphase time intervals were estimated. The respective periods were measured in this manner, rather than at the level of 50 per cent labeling, because the ascending limbs of all of the mitotic class curves were not parallel.

Inasmuch as the time interval occupied by a class of mitoses is proportional to the relative frequency of that class, telophase time was estimated from the ratio:

$$\frac{\text{Anaphase time}}{\text{Per cent anaphase population}} = \frac{\text{Telophase time}}{\text{Per cent telophase population}}$$

The interval for DNA synthesis was determined from the time between the 50 per cent labeling intercepts of the ascending and descending limbs of the curve for prophase labeling. The time for the postmitotic gap (G_1) was obtained by subtracting the sum of the times for G_2 + Mitosis + DNA synthesis from the total replication time.

RESULTS

The intraperitoneally administered H^3TDR is absorbed rapidly. Peritoneal washings show that after 0.25 hour, more than 85 per cent of the H^3TDR is removed, after 0.5 hour more than 90 per cent, and after 1 hour greater than 96 per cent. A sustained and fairly stable level of interphase nuclear labeling (about 4 per cent) occurs within 0.25 hour, and persists for about 22 hours (Table I). The labeling of the respective mitotic classes shows a waxing and waning pattern (Figs. 1 to 4). The curves for prophase nuclei are the most symmetrical of all of the mitotic classes (Fig. 1). The first prophase curve shows that 23 per cent of these cells are labeled within 0.5 hour. Relatively few grains are found over prophase at

this time but the interphase nuclei are heavily labeled (Fig. 5). At 6 hours almost 100 per cent of the prophase nuclei are labeled. The descending limb falls to zero between 14 and 16 hours. Labeling begins again at 22 hours and the cycle is repeated. However, in the second cycle the peak of labeling rises to only 43 per cent at 30 hours and first curve shows some skewing of its descending limb, and this becomes more marked with the curves of each of the succeeding mitotic stages (Figs. 2 to 4). The skewing reflects the variations among the many dividing cells. Accordingly, it is to be expected that the prophase curve of the second cycle would show more skewing than that of

TABLE I

Summary of Nuclear Labeling

Time after H^3TDR	No. rats	No. nuclei scored	Pro / 100 Mit	Lab Pro / 100 Pro	Met / 100 Mit	Lab Met / 100 Met	Ana / 100 Mit	Lab Ana / 100 Ana	Tel / 100 Mit	Lab Tel / 100 Tel	Lab Mit / 100 Mit	Mit / 100 Cells	Lab Int / 100 Int
hrs.													
0.25	3	226	32.8	0	43.7	0	13.2	0	10.3	0	0	0.9	4.6
0.50	3	158	41.0	23.3	36.2	0	15.2	0	7.6	0	9.5	0.8	4.4
1	3	192	32.9	23.4	49.0	1.4	14.0	5.0	4.2	0	9.1	1.1	2.8
2	4	228	53.1	57.9	27.6	4.8	10.1	0	9.2	0	32.0	1.1	4.2
3	2	132	40.9	44.4	40.0	20.5	14.6	6.3	5.5	0	27.3	0.8	3.1
4	3	153	39.1	67.3	39.9	45.3	14.3	31.6	6.8	33.3	51.1	0.4	3.4
6	2	216	49.5	99.1	31.5	83.8	10.2	90.9	8.8	70.0	91.2	1.5	4.5
7	3	210	49.3	83.6	32.4	87.5	9.5	85.7	8.8	84.6	85.1	1.2	4.5
9	3	214	30.3	81.8	30.3	100.0	22.0	95.8	22.9	100.0	93.6	0.7	3.4
12	3	169	11.0	44.4	50.0	65.9	25.6	47.6	14.6	100.0	64.6	0.6	6.7
14	3	264	13.4	19.1	54.8	27.9	17.8	28.6	14.0	54.6	30.6	1.0	5.1
16	3	217	22.4	0	47.0	7.9	14.2	26.3	16.4	27.3	11.9	1.0	4.4
20	3	130	5.7	0	37.1	0	35.7	12.0	21.4	33.3	11.4	0.6	4.1
22	3	282	39.1	5.6	33.0	0	13.0	3.3	13.9	15.6	4.8	1.2	2.5
24	4	322	31.2	7.0	47.5	12.6	13.1	4.2	8.2	20.0	10.4	2.2	7.1
26	3	560	25.9	21.3	50.4	4.1	15.2	0	8.6	0	7.6	2.5	7.1
28	3	290	21.2	26.2	46.2	2.2	19.6	12.8	13.1	11.5	10.6	1.4	5.8
30	3	254	39.6	43.1	34.8	12.3	12.8	0	12.8	9.5	22.6	1.3	5.8
32	2	214	36.6	22.5	41.0	9.1	12.7	0	9.7	30.8	15.7	1.6	5.5
34	3	209	18.5	36.4	52.9	6.4	16.0	9.5	12.6	40.0	16.8	0.9	4.1
36	3	172	34.2	30.8	43.9	6.0	11.4	0	10.5	33.3	16.7	0.7	6.0
40	3	214	48.4	4.9	40.5	15.7	7.1	0	4.0	20.0	4.5	2.0	5.2
42	5	426	27.2	9.2	58.1	12.9	13.1	19.2	4.4	18.8	13.3	2.9	5.6
48	6	341	34.4	33.1	49.2	26.3	11.0	31.4	5.4	3.5	29.3	0.4	8.3
72	2	80	36.9	0	38.5	0	15.4	0	9.2	0	0	0.4	1.9

Abbreviations: Lab, labeled; Pro, prophase; Met, metaphase; Ana, anaphase; Tel, telophase; Mit, mitoses; Int, interphase.

then the curve declines. At 40 hours it is at its low point. The labeling per cent increases again at 42 hours, and at 48 hours about 30 per cent of the prophase mitoses are labeled.

Ideally for the estimation of the replication time and its several compartments, the curves of each cycle of prophase labeling should be symmetrical and should reach 100 per cent at their peaks. It is obvious that such is not the case. The the first (Fig. 1). The lower peak of the second curve of prophase labeling may be explained by the "loss" of some of the labeled daughter cells, in the autoradiograph, due to dilution of labeled DNA with unlabeled DNA. This loss is marked in the metaphase and anaphase second cycles (Figs. 2 and 3). In addition, since hepatic cell polyploidization is known to occur in rats of this age (19), some labeled diploid cells may be removed

from the population of dividing cells by becoming tetraploids which do not divide. This would tend to lower the peak of the second curve. The number of cells so involved cannot be estimated from these data.

The interval between the initiation of labeling in the first curve (0.5 hour) and in the second curve (22 hours) is about 21.5 hours; that between the second curve (22 hours) and the third curve (42 hours) is 20 hours. The interval between the peak of the first curve (6 hours) and that of the second curve (30 hours) is 24 hours. Finally, the midpoint of labeling of the first cycle (ascending limb) is 2 hours and the comparable ascending limb midpoint in the second curve is about 25 hours. Their difference is about 23 hours. These several estimates of replication time are similar. For the purposes of this paper, the time of about 21.5 hours will be used, because the points of initiation of labeling are more accurately determined from these data than are the other points employed for estimating this interval.

The validity of these estimates requires that the labeled cells in each successive cycle be derived from those of the preceding cycle. This sequence is not demonstrable with certainty for each cell, because the continuity of the cell line from one generation to the next cannot be established by the methods herein employed. Cinematographic records would be required, and these are not adaptable to this type of *in vivo* experiment. However, an identifying characteristic of the cells of each cycle is the grain count. This estimation is subject to error, particularly in sectioned tissue

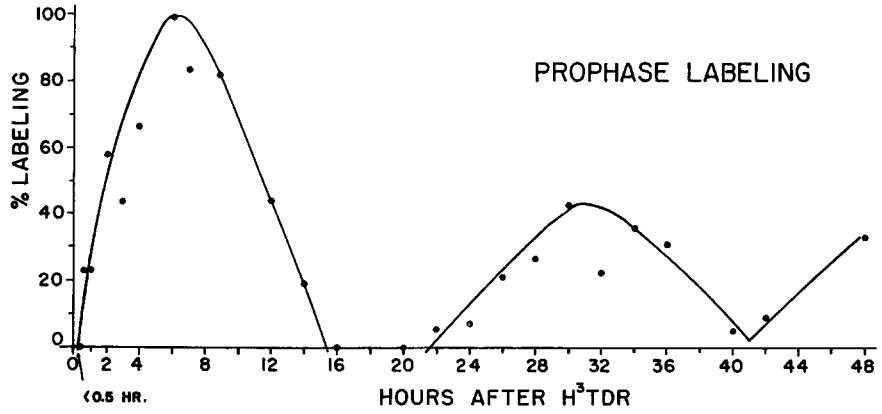

FIGURE 1

Prophase labeling after H³TDR.

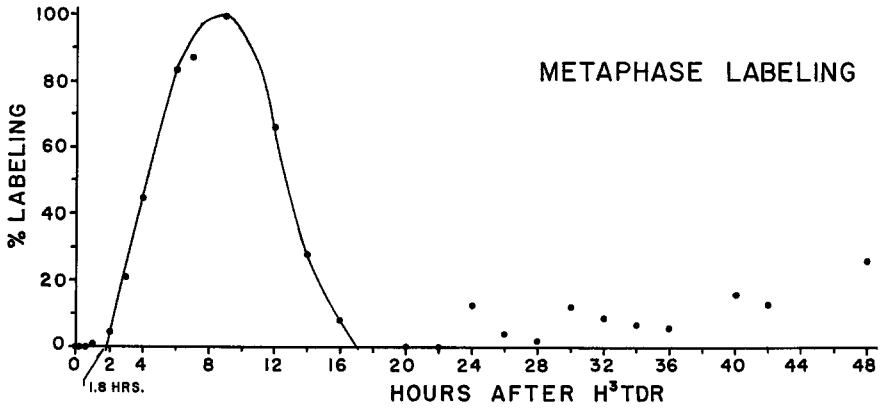

FIGURE 2

Metaphase labeling after H³TDR.

where whole nuclei are not available. If the sampling is large, useful information may, nevertheless, be obtained for the comparison of the respective populations of the several cycles. When cell division has occurred the daughter cells will have fewer grains than the parent cells, ideally 50 per cent fewer. Accordingly, grain counts have been made of interphase and prophase nuclei at the peaks of prophase labeling in each of 3 cycles (Table II, Figs. 6 to 9). These times were chosen to assure the sampling of the largest numbers of labeled prophase nuclei. In addition, grain counts have been made of interphase nuclei 72 hours after H^3TDR administration. At this time no prophase nuclei are labeled.

There is a significant decrease of the mean grain counts of interphase nuclei with each successive cycle. At 72 hours there is a further decrease indicating that this is part of a fourth cycle. The means of each group are probably weighted on the high side, because many of the lightly labeled nuclei are "lost" in the autoradiograph. There is narrowing of the range of grain counts with each successive cycle.

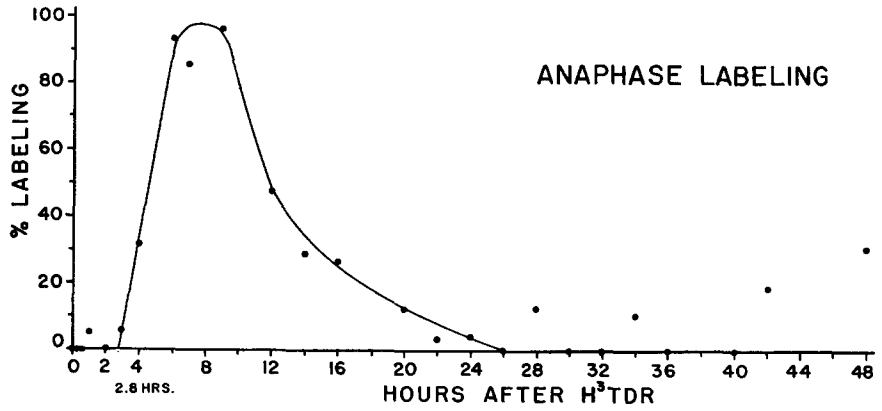

FIGURE 3

Anaphase labeling after H^3TDR.

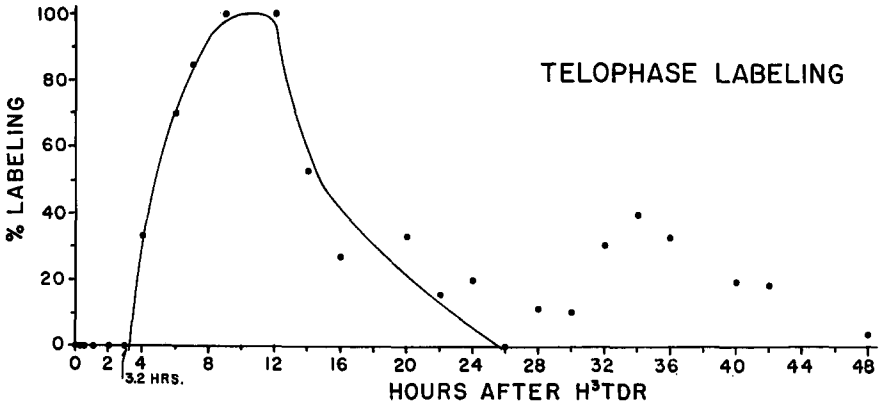

FIGURE 4

Telophase labeling after H^3TDR.

Theoretically, the prophase grain counts should be the same as those of their precursor interphase nuclei. However, there is probably a larger "loss" of prophase cells in the autoradiograph because of the more uneven distribution of the radioactive material, in comparison with that of the interphase nuclei. Nonetheless, there is a decrease in prophase grain counts with each cycle. By 72 hours there are no labeled prophase nuclei detectable in the autoradiographs.

These results support the concept that the labeled cells of the second, third, and fourth cycles are derived from parent cells of the respective preceding generation. The curve which rises from zero and then returns to that level indicates that the labeled cells are passing through cell division in phase.

The DNA synthesis time is estimated from the first prophase labeling curve and is about 9 hours (Fig. 1). Since prophase labeling appears 0.5

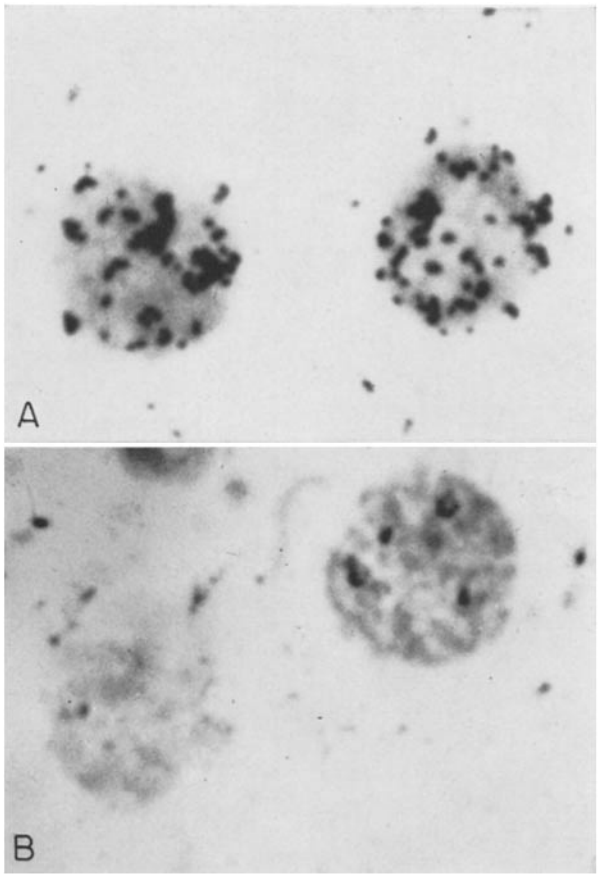

FIGURE 5

Labeled interphase (*A*) and prophase (*B*), 0.5 hour after H^3TDR. × 1125.

TABLE II

Summary of Interphase and Prophase Grain Counts

Group	Interphase				Prophase				Background (per average nuclear area)
	No. rats	No. nuclei counted	Mean	SE Mean	No. rats	No. nuclei counted	Mean	SE Mean	
6–7 hours after H^3TDR	3	300	38.7	0.8	3	59	29.8	1.5	2
30 hours after H^3TDR	3	300	27.0	0.8	3	52	7.4	0.3	<1
48 hours after H^3TDR	3	300	16.0	0.6	3	63	3.5	0.2	0
72 hours after H^3TDR	3	300	11.1	0.04	—	—	—	—	<1

hour after H^3TDR administration but not at 0.25 hour, the former is an approximation of the postsynthesis gap (G_2).

The estimation of the mitotic intervals from the intercepts of the abscissae yields the following results: prophase, 1.8 minus 0.5 (G_2) = 1.3 hours; metaphase, 2.8 minus 1.8 = 1.0 hour; anaphase, 3.2 minus 2.8 = 0.4 hour. Telophase is calculated from the population ratios of anaphase and telophase. The respective population percentages were derived from the distribution of 1729 mitoses scored during the first 9 hours of the first cycle. The respective means and their standard errors are as follows: prophase, 41.0 ±

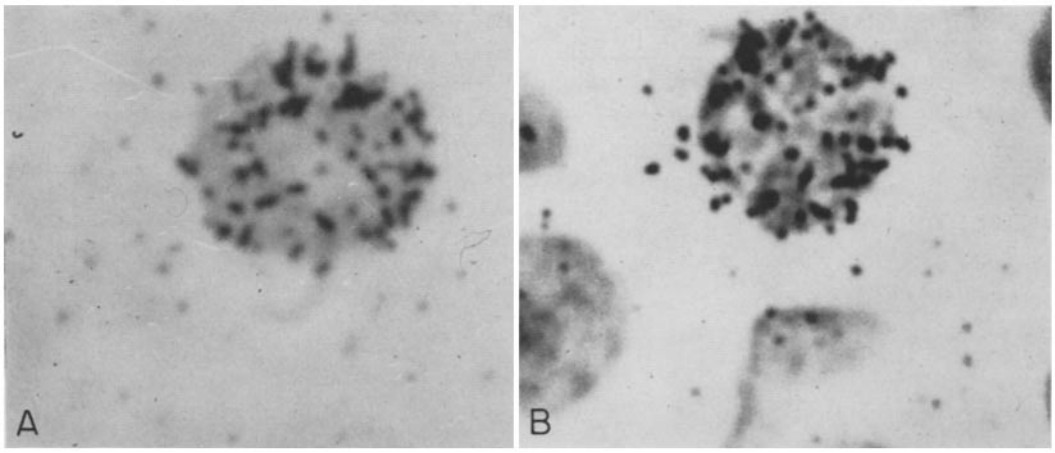

FIGURE 6

Labeled interphase (*A*) and prophase (*B*), 6 and 7 hours after H^3TDR. × 1125.

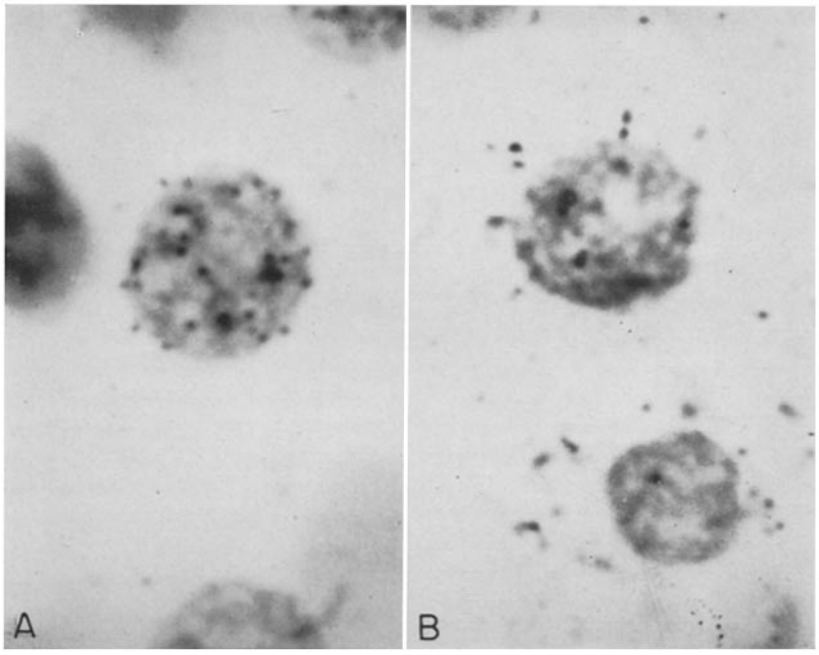

FIGURE 7

Labeled interphase (*A*) and prophase (*B*), 30 hours after H^3TDR. × 1125.

3.1; metaphase, 36.7 ± 2.6; anaphase, 13.7 ± 1.6; and telophase, 9.3 ± 2.1. From these data the time for telophase is estimated as 0.3 hour.

The ratios of the observed time intervals for the several stages of mitosis are in excellent agreement with those of the observed respective population percentages. The ratio of prophase time to metaphase time is 1.3 and the ratio of their populations is 1.1. The ratio of prophase time to anaphase time is 3.2 and their populations ratio is 3.0. For prophase to telophase the time intervals ratio is 4.3 and the populations ratio, 4.4. The closeness of these figures is a check on their accuracy.

DISCUSSION

The replication time and pattern of the liver cell in the growing rat have been studied by the use of H^3TDR labeling of DNA and autoradiography. The results show that at any one time only about 4 per cent of the liver cells are engaged in DNA synthesis preliminary to cell division. These cells appear to alternate with waves of other cells, similarly involved, since the per cent of mitoses remains fairly stable but the proportion of mitotic labeling rises and falls. The replication time is about 21.5 hours. DNA synthesis occupies about 9 hours, a time similar to the 8 hours found by

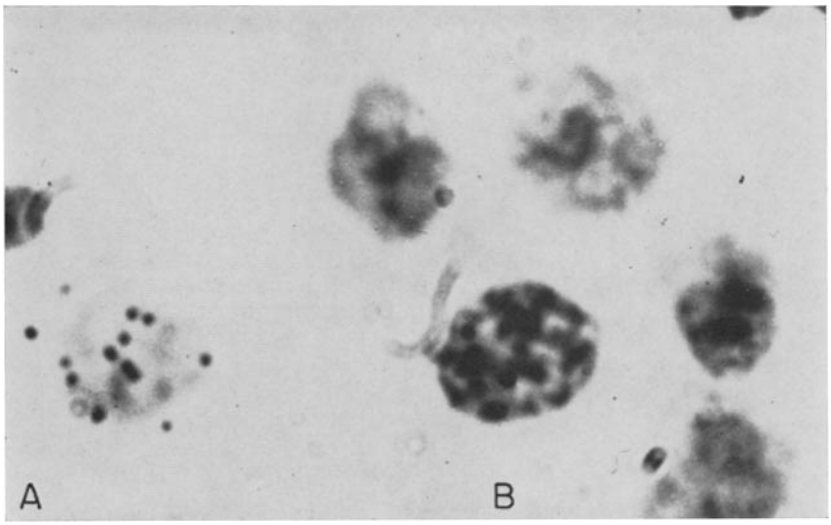

FIGURE 8

Labeled interphase (*A*) and prophase (*B*), 48 hours after H^3TDR. × 1125.

The value for the postmitotic interval (G_1), 9 hours, is determined by subtracting the sum of the times for DNA synthesis, G_2, and mitosis from the replication time.

There is a statistically significant rise in the per cent of interphase labeling during the second 24 hours after H^3TDR injection. This is in keeping with the occurrence of cell division. There is a sharp decline in labeling at 72 hours (Table I). This phenomenon indicates dilution of labeled DNA with unlabeled DNA and the "loss" of labeled cells in the autoradiograph. The mitotic rates remain relatively unchanged except for increases above 2 per cent at 24, 26, and 42 hours (Table I). After 24 hours there is a tendency for metaphase nuclei to exceed prophase in number.

Looney (13) in the regenerating liver of the adult rat after partial hepatectomy. The post-DNA synthesis time (G_2) is 0.5 hour or less, and mitosis occupies about 3.0 hours with the following times for its stages: prophase, 1.3 hours; metaphase, 1.0 hour; anaphase, 0.4 hour; and telophase, 0.3 hour. The postmitotic interval (G_1) is about 9.0 hours. There is probably much variation in these time intervals, within this dividing population.

Second and third cycles of cell division follow immediately after the first, and the replication time of the second cycle is about 20 hours. This is close to the 21.5 hours estimate for the replication time of the first cycle. As judged by the decrease in interphase grain counts at 72 hours, it

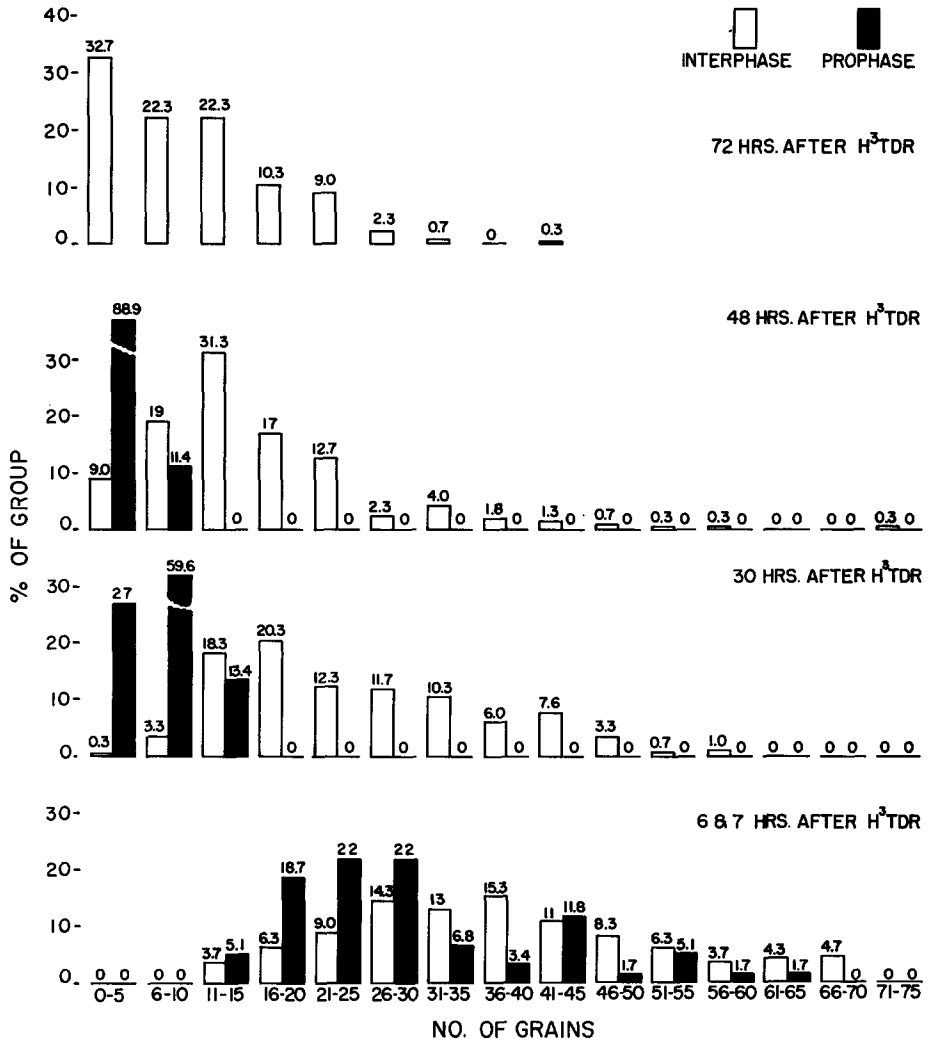

FIGURE 9

Frequency distribution of grain counts in interphase and prophase nuclei during cycles of cell division.

is likely that a fourth cycle of cell division has occurred.

One may conclude that, in the growing animal, new liver cell formation is an orderly phenomenon confined to a very small population of cells. From the formula presented by Kisieleski *et al.* (8), the "proliferating pool" may be derived. Thus

$$P_c = \frac{I \times G_t}{T_s},$$

where P_c = proliferating pool,

G_t = replication time,

t_s = DNA synthesis time,

I = per cent labeled interphase nuclei.

When the formula is applied to these data, the proliferating pool is estimated at about 10 per cent. These findings indicate that only certain liver cells are concerned with organ growth. It has not been possible to characterize these cells either by their appearance or geographic distribution within the liver lobule. It is possible that a new group of cells takes over after one group has been through several divisions. Further work is needed to

determine whether this is the case, as well as whether growth in other organs follows a similar pattern. Beyond the third cycle, autoradiographic studies of replication time are useless because of the "loss" of labeled cells by dilution. Increasing the dose of the label carries the risk of changing the ploidy pattern of the liver cell nuclei (19).

These findings were unexpected, since it had been anticipated that during growth, in an organ whose cells were not being lost by exfoliation (gut, skin) or by lysis (blood cells), the recently divided cells might remain in a resting state throughout a particular life span. A new group of cells might take up cell division thereafter. Indeed, by assuming this to be the case, the life span of liver cells has been estimated by dividing the per cent of labeled interphase nuclei into 100 (14). While this may be valid in fully grown animals, it is not so in growing animals. Unpublished data (20) from this laboratory show that in the mature rat of 6 months of age, interphase labeling is less than 0.2 per cent, 2 hours after the administration of 50 μc H^3TDR. Mitoses are equally sparse. From these observations it seems reasonable to conclude that, after full growth has been achieved, there is a very low level of liver cell turnover and that the life span of the liver cell is similar to the remaining life span of the animal.

A comparison of the data in this report with other data on replicating normal mammalian cells, studied *in vivo*, is given in Table III. Blood and sperm cells have been omitted since these have a more complex origin from stem cells, and pass through several developmental stages.

There is a close similarity of the replication times and of their component intervals of the gut lining cells (11, 12, 21). The skin cells have a remarkably long replication time (23), the major part of which is G_1. Considerable variation occurs in the time intervals of the bone cells (28) and, again, the major time compartment is G_1. Mitosis time and G_2 are relatively short for most of the cells. It is noteworthy that DNA synthesis is of similar duration in cells with as widely different replication times as the mouse colon cell (11), with a replication time of 16 hours, and the periosteal cell of the growing rat, with a replication time of 114 hours (28). The major difference between their replication times is in the respective G_1 periods.

TABLE III

Replication Times of Mammalian Cells (In Vivo) (In Hours)

Cell type	Replication time	DNA synthesis	G_2	Mitosis	G_1	Reference
Mouse jejunal epithelial cell	18.75	7.5	about 0.75	1.0	9.5	(21)
Mouse duodenal mucosal cell	11.5	—		1.38	—	(4)
Mouse ear epidermis cell	24.4 days	30	4.6	3.8	>22 da.	(23)
Mouse colon epithelial cell	16	6.5	1.5		8	(11)
Human colon epithelial cell	24	11–14	0.25+	—	—	(12)
Growing rat, metaphysis cell	36	8	1.0–1.5	1.5	25	
Growing rat, endosteum cell	57	8	1.0–1.5	2.0–2.5	47	
Growing rat, periosteum cell	114	8	1.5–2.0	1.5–2.0	102	(28)
Growing rat, liver cell	21.5	9	about 0.5	3.0	9.0	(this report)

Unfortunately, the results published to date do not permit one to judge whether, in other mammalian tissues, the same group of cells divides time after time. As derived from the data on mouse jejunal (21) and colon (11) mucosal cells as well as on human colon cells (12), the curve of mitotic labeling suggests a sinusoidal pattern which is consistent with a second cycle of division in crypt cells. It would be of interest to extend this information to dividing cells of other organs.

The question arises as to whether the labeling dose produces changes in the cell behavior, inasmuch as a dose of H^3TDR twice as large as

that used here caused changes in the nuclear ploidy patterns (19). Fry *et al.* (4), in studies on the mouse duodenal lining cells, showed that a label dose of 1 μc/gm, similar to the one employed here, did not alter either the mitotic rate or the replication time. The data in the present report indicate that the first 2 labeled cycles have similar replication times. The mitotic rate remains fairly stable throughout the period studied, except for the increases at 24, 26, and 42 hours. In addition, there is more variability of frequency distribution of the several mitotic types following the first 9 hours. Whether or not these changes are of significance in relation to effects of the radioactive label is not clear. The possibility of H^3TDR-induced cell change, at this dosage level, has not been excluded.

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