

## Some Characteristics of DNA Synthesis and the Mitotic Cycle in Ehrlich Ascites Tumor Cells\*

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### ABSTRACT

*In vivo* studies of Ehrlich ascites tumor cells during the first 5 days of growth in peritoneal cavities of mice consisted of the following:

1. Determination of growth curves by direct enumeration of cells.
2. Estimation of the duration of each phase of the mitotic cycle based on incidence of cells in different phases.
3. Radioautographic studies to determine the proportion of cells in different phases of the mitotic cycle that incorporate tritiated thymidine during a single brief exposure to this precursor of DNA.
4. Estimation of the rate of incorporation of tritiated thymidine at different times during the period of DNA synthesis by comparison of mean grain counts over nuclei in radioautographs at different times following exposure to tritiated thymidine.

The assumptions underlying these experiments and our observations concerning the duration of the period of DNA synthesis and its relation to the mitotic cycle are discussed. It is concluded that DNA synthesis is continuous, occupying a period of 8.5 hours during the interphase and that the average rate of synthesis is approximately constant.

### INTRODUCTION

It is generally known that the synthesis of DNA is intimately related to or may, indeed, direct the process of cell division. Quantitative microspectrophotometric studies, notably those of Swift (1), Pasteels and Lison (2), Richards (3, 4), and Walker (5, 6) have shown that DNA synthesis occurs during a limited portion of the interphase prior to the onset of mitosis in mammalian cells. Richards, Walker, and Deeley (4) also have calculated synthesis curves from the frequency distribution of amounts of DNA found in interphase cells. The observations of Klein, Klein, and Klein (7) based on chemical analyses of DNA in cells provided

some evidence, however, that synthesis may extend into the metaphase.

Radioautographic studies by Howard and Pelc (8), Lajtha (9), Lajtha, Oliver, and Ellis (10), and the quantitative studies of Hornsey and Howard (11) using  $C^{14}$  or  $P^{32}$  and radioautographic techniques with a variety of cell types including ascites tumor cells, all agree in respect to the location of the period of synthesis in interphase ending prior to mitosis. On the other hand, conclusive evidence that the process is uninterrupted and that the rate is constant we believe remains to be presented.

More precise measurements of timing and additional evidence concerning the rate and continuity of DNA synthesis have been obtained in the studies reported here using a combination of methods which include employing a relatively short labeling time with tritiated thymidine as the DNA precursor. Precision of this order is essential for study of the growth cycles of cell types in sections of complicated tissues.

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The experiments reported here were carried out with ascites tumor cells, as part of a study of the temporal relationships of DNA synthesis to the growth cycle in cells of mammalian tissues.

From a combination of data obtained from cytologic studies and measurements of radioactivity of cells in different phases of the mitotic cycle at various times after a short labeling period with tritiated thymidine, estimates of the following were obtained:

1. The mean generation time.
2. The duration of each phase of the mitotic cycle.
3. The duration of the period of DNA synthesis and its relation to the mitotic cycle.
4. The constancy and rate of DNA synthesis within the period of synthesis.

#### Materials and Methods

*Ehrlich Ascites Tumor Cells and Host Mice.*—The Ehrlich ascites tumor cells (E.A.T. cells) employed in all experiments were mononuclear cells. The mean area of individual cells in smears was 548 (s.d.<sup>1</sup> 53) square micra and the mean number of chromosomes was 51.5 (s.d. 13.4). The frequency distribution of chromosome numbers was not bimodal in contrast to certain other strains of ascites tumor cells (12, 13). The cell line was obtained from the laboratories of Dr. Charles Heidelberger through the kindness of Dr. Lois Griesbach and subsequently maintained in a hybrid strain of Swiss white mice by weekly transfer of approximately  $2 \times 10^6$  cells. Cells used for all experiments were obtained fresh from populations in their 4th day of growth in the peritoneal cavity. Dilutions suitable for injection were made in sterile cell-free ascitic fluid.

*Measurement of Growth of the Tumor Cell Population—Experiment 1.*—Sixteen mice received a single intraperitoneal injection of  $1.8 \times 10^6$  E.A.T. cells. At 16, 32, 64, and 128 hours after inoculation, the total number of E.A.T. cells in the peritoneal cavity of each of 4 mice was determined.

The total volume of ascitic fluid was measured both by the Evans blue dilution method described by Patt *et al.* (14) and by quantitatively rinsing and draining the cavity of the killed mouse.

On each sample total cell counts were done on the fresh ascitic fluid diluted in 1.0 per cent sodium chloride and counts of nucleated cells were done in dilutions of dilute acetic acid (erythrocytes lysed). Approximately 1,000 cells were counted over 8 square mm. in a Spencer bright-line hemocytometer by one and the same observer.

Differential counts of tumor cells, leukocytes, erythrocytes, and macrophages were made on wet prepara-

tions of the ascitic fluid fixed and stained in 4 parts of a solution of 1 per cent aceto-orcein prepared as described by Ritter (15).

The extent of invasion of lymph vessels and tissue spaces by tumor cells was assessed in sections of omentum, abdominal wall, pancreas, spleen, and mesenteric lymph nodes.

Estimations of cell viability were made by Schrek's method (16) and by direct observations with phase microscopy.

*Counting of Phases of the Mitotic Cycle—Experiment 2.*—Samples of the tumor cell population of 20 mice inoculated with  $1.8 \times 10^6$  cells were aspirated at successive intervals throughout the phase of logarithmic growth. The freshly aspirated ascitic fluid was mixed with four parts of the solution of aceto-orcein. A drop of this wet, fixed, and stained suspension on a glass slide was covered with a coverglass and examined directly with the light microscope. Chromatin patterns were clearly demonstrated in these preparations and cells in different phases of the mitotic cycle were readily distinguished. The proportion of cells in different phases of the mitotic cycle was determined by differential counting of 500 cells from each sample.

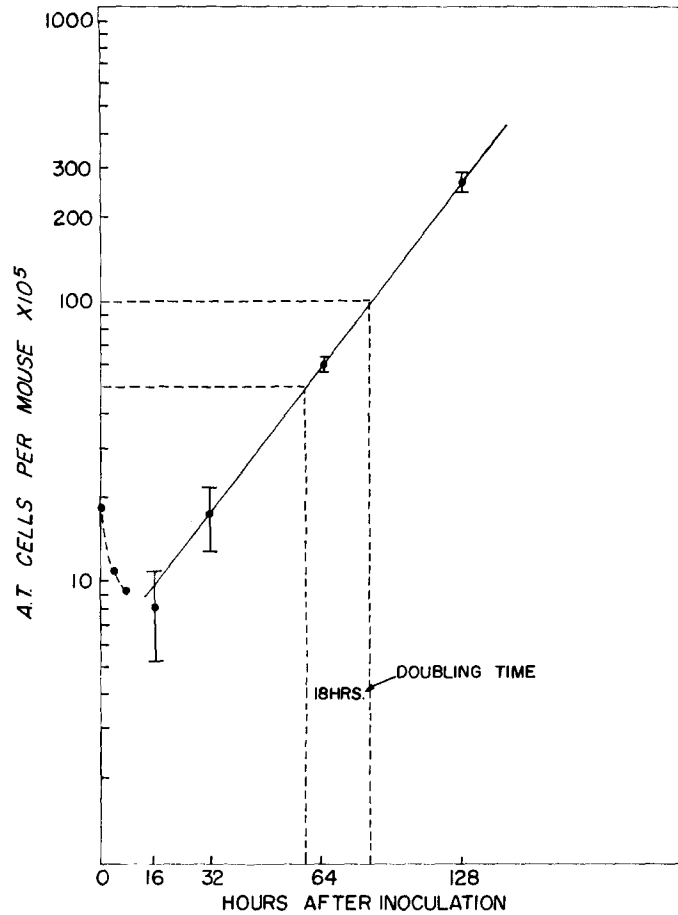
*Incorporation of Tritiated Thymidine ( $H^3TDR$ )—Experiment 3.*—The  $H^3TDR$  used in all experiments had a specific activity of 360 curies per mole, was labeled in the pyrimidine ring and was free of labeled impurities and labile tritium atoms. It was prepared, purified, and supplied by Schwarz Laboratories. Dilutions suitable for injection were made in 1 per cent sodium chloride.

In order to expose a population of E.A.T. cells to  $H^3TDR$ , the material was injected intraperitoneally, the injection site was coated with collodion and the abdomen of the mouse was massaged to insure rapid mixing with the ascitic fluid. Samples of the population were removed by either careful aspiration with a small needle or by opening the abdominal cavity, draining, and rinsing it with a glass pipette. Smears of the cell suspensions were made on glass slides previously coated with a thin, dry film of chrome alum gelatin. Smears were fixed while wet in formaldehyde vapor, then quickly air-dried. Radioautographs of these were prepared by the method of Doniach and Pelc (17) using AR10 stripping film obtained from Kodak, Ltd., London. After exposing the film to the smear for either 6 or 19 days under standard, dry conditions the preparations were developed in D19 for 10 minutes, processed further under standard conditions, and finally stained through the gelatin film.

#### OBSERVATIONS

*Characteristics of the Growth Curve—Experiment 1.*—Changes in the total number of E.A.T. cells in the peritoneal cavity of mice at different times following an inoculation of  $1.8 \times 10^6$  tumor cells (Experiment 1) are shown in Text-fig. 1. That this

<sup>1</sup> Standard Deviation.



TEXT-FIG. 1. Growth curve of ascites tumor cells.

time curve represents a true growth curve is strongly suggested by the observation that all cells appear to remain viable throughout the first 128 hours of the growth period. Moreover, studies of histologic sections reveal no loss of tumor cells from the peritoneal cavity by tumor cell invasion of lymph vessels, veins, or tissue spaces.

Since the rate of growth of the population is exponential between 16 and 128 hours, the observed doubling time in this interval is equivalent to the average life span of all cells of the population. As indicated in Text-fig. 1, this is calculated to be 18 hours under the conditions of these experiments.

Measurement of the total number of cells in mouse peritoneal cavity within 16 hours after the inoculation of tumor cells is highly inaccurate due to the difficulty of precisely measuring the small volume of fluid in the cavity. After 128 hours accuracy of counts decreases progressively due to

the adherence of layers of tumor cells to peritoneal surfaces, thus making quantitative recovery impossible (Fig. 3). After 6 or 7 days total population counts are entirely unreliable because many cells are dead or dying and others are invading abdominal structures (Figs. 4 and 5).

*Duration of Phases of the Mitotic Cycle—Experiment 2.*—Mean values for the numbers of EAT cells in different phases of the division cycle at different times following inoculation of the peritoneal cavity with tumor cells are shown in Table I. Throughout the logarithmic phase of growth the mitotic index and the proportion of cells in different phases of the division cycle show no significant variation. Accurate values for the incidence of each phase in the mitotic cycle can thus be assigned.

When a population of cells, whose mitotic index is constant, increases exponentially without loss of cells from the population, the incidence of cells in a

TABLE I  
Incidence of Phases of Mitotic Cycle During Log Phase of Growth

Time	No. of mice	Interphase		Prophase		Metaphase		Anaphase		Telophase	
hrs.		per cent		per cent		per cent		per cent		per cent	
4	4	79.3		18.1		1.0		0.6		1.0	
32	4	78.0		18.4		1.1		0.8		1.7	
64	4	78.1		17.5		1.7		1.1		1.7	
128	4	77.9		18.5		1.2		0.9		1.5	
		Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
4 to 128	16	78.2%	0.68	18.1%	0.49	1.3%	0.34	0.9%	0.24	1.5%	0.34

given phase of the mitotic cycle is a measure of the fraction of the life span occupied by that particular phase of the division cycle. If, at division, one of the two daughter cells were removed from the population then the proportion of cells in a given phase at any time would be directly proportional to the length of time the cell spends in that morphological phase. In a growing population the duration of phase is, however, not directly proportional to incidence of the phase since upon division, two young daughter cells replace one old parent cell. Thus, allowance must be made for the unequal size of the age groups in computing the duration of interphase, prophase, etc., from the incidence of each. This was done in the following manner:

Let the fraction of all the cells that are in the phases of growth between division and a particular phase of growth be called the cumulative phase index (C.P.I.). Let  $X$  be the fraction of time of the division cycle spent since the last division up to the particular phase of growth in question. As indicated above, these two quantities (both ratios) would be equal if net growth did not take place. In growing populations the relationship between the two is:

$$C.P.I. = 2(1 - 2^{-X}) \quad (1)$$

and is plotted in Text-fig. 2. This relationship may be readily derived as follows: let  $D_o$  be the division rate at the time of sampling. Since the population has been growing exponentially, the division rate  $t$  hours earlier will be given by

$$D = D_o 2^{-t/T} \quad (2)$$

in which  $T$  is the doubling time, also in hours. Now the rate at which cells are entering the  $t^{\text{th}}$  hour of the mitotic clock is twice the rate,  $\left(\frac{dN}{dt}\right)$ , that cells were dividing  $t$  hours before, when these cells were produced by mitosis:

$$\frac{dN}{dt} = 2D_o 2^{-t/T} \quad (3)$$

By integrating this expression from  $t = 0$  to  $t = \theta$ , the total number of cells between division and the  $\theta^{\text{th}}$  hour of the mitotic clock,  $N_\theta$ , is obtained:

$$N_\theta = \int_0^\theta 2D_o 2^{-t/T} dt = 2 \frac{D_o T}{\ln 2} [1 - 2^{-\theta/T}] \quad (4)$$

If  $\theta$  is set equal to  $T$ , Equation 4 becomes:

$$N_T = 2 \frac{D_o T}{\ln 2} [1 - 2^{-1}] = \frac{D_o T}{\ln 2} \quad (5)$$

in which  $N_T$  is the number of cells in all phases of the mitotic cycle, *i.e.*, the total number of cells in the population. Dividing (4) by (5) the cumulative phase index (C.P.I.) =  $\frac{N_\theta}{N_T}$  is obtained:

$$C.P.I. = 2[1 - 2^{-\theta/T}] \quad (6)$$

Replacing  $\theta/T$  by  $X$ , the relationship given in Equation 1 is obtained.

Frequently, on the other hand, interest is directed at events ending with cell division; if  $R$  is defined as the fraction of cells from a certain stage up through the completion of cell division, then  $C.P.I.$  may be replaced by  $1 - R$ , and if  $Y = (1 - X)$ , is defined as fraction of time occupied by these stages, then

$$[1 - R] = 2[1 - 2^{-1+Y}] \quad (7)$$

then

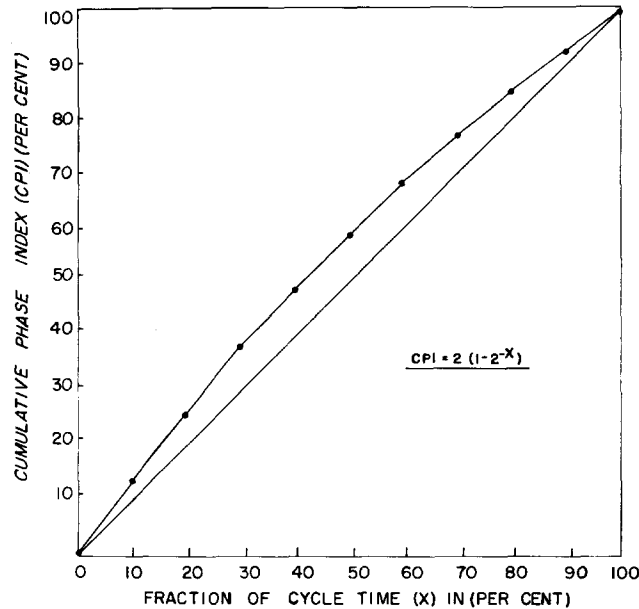
$$-1 - R = -2 \frac{2^Y}{2} \quad (8)$$

$$1 + R = 2^Y \quad (9)$$

or

$$Y \ln 2 = \ln(1 + R) \quad (10)$$

Another formula for this case has been quoted by Hughes (18) as



TEXT-FIG. 2. Relation of phase index to time spent in phase for populations growing exponentially.

$$Y \ln 2 = \ln \left[ \frac{1 + 2R}{1 + R} \right]. \quad (11)$$

It can readily be seen that this formula is not applicable to the entire division cycle, for if  $R = 1$  then  $Y = 1$ , and Equation 11 becomes  $\ln 2 = \ln \frac{3}{2}$  whereas Equation 10 becomes  $\ln 2 = \ln 2$ .

Equation 11 is very nearly correct for small values of  $R$  and becomes  $Y = 1.44R$ , as does Equation 10 when  $R$  is very small; *i.e.*, when the phase under consideration occupies only the last small proportion of the entire mitotic cycle.

The underlying assumptions in this derivation are that (a) cells are randomly entering mitosis (no synchrony) and (b) the fraction of time spent in each phase is the same for all cells even though the doubling time for the individual cells is not the same.

The equation given has been previously derived by Scherbaum and Rasch (19). Our derivation is presented since it is accomplished with simpler mathematical tools.

Text-fig. 2 allows the rapid computation of the fraction of the cycle to the beginning and to the end of a particular morphological phase and, hence, the duration of that phase. In Table II values for the *C.P.I.* and duration of different phases in the mitotic cycle are shown.

*Nuclear Incorporation of H<sup>3</sup>TDR—Experiment 3.*—When a population of E.A.T. cells, 72 hours of

age, is exposed to a single intraperitoneal injection of H<sup>3</sup>TDR of from 1 to 32 microcuries, some of the cells incorporate the thymidine into their nuclei. The tritium is manifest in radioautographs by the presence of reduced silver grains in the film overlying the nuclear chromatin as is shown in Figs. 1 and 2. These are hereinafter referred to as "labeled cells."

*1. Proportion of the E. A. T. Cell Population Incorporating H<sup>3</sup>TDR.*—Six mice received a single I.P. injection of from 1 to 32 microcuries of H<sup>3</sup>TDR, 72 hours after inoculation with tumor cells. Samples from each mouse were taken at intervals of  $\frac{1}{2}$ , 1, 2, 4, 8, and 24 hours after the injection of H<sup>3</sup>TDR. When 8 or more microcuries of H<sup>3</sup>TDR were injected into the peritoneal cavity, tumor cell nuclei incorporating thymidine, in a sample removed 1 hour after injection, were so heavily labeled that grains over nuclei in radio-autographs exposed for 6 days overlap and accurate grain counts are impossible. Chromatin patterns in these cells are obscured by the large numbers of reduced silver grains overlying nuclei so that phases of the mitotic cycle are not readily identified (Fig. 1). Background under these conditions is insignificant, and "labeled" and "unlabeled" cells are easily distinguished.

In samples from mice receiving 1 to 4 microcuries careful analysis was required to distinguish labeled from unlabeled cells. There was no significant dif-

TABLE II  
Duration of Phases in the Mitotic Cycle

Phase	Phase incidence	S.E.	Cumulative phase index (C.P.I.)	Fractions of life span from division to end of phase	Duration of phase	Time from beginning of cycle
	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	<i>hrs.</i>	<i>hrs.</i>
Interphase.....	78.2	0.68	78.2	71.5	12.9	12.9
Prophase.....	18.1	0.49	96.3	94.6	4.16	17.06
Metaphase.....	1.3	0.34	97.6	96.7	0.38	17.44
Anaphase.....	0.9	0.24	98.5	98.0	0.23	17.67
Telophase.....	1.5	0.34	100.0	100.0	0.36	18.03

ference, however, in the incidence of labeled cells when the dose of H<sup>3</sup>TDR injected was over the entire range 1 to 32 microcuries, and, as seen in Table III, no significant difference between samples taken at different times.

2. *Incidence of Labeled Cells in Different Phases of the Mitotic Cycle.*—When H<sup>3</sup>TDR in the range of 1 to 4 microcuries was injected into the peritoneal cavities of 3 mice with tumor populations aged 72 hours, grain counts over tumor cell nuclei in radioautographs exposed for 19 days ranged from 0 to 75. Grains were discrete and did not obscure the chromatin patterns (Fig. 2). The phase of division cycle in these cells can, therefore, be correlated with the numbers of grains overlying the nuclei. Samples of the tumor cell populations were taken at ½, 1, 2, 4, 8, and 24 hours.

Text-fig. 3 shows the proportion of labeled cells in each phase of the mitotic cycle at different intervals following the injection of H<sup>3</sup>TDR.

In each of 108 radioautographs prepared from 30-minute and 1-hour samples, all the cells labeled were in the interphase; *i.e.*, no cells in the process of division had incorporated H<sup>3</sup>TDR during this 1-hour period. Labeled cells in early prophase are first encountered in small numbers in those samples removed 2 hours after injection of H<sup>3</sup>TDR. Labeled cells in metaphase are first seen in the 4-hour samples which, however, contain no labeled cells in anaphase or telophase. These appear later, but sampling times were too far apart to accurately assess when they first appear.

3. *Grain Counts over Nuclei.*—Table IV shows the mean grain counts over all labeled nuclei at different times following exposure of a mouse to H<sup>3</sup>TDR. The numbers of grains overlying nuclei do not increase significantly after 30-minute exposures to H<sup>3</sup>TDR, signifying that within 30 minutes after the injection all of the H<sup>3</sup>TDR in the

TABLE III  
Incidence of Labeled Cells at Different Times Following Injection of H<sup>3</sup>TDR

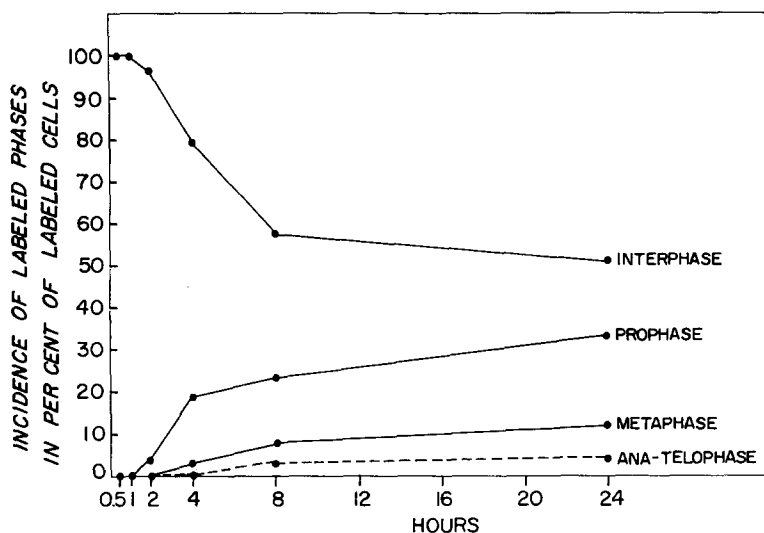
Time after injection	Per cent labeled	S.E.
<i>hrs.</i>		
½	45.4	9.3*
1	48.6	13.0*
2	51.5	9.6*
4	43.6	4.6*
8	47.1	5.7*
24	44.7	8.8*
All	46.8	3.1‡

\* Standard deviation of means obtained by different observers, each counting approximately 500 cells.

‡ Standard error of all means.

ascitic fluid has been either incorporated into the nuclei or metabolized. Thus the labeling period is, at most, one thirty-sixth or 2.7 per cent of the generation time of 18 hours. The mean number of grains over nuclei in samples taken 24 hours after injection of H<sup>3</sup>TDR is approximately one-half the value obtained in samples taken after 1 hour indicating that all cells have undergone one cell division within 24 hours. These observations show that label is taken up during interphase, remains intact through mitosis, and is divided between the daughter cells.

Table V shows that the mean number of grains overlying nuclei in samples taken at 2 hours (all interphase nuclei) does not vary significantly from the grain count over labeled prophase nuclei in samples taken 4 and 8 hours following the exposure to H<sup>3</sup>TDR. The relation of this observation to the rate of DNA synthesis is discussed below.



TEXT-FIG. 3. Movement of labeled cells through mitosis.

TABLE IV  
Mean Grain Counts over Nuclei of Labeled Cells at Different Times Following Injection of  $H^3TDR$

Time after injection $H^3TDR$	Labeled cells counted	Total No. grains	Mean No. grains
hrs.			
$\frac{1}{2}$	177	2172	12.27
1	191	2537	13.28
2	192	2648	13.79
4	192	2390	12.44
8	143	1929	13.48
24	135	903	6.69

TABLE V  
Mean Grain Counts over Labeled Nuclei in Different Phases of the Mitotic Cycle at Different Times Following Injection of  $H^3TDR$

Mouse	Dose	Interphase at 2 hrs.		Prophase at 4 hrs.		Prophase at 8 hrs.	
		Mean	S.E.*	Mean	S.E.	Mean	S.E.
	$\mu c.$						
2	2	33.6	7.6	41.8	9.8	33.8	8.4
3	4	34.0	7.1	44.1	13.0	36.2	9.2
4	8	64.6	15.0	63.8	19.1	53.4	15.8

\* Standard deviation of different observer's means.

#### DISCUSSION

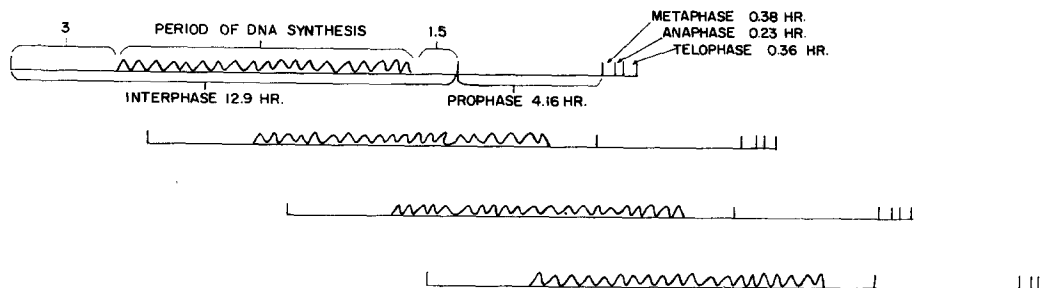
Formulation of the events of an average mitotic cycle (Text-fig. 4) depends upon the following seven premises:

1. The cell population is not synchronized.
2. All cells in the population continue to grow and divide.
3. Tritiated thymidine is stably and exclusively incorporated into DNA being newly synthesized.
4. The isotope is not lost from the nucleus except through redistribution of chromatin at division.
5. The period of exposure to  $H^3TDR$  is brief relative to the period of DNA synthesis.
6. The intensity of incorporation is proportional to the intensity of DNA synthesis and the proportion of the period of synthesis occupied during incorporation of the label.
7. The absence of labeling indicates the absence of DNA synthesis.

Strong evidence supports the validity of these premises. The data presented showing that the population increases exponentially for a time, and that the mitotic index does not vary significantly during this time argues strongly that this is a homogeneous asynchronously dividing population.

The fact that the mean grain counts over nuclei of all labeled cells is approximately halved after all cells in the population have undergone one, and only one, division strongly suggests that all but a small percentage of the cell population, proceeds through the complete growth cycle.

The studies of Reichard and Estborn (20) and Friedkin *et al.* (21) have established that thymidine is incorporated exclusively, efficiently, and stably into new DNA being synthesized. Davidson and Smellie (22) have further shown that this is the case with  $H^3TDR$  in Ehrlich ascites tumor cells.



TEXT-FIG. 4. Diagram of relation of DNA synthesis to mitotic cycle in Ehrlich ascites tumor cells.

That  $H^3TDR$  so incorporated is not lost from nuclei except through cell death is strongly confirmed by the studies of Taylor, Woods, and Hughes (23) in their study of chromosomal replication.

Our data concerning length of the labeling period (premise 5) are supported by those of Hughes *et al.* (24) indicating that the effective concentration of  $H^3TDR$ , injected intraperitoneally, is available to cells for a period of less than 1 hour. Thus, the mean grain count over all labeled nuclei 1 hour following exposure to  $H^3TDR$  is an expression of the mean rate of DNA synthesis throughout the entire period. From Text-fig. 4 it can be seen that labeled cells in prophase from samples taken 4 hours after exposure to  $H^3TDR$  were labeled near the end of their synthetic period. On the other hand, labeled cells in prophase from samples taken 4 hours later, 8 hours after their exposure to  $H^3TDR$ , were labeled early in their synthetic period. Thus, mean grain counts over prophase nuclei at these times represent the rate of synthesis near the beginning and at the end of the synthetic period.

That synthesis is approximately constant during this synthetic period can be adduced from these observations. Consider the mean grain count over prophase cells at 4 and at 8 hours. These cells were being labeled during the 9th, 10th, and the 1st half of the 11th, and the 5th, 6th, 7th, and 8th hours of the division cycle, respectively. Since the grain counts are equal, the synthetic rates must be equal at these two times. Furthermore, since the mean grain counts are equal to the mean counts of all cells (sampled at 2 hours following exposure to  $H^3TDR$ ), this rate of synthesis must be constant throughout the 8.5 hours during which synthesis is taking place.

The slopes of growth curves of mouse ascites tumor are known to vary with the line and strain of cell, the host, the size of the inoculum, and other factors (Klein and Révész, 25). Our growth curves

showing an immediate lag, then an exponential phase of growth during which the generation time is approximately 18 hours are comparable to those obtained by Patt and associates (1), Straube *et al.* (26), Lucké and Berwick (27), and Hornsey and Howard (11).

These results provide a more precise characterization of the mitotic cycle of the Ehrlich ascites tumor cells and data which allow an estimate of the mean rate and constancy of the process of DNA synthesis in these cells. It is reasonable to suppose that the techniques employed, with appropriate modifications, can be used to determine characteristics of the growth cycle of cells in sections of tissues and solid tumors.

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## EXPLANATION OF PLATE 136

*Abbreviations Used in the Figures*

<i>T.C.</i> , tumor cells.	<i>LN</i> , lymph node, parenchyma.
<i>P</i> (Fig. 3), peritoneum.	<i>P</i> (Fig. 4), pancreas.
<i>LNC</i> , lymph node capsule.	<i>M.M.</i> , muscularis of intestine.

FIG. 1. Radioautograph of smear of ascitic fluid removed 1 hour following intraperitoneal injection of 16 microcuries of tritiated thymidine. The formaldehyde vapor fixed smears exposed to Kodak, Ltd., AR10 radioautographic stripping film for 16 days were stained with hematoxylin and eosin. Cells that have incorporated  $H^3TDR$  are readily distinguished from those cells not so labeled by the overlying grains of reduced silver. The intensity of labeling and overlapping of grains obscures nuclear structure.  $\times 1,150$ .

FIG. 2. Radioautograph of smear prepared as above of cells removed 1 hour following injection of 4 microcuries of tritiated thymidine and exposed to film for 6 days. The numbers of grains overlying nuclei are sufficient to distinguish labeled and unlabeled cells but in this concentration do not obscure nuclear structure. Different phases of the mitotic cycle can, therefore, be distinguished in labeled cells.  $\times 800$ .

FIG. 3. Histologic section of mesenteric lymph node and overlying peritoneum taken 7 days after inoculation of peritoneal cavity of the mouse with  $1.8 \times 10^6$  Ehrlich ascites tumor cells. Multiple layers of ascites tumor cells are densely adherent to the peritoneum.  $\times 180$ .

FIG. 4. Histologic section of pancreas taken 11 days following inoculation of  $1.8 \times 10^6$  cells. Extensive invasion and destruction of pancreatic acini by Ehrlich ascites tumor cells are shown.  $\times 140$ .

FIG. 5. Histologic section of small intestine taken 16 days following inoculation of  $1.8 \times 10^6$  ascites tumor cells. There is invasion of the muscularis and submucosa by ascites tumor cells.  $\times 140$ .

