TIMING OF DNA SYNTHESIS IN THE MITOTIC CYCLE *IN VITRO*

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ABSTRACT

A study was made of the timing of DNA synthesis in the mitotic cycle under conditions where the average mitotic cycle of populations of human amnion and kitten lung cells in culture was variable. Three types of experiments were performed: (*a*) Autoradiographs were made of incorporated tritiated thymidine in cells whose mitotic histories were recorded microcinematographically allowing the measurement of telophase + G1 along with the total length of the mitotic cycle. (*b*) Measurement of the G2 + prophase part of the mitotic cycle was performed under various conditions by exposing cells to tritiated thymidine and observing the increase in labeled metaphases plus anaphases as a function of time. (*c*) The effect of a change in pH on parts of the mitotic cycle was tested by continuously photographing a single colony of cells first at pH 7.8 and then at pH 7.1. All of our data point to the same conclusion; namely, that within a population of cells with a given generation time, the length of each of the measurable parts of the mitotic cycle has a particular distribution of values and that, when there is a change in the generation time, under our conditions only the T + G1 distribution changes.

When studying variations in the length of the mitotic cycle of cells in culture, two kinds of variability must be dealt with. The first is variation among individual cells within a single culture and may be classified as the internal variability of cultures. In addition to this, there are changes in the average generation time of entire cultures in response to extracellular conditions and this type of variability may be considered physiological. The present study deals mainly with the second of these and shows that, under our conditions, virtually all of any change in the average length of the mitotic cycle can be accounted for in the period from division to the beginning of DNA synthesis. The internal variability of cultures is also demonstrated.

The results were obtained in part by a method which allows the autoradiographic detection of incorporated radioisotopes in individual cells whose mitotic histories had been previously recorded by means of time-lapse microcinematography. This technique is essentially a modification of one first used by Walker and Yates (23) who carried out microphotometric measurements on cells after having recorded their mitotic histories by microcinematographic methods.

Confirmatory data were obtained by more conventional methods.

MATERIALS AND METHODS[1]

Studies were carried out on Nakanishi's kitten lung strain (12) and Fernandes' human amnion strain (4). The modified Eagle's medium and the general culture methods used have been described (19). Experiments involving cinematography were carried out with cells grown in Rose chambers. For these cul-

[1] Abbreviations of Howard and Pelc (6) are used in this paper. G1 indicates the period of the mitotic cycle from the end of telophase to the beginning of DNA synthesis; S, the period of DNA synthesis; and G2, the period from the end of DNA synthesis to prophase.

tures, cells were passed gently through a syringe so that they were left in small clumps, and the volume of cell suspension so adjusted that only a few clumps grew in each chamber.

Experiments were begun when Rose chamber cultures were 18 to 24 hours old. Microcinematography was carried out at 37°C. ± 0.5° at the rate of one frame per minute for a period of approximately 30 hours. At the end of this time, the medium was exchanged for fresh medium containing 1 microcurie per ml. of tritiated thymidine (Schwartz Laboratories, Mt. Vernon, New York, sp. act., 1.9 curies per mM). Photography of the same colony was continued for 10 minutes after which the medium was exchanged for fixative (3 absolute ethanol:1 acetic acid).

The cells *in situ* were washed with 100 per cent ethanol, and after disassembly of the chamber and air drying, they were carried through the Feulgen staining procedure. Stripping film was then applied and allowed to expose for approximately 14 days.

After development of the autoradiograph, individual cells which had been previously photographed were located both in the autoradiograph and in the last frame of the motion picture recording. Each cell was followed on a motion picture editor until the time that it began its previous anaphase was observed, the number of frames being recorded by a Synchromaster film measuring machine. In this way, it was possible to relate isotope incorporation in a single cell, as observed in the autoradiograph, to the time since the previous anaphase of that same cell, as determined by analysis of the motion picture. In order to determine the length of the entire mitotic cycle, individual cells in the same colony were followed from one anaphase to the next.

To measure the time from the end of DNA synthesis to metaphase, *i.e.*, $G2^1$ + prophase, amnion cells were grown on 12 × 50 mm. coverslips in test tubes. Coverslips with adherent cells were placed in fresh medium containing 1 μc./ml. of tritiated thymidine and were removed periodically and fixed immediately, or were allowed to remain in the isotope-containing medium for 20 minutes and were then returned to unlabeled medium until the time of fixation. Only metaphases (M) and anaphases (A) were scored and a total of 50 to 100 cells was counted for each sample. Since anaphase generally accounted for 10 to 20 per cent of the total, the curve obtained by plotting the percentage of labeled M + A as a function of time since exposure to isotope describes essentially the time required for the progression of cells from the end of DNA synthesis to metaphase.

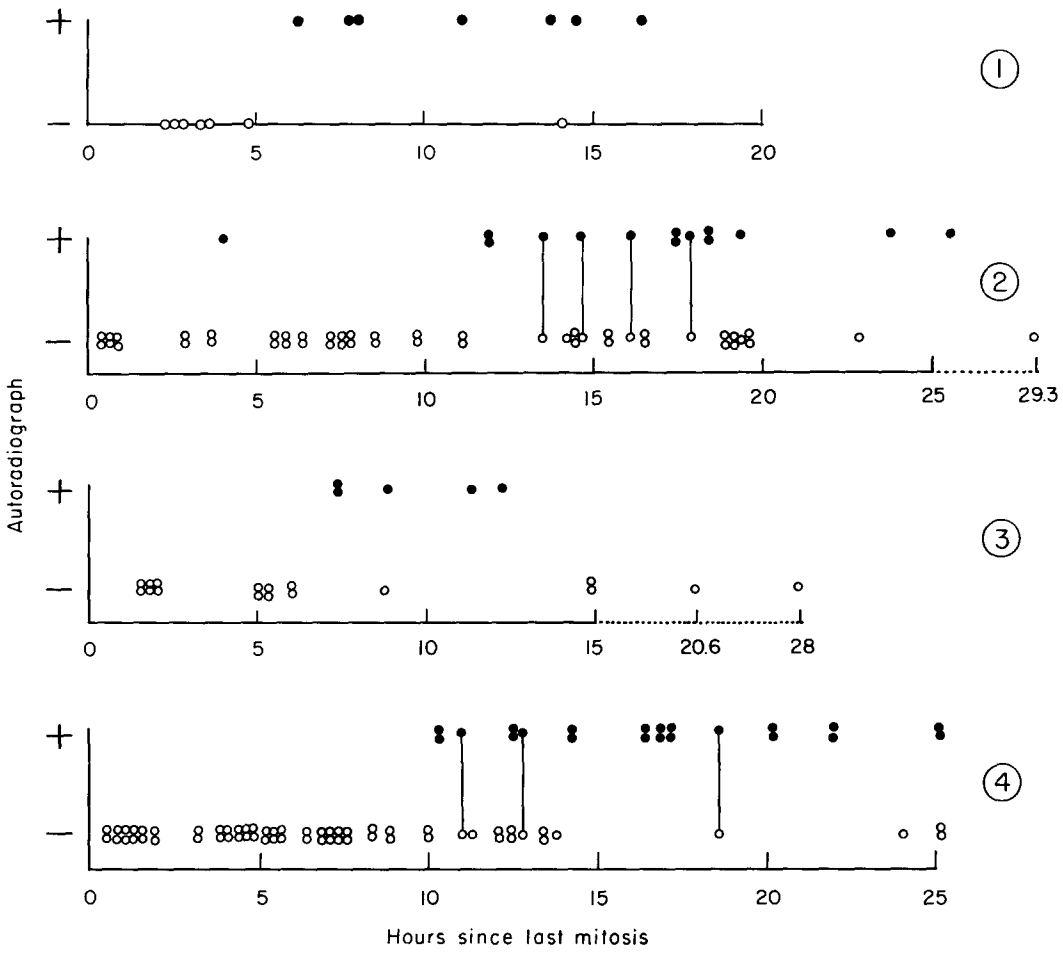
RESULTS

Measurements of the period from the beginning of anaphase to DNA synthesis (A + T + G1) in the kitten lung strain are shown in Figs. 1 and 2. In the colony observed in Fig. 1, the shortest time observed between a cell's division and the incorporation of tritiated thymidine into one of its daughters was 6.3 hours. In another colony from a later subculture of the same line of cells (Fig. 2), DNA synthesis did not start until 11.9 hours after the previous anaphase. This difference can be explained by the data on cell cycle length in these same colonies. The culture which began DNA synthesis 6.3 hours after anaphase had an average total mitotic cycle time of 18.3 hours. The second colony, in which DNA synthesis did not start until 11.9 hours after anaphase, had an average total cycle time of 23.6 hours. In other words, the increase in total length of the mitotic cycle can be accounted for by an increase in T + G1 (since anaphase takes at the maximum about 10 minutes, it may be disregarded). The time between the beginning of DNA synthesis and the beginning of the next anaphase (S + G2 + P + M) as determined by simple subtraction was practically constant; 12 hours in the first case and 11.7 hours in the second. Similar results were obtained when two colonies of human amnion were analyzed in the same way (Figs. 3 and 4). The results of all four experiments are summarized in Table I.

Theoretically, the culture conditions were identical but there were obviously some differences. In any case, the data indicate that under the unknown conditions which caused variations in the length of the mitotic cycle, virtually all of the variation is confined to the period between anaphase and the beginning of DNA synthesis.

Measurements of G2 + P in the human amnion line of cells is shown in Figs. 5 and 6. The determinations in Fig. 5 were carried out at pH 7.2 and pH 7.8, the approximate limits of the physiological range for these cells. This difference had no detectable effect on the progression of cells through this part of the mitotic cycle even though it can be shown to affect the mitotic cycle (see below). Since most cells including our own lines (unpublished results) go through a lag period when placed in fresh medium, the effect of an unadapted medium on the progression of cells from the end of DNA synthesis to metaphase was also tested. In one series, cells were transferred to fresh medium after exposure to isotope, while in the control they were returned to their original medium. The unadapted medium caused no increase in the length of G2 + P (Fig. 6).

By combining the data from the two previous



FIGURES 1 to 4

These figures show whether or not individual cells incorporated tritiated thymidine in relation to the time since their last division. ○ = unlabeled, ● = labeled. Vertically paired points are sister cells. Fig. 1, KL 38; Fig. 2, KL 51; Fig. 3, A 152; Fig. 4, A 160.

sets of experiments, it is possible to calculate the length of the S period and hence to predict the percentage of cells synthesizing DNA at any given moment in the amnion cells. Since this percentage can also be determined by direct counting, it can serve as a check of the validity of the measurements. These calculations were made in two different ways for the two amnion cultures in Figs. 3 and 4, and Table I. In the first case, the data for total cycle time and A + T + G1 were used directly as shown in Table I. As a measure of G2 + P, we used the time at which 50 per cent of the mitotic cells were labeled in experiments such as those shown in Figs. 5 and 6. In these ex-

TABLE I

Summary of Measurements of the Time from the Beginning of Anaphase to the Beginning of DNA Synthesis (A + T + G1) in Relation to the Length of the Mitotic Cycle

Culture	Number of cells	Average mitotic cycle time	S.E.	A+T+G1	Mitotic cycle minus A+T+G1
KL 38	7	18.3	±.9	6.3	12
KL 51	10	23.6	±.9	11.9	11.7
A 152	28	18.2	±.3	7.3	10.9
A 160	14	21.4	±.7	10.3	11.1

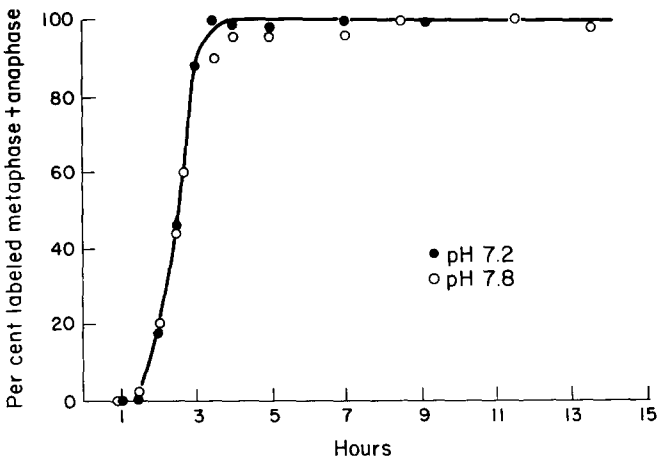


FIGURE 5

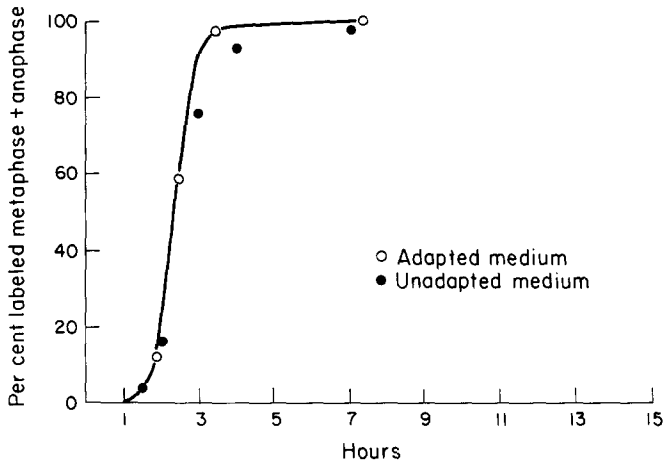


FIGURE 6

FIGURES 5 and 6

These figures show the rate at which cells can pass through the period of the mitotic cycle from the end of DNA synthesis to metaphase under the indicated conditions.

periments and three other unpublished determinations, this always occurred between 2.3 and 2.5 hours (average, 2.4 hours). Disregarding M, which takes about 20 minutes as determined by microcinematography, the length of S was calculated by subtracting A + T + G1 and G2 + P from the total mitotic cycle time. We then determined the percentage of cells which should be in S by redrawing the curve of Scherbaum and Rasch (Fig. 2 in (17)) on a larger scale, relabeling the axes to fit our data, and measuring the percentage of the total area under the curve which lies over S. A sample diagram is shown in Fig. 7. The actual percentage of labeled cells was determined directly in the autoradiographs in the same cultures and these data are compared in columns 1 and 2 of Table II.

Although the predictions are close, within less than 4 per cent of the observed, both are slightly high. This is to be expected since our measure of A + T + G1 would tend to be somewhat lower than an average, assuming some degree of variation between individual cells, because it is determined in each culture by that cell which shows the shortest interval between division and labeling (with one exception: Fig. 2).

In the second series of predictions, we considered our measure of A + T + G1 a minimum, and we

used as a minimum G2 + P the time it takes the quickest cell to get from the end of S to M, which in six out of seven experiments was between 1.3 and 1.5 hours (average, 1.4 hours); and as a measure of a minimum cycle time, we used the average mitotic cycle time minus one standard deviation. In this case, the predictions are within less than 1 per cent of the observed values (column 3, Table II). When similar calculations were also made for the kitten lung culture of Table I, using the data for G2 + P as determined in the amnion line, the predictions again turned out to be reasonably close.

In the next experiment, an attempt was made to measure directly, in a single colony of cells, the effects of a change in pH on the length of the mitotic cycle. Before beginning cinematography of a colony of cells in a Rose chamber, the medium was removed and replaced with a preadapted medium (one in which cells had previously grown),

TABLE II

Per Cent Labeled Cells

Culture	Observed	Calculated	
		From averages	From minimums
A 152	38.2	41.5	37.7
A 160	31.4	35.2	30.5
KL 38	40.8	48.1	45.3
KL 51	27.4	32.7	29.2

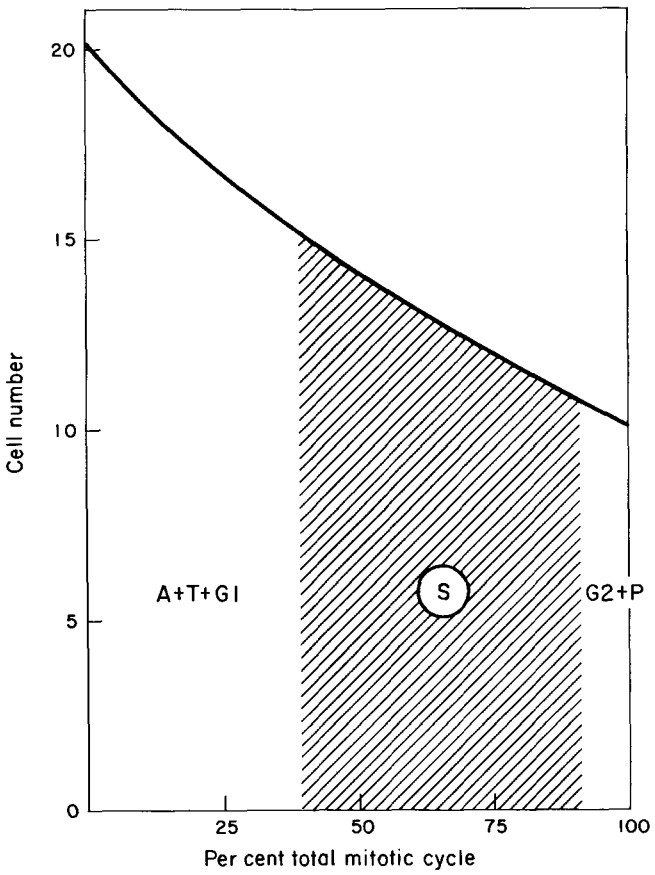


FIGURE 7

A sample diagram representing the percentage of cells synthesizing DNA at a given instant (redrawn from Scherbaum and Rasch (17)). The length of the mitotic cycle represented on the abscissa by 100 per cent is divided into three component parts. Ordinate represents cell number. The percentage of the total area of each part of the mitotic cycle equals the percentage of cells in that part of the mitotic cycle.

TABLE III

Effect of Low pH on the Length of the Mitotic Cycle in a Single Colony of Human Amnion Cells

Cells were photographed for 29.8 hours in a medium adjusted to pH 7.8, after which the medium was exchanged for one at pH 7.1. Photography continued for a total of 63 hours.

Completed cycle prior to change		Completed cycle within 10 hrs. after change		First division prior to change, second division 10+ hrs. after change	
Cycle length	S.E.	Cycle length	S.E.	Cycle length	S.E.
21.6	±.5	20.6	±.7	25.1	±.9

adjusted to pH 7.8. After 29.8 hours, this medium was removed and replaced with an aliquot of the same preadapted medium which had been adjusted to pH 7.1. The data in Table III show that in those cells which divided within 10 hours after the medium was exchanged for one of lower pH, *i.e.* cells which had reached the S period or beyond, the mitotic cycle was identical with that of cells which had completed their entire cycle before the medium was changed. However, those cells which had their first division prior to the medium change but their second division more than 10 hours after the medium change, *i.e.* cells which were at the early part of the cycle, had a mitotic cycle time which was significantly higher. Therefore, in agreement with the previous experiments, it was the early part of the mitotic cycle which was affected when the total length was increased by a change in pH. Since the entire motion picture recording lasted only 63 hours, it was not possible to get a good sample of the cells which had undergone both divisions after the medium was changed for one of lower pH.

It will be noticed in Figs. 1 to 4 that in each culture there are cells which remain unlabeled, even though the period since their previous division was long enough for a number of other cells in the same colony to begin incorporating tritiated thymidine. In fact, in a total of seven cases, there occur differences between two sister cells of a single division, one incorporating isotope and the other not. This phenomenon in KL 51 is illustrated in Figs. 8 to 11. Figs. 8 to 10 are enlargements of selected frames from the 16 mm. motion picture film tracing the development of one pair of daughter cells (labeled 58 and 59), and Fig. 11 is the autoradiograph of part of this same colony. In each of three pairs, including cells 58 and 59, one sister is labeled and the other is not. Other unlabeled pairs are so designated. Another indication of variation between individual cells, *i.e.* internal variability, is seen in Figs. 5 and 6. A few cells were able to progress from the end of S to M as quickly as 1.3 hours, while others took as long as 3 to 4 hours. Similar curves have been reported in a wide variety of materials (2, 5, 6, 11, 14, 15, 18).

DISCUSSION

In these studies, the emphasis was placed upon the response of entire populations to a change in environmental conditions. Data from the combination microcinematography-autoradiography experiments show that, while the progress of cells through the early part of the mitotic cycle will vary in different populations, once DNA synthesis commences the cells on the average proceed to division at a constant rate. A recent report by Mendlesohn *et al.* (11) indicates the same phenomenon in tumor cells.

In the experiments shown in Figs. 5 and 6, attempts were made to shift the curve representing the population's progress through G2 + P but neither the change in pH nor the transfer of the

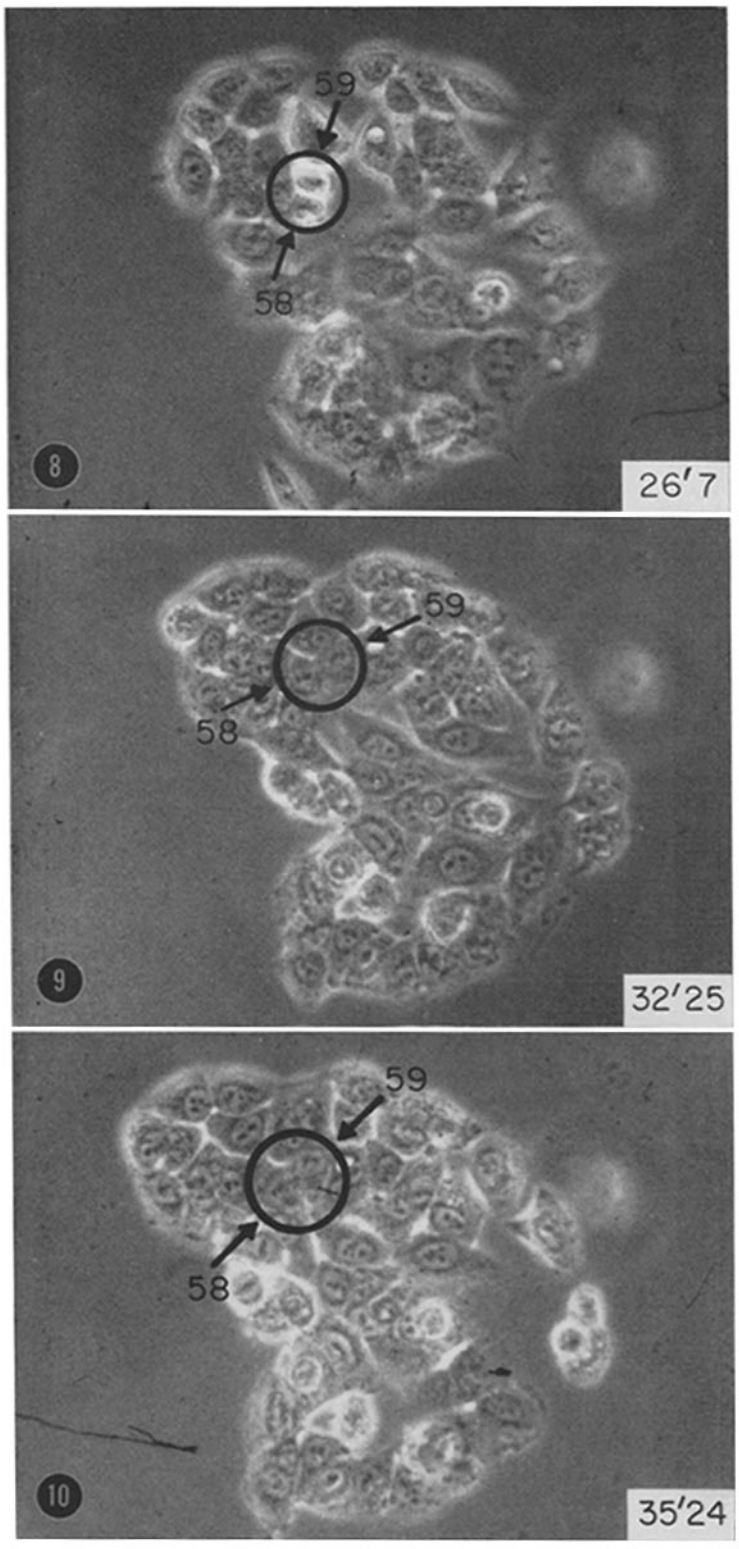
FIGURES 8 to 10

Selected frames of KL 51 enlarged from 16 mm. motion picture film, tracing a single pair of cells from division to fixation. Numbers in corners represent distance in feet and frames. × 340, approximately.

Figure 8. Cells in telophase.

Figure 9. Cells in interphase.

Figure 10. Cells in interphase just before fixation.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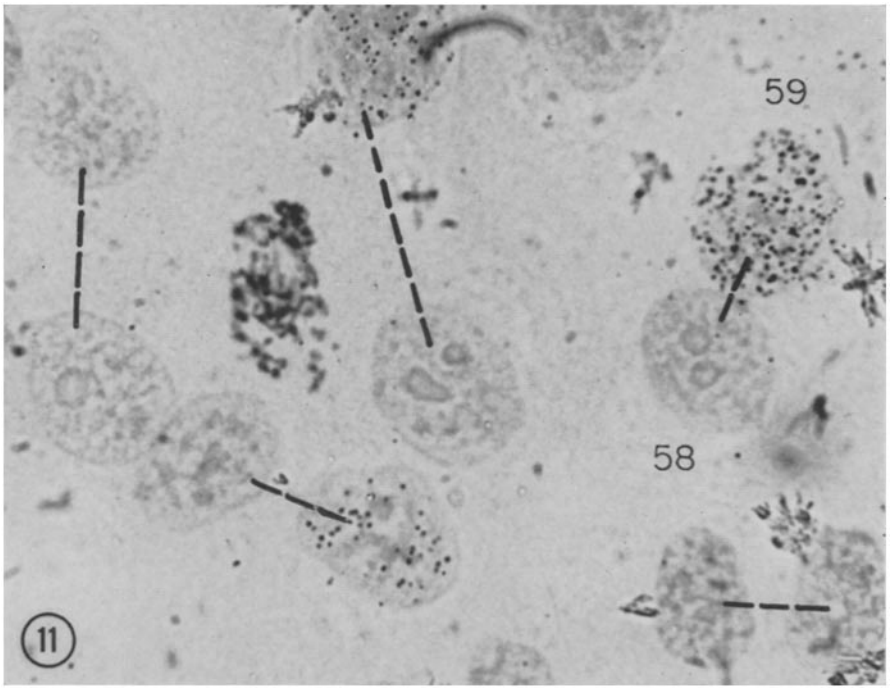

FIGURE 11

An autoradiograph of a portion of the same colony showing the two cells which were followed in Figs. 8 to 10, as well as other pairs of cells which are designated by a connecting dotted line. Note that in some cases only one cell of a pair is labeled. × 1275, approximately.

cells to unadapted medium was effective. That a pH change does affect the mitotic cycle and in fact affects only the early part of the cycle is shown by the experiment in Table III.

All of our data, therefore, point to the same conclusion; namely, that within a population of cells with a given generation time, the length of each of the measurable parts of the mitotic cycle has a particular distribution of values and that when there is a change in the generation time, only the T + G1 distribution changes.

If G2 + P is constant and S + G2 + P is constant, then S must also be constant, although there is evidence of individual variation among cells (14). An alternative to a constant S period is that its length varies in proportion to the length of the mitotic cycle (22). This implies that a constant percentage of cells is synthesizing DNA at any given time, but this is not supported by the data in Table II.

A period of variability followed by a period of constancy indicates that a cell destined to divide first goes through a period of preparation, the length of which depends upon the environment of the cell and during which there may be a collection and synthesis of precursors, enzymes, etc. Once it reaches a critical point at which these preparations are complete, it can carry on whatever other syntheses may be necessary for mitosis (9, 20, 21) at a rate which is constant and relatively unaffected by its environment. Our data and those of Mendelsohn *et al.* (11) indicate that the critical point occurs either at or prior to the commencement of DNA synthesis. Nygaard *et al.* (13) did not consider it likely that DNA synthesis *per se* could be a trigger for mitosis, because of its remoteness from the next division in the slime mold with which they worked (a short G1 and a long G2). On the other hand, Mazia *et al.* (10) have shown that the critical point may occur prior to DNA synthesis since in the sea urchin, at least, centriole duplication occurs at the end of the previous division and before DNA synthesis. Under experimental conditions, however, DNA synthesis can occur even though centriolar duplication has been blocked (1) so that the latter

cannot be considered an obligate trigger for the former. Triggers for DNA synthesis itself have been suggested by Lajtha *et al.* (8) to explain the effects of x-rays on DNA synthesis in bone marrow cells, and have also been discussed by Nygaard *et al.* (13). Although we have no data on this last topic, the methods herein presented appear useful for such studies.

Variation in the length of the mitotic cycle of individual cells in culture has been noted (3, 7, 19). One probable result of this is shown in Figs. 1 to 4 where many cells which, on the basis of the time since their last division, should have been labeled, were not. Similar observations were made by Walker and Yates (23) and Richards *et al.* (16) using photometric methods. An alternative explanation might be that these cells are no longer reproductive and will eventually die without further DNA synthesis. This seems unlikely, however, since time-lapse motion picture recordings carried out for several days show that only a small percentage of cells in our cultures die. It is more likely that such observations result from variations in the lengths of the mitotic cycle with a concomitant increase in G1.

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