DNA SYNTHESIS IN INDIVIDUAL L-STRAIN MOUSE CELLS

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SUMMARY

The time relationship between DNA synthesis and mitosis has been determined for L-strain mouse cells cultivated *in vitro*. DNA synthesis was detected autoradiographically by following the uptake of [^{3}H]thymidine into the cell nucleus. DNA synthesis in this cell system was found to take place in an approximately linear fashion over a single period of six to seven hours, ending three to four hours before mitosis, for a total generation time of twenty hours.

A mathematical treatment for exponentially multiplying cultures is presented.

INTRODUCTION

It is obvious that a cell must double its content of deoxyribose nucleic acid (DNA) before it divides. For many years it was believed that the cell synthesized this DNA during prophase and metaphase, so that when the two sets of chromosomes separated at anaphase, each had a full complement of DNA. Recently, many workers have shown, by the use of photometric and autoradiographic techniques, that DNA synthesis actually occurs during interphase over a period which ends at a certain time before mitosis, and which has a definite length[1–7]. This was found to be true for many different types of cells, both animal and plant, but the exact timing of the DNA synthesis period appeared to vary with the cell type. We have investigated, by the method of autoradiography, DNA synthesis in exponentially multiplying mouse cells, cultivated *in vitro*. This investigation is preliminary to studies of the perturbing influence of various chemical and physical agents upon DNA metabolism in the cell.

MATERIALS AND METHODS

Detection of DNA synthesis

Since thymidine has been shown to be a specific precursor of DNA[8], the uptake of [^{3}H]thymidine (^{3}HTDN) into the cell nucleus was used as a measure of DNA synthesis. The β-decay of tritium was detected autoradiographically. The low energy of tritium β-particles affords excellent resolution on the autoradiographs[9] (see Fig. 1).

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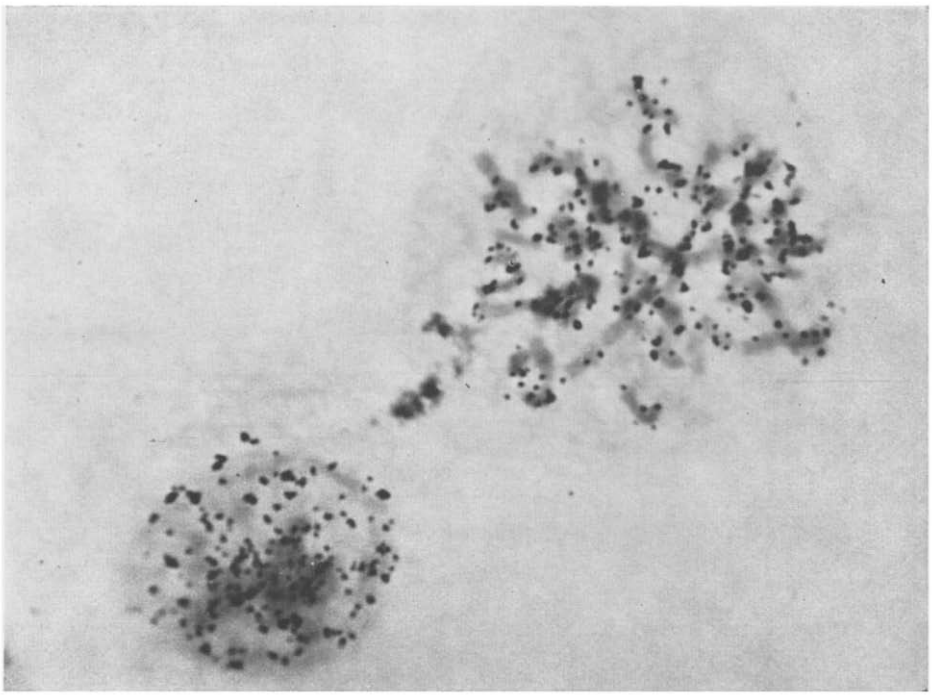

Fig. 1. Autoradiograph of two L cells after 16-h exposure to [^{3}H]thymidine. One cell is in metaphase, the other in interphase. The metaphase cell has synthesized one complement of DNA in the presence of label. Film exposure time was 15 days.

Cells

EARLE's L-strain mouse cells (NCTC Clone 929) were used[10]. Cells were cultivated continuously in the logarithmic growth phase, using suspension cultures[11]. It has been shown that under these conditions, almost 100 % of the cells are viable[12]. Growth medium for stock cultures consisted of a 20 % solution of horse serum in CMRL 1066[13]. For labelling of cellular DNA, ^{3}HTDN at a specific activity of 360 mC/mmole* was added to the growth medium to a concentration of 0.25 μC/ml. In one experiment a concentration of 0.5 μC/ml was used. The effective specific activity of the ^{3}HTDN was reduced 40-fold due to the presence of 10 μg/ml of unlabelled thymidine in the growth medium. The concentration of ^{3}HTDN used was safely below the radiation toxic level reported by PAINTER, DREW AND HUGHES[14] for HeLa cells, and no effect of addition of ^{3}HTDN on the growth rate of the cultures was observed.

Preparation of slides for autoradiography

After addition of ^{3}HTDN, the culture was sampled at hourly intervals. The samples taken were small, so that a single suspension culture could be used for an entire experiment. The cells of each sample were immediately dilated in hypotonic saline and fixed in acetic–alcohol, whereupon aliquots were smeared on duplicate slides and allowed to dry. The slides were then washed in acetic–alcohol, stained with 2 % acetic orcein, washed again in alcohol to remove excess stain, and then washed in running water for 3 to 4 h to prepare them for autoradiography.

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Autoradiography

After washing, the slides were ringed with egg albumin to prevent lifting of the photographic emulsion, and the emulsion was applied, using the method of MESSIER AND LEBLOND[15], in which the slides are dipped directly into liquid bulk emulsion*. The emulsion was placed in direct contact with the cells, without an interposed film of celloidin, in order to ensure maximum efficiency of detection of the low energy tritium β-particles[16]. Direct contact between emulsion and cells did not introduce spurious grains, as unlabelled cells showed a normal background grain count over their nuclei. In one experiment, the stripping film technique of PELC[17] was used.

For exposure, the slides were stored in light tight boxes at 4°. Exposure time was usually two weeks.

Counting methods

Fifty metaphases were scored to determine the percentage of metaphases which were labelled in each sample. The percentage of labelled cells was determined by scoring 500 interphase nuclei. An interphase nucleus or a metaphase was considered to be labelled if it was covered with 5 or more grains, the average background being about 2 grains/nucleus. The generation time of the cells was determined by measuring the cell concentration in the growing culture at various times with a hemocytometer. Mitotic indices were measured by counting the number of cells in mitotis in 3000 cells. In our material, the great majority of cells in mitosis were found to be in metaphase; one slide showed, for example, 73 metaphases, 7 prophases, and 2 anaphases. Our mitotic indices are therefore, to a close approximation, the percentage of cells seen in metaphase. Any cell showing a single group of clearly discernible chromosomes in the absence of a nuclear membrane was considered to be in metaphase.

RESULTS

From the recent reports of interphase DNA synthesis in various types of cells referred to in the introduction, it was assumed at the outset that every cell in the exponentially growing culture passes repetitiously through a mitotic cycle of the type illustrated in Fig. 2. If cells are considered to move clockwise around the cycle, they pass successively through a postmitotic non-synthetic period (G_1), a period of active synthesis of DNA (S), and a premitotic non-synthetic period (G_2)[1,3,4,7]. It remained to verify this pattern of DNA synthesis in our cells and to measure the actual values of G_2, S, and G_1. We also investigated how DNA synthesis proceeds with time over the S period.

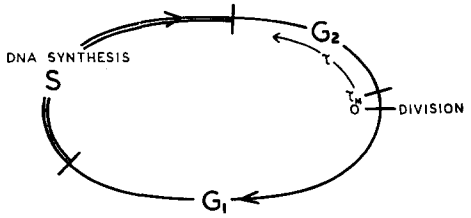


Fig. 2. The mitotic cycle of actively multiplying cells, showing the DNA synthetic period and its time relation to division (cells move clockwise around the cycle).

* Ilford Nuclear Research Emulsion, type G5, in gel form, Ilford, Inc., Ilford, Essex.

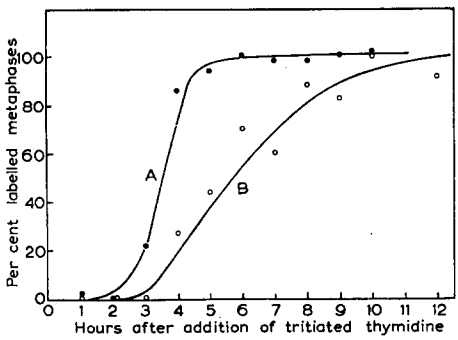


Fig. 3. The percentage of labelled metaphases for two cultures (denoted A and B) at various times after addition of [^{3}H]thymidine.

Determination of the G_2 *period*

The time required for labelled DNA to show up in metaphase chromosomes should give a measure of the length of the G_2 period. The ratio of labelled metaphases to total metaphases, labelled or unlabelled ("percent labelled metaphases") was measured for each hour after addition of ^{3}HTDN. Fig. 3 gives the results of two experiments. It is to be seen that in both cases virtually no labelled metaphases are seen for 3 h after addition of label. That this is not a lag in the uptake of label is shown by the observation that many *interphase* nuclei have taken up label within 1 h after addition of ^{3}HTDN (see Figs. 8 and 10). Indeed, the lag in uptake of label is probably negligible[7]. The 3-h delay in the appearance of label in cells in metaphase means that the period between completion of DNA synthesis and metaphase, *i.e.*, the G_2 period (see Fig. 2), is at least 3 h long. This, however, is a minimum period only. If all the cells had the same 3-h G_2 period, all metaphases would be labelled, though only slightly so, when this period had elapsed; that is, all metaphases seen at time G_2 after the addition of label would have just been completing DNA synthesis when ^{3}HTDN was added, and would thereby all be labelled. The percent labelled metaphases curve would, after a 3-h interval, rise abruptly to 100 %. The fact that it does not indicates that there is a variation among cells in the length of the G_2 period. Since, as will be shown, the duration of metaphase is short, about 0.5 h, the variation seen in Fig. 3 is only in a small part due to the time required for cells to pass through metaphase. A larger variation was observed in experiment B than in experiment A.

By taking the derivative of the percent labelled metaphase curves, the frequency distributions relating the numbers of cells with a given G_2 period are obtained. These are presented in Fig. 4. In experiment A the result is a sharp Gaussian curve with a peak at 3.5 h; in experiment B it is a more flattened curve skewed to the right, with a peak near 4 h. The reason for the difference between the two experiments has not been ascertained, but is probably due to differing culture conditions.

The generation time was found to be 20 h for both experiments. Thus, for a generation time of 20 h, the G_2 period has a most probable value of from 3 to 4 h.

Determination of the S *period*

The length of the G_2 period has now been found. The length of the S period can be determined in two ways.

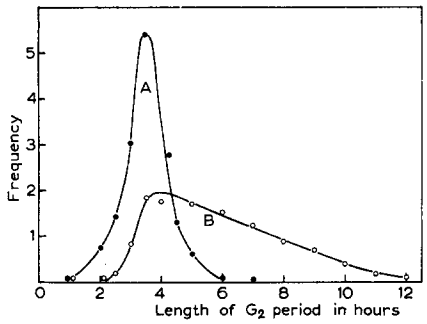


Fig. 4. Frequency distributions giving the relative number of cells with a given G_2 period for cultures A and B.

Grain counts over metaphases: The grain count over metaphases seen at increasing times after addition of label, should be zero (neglecting background) until the G_2 period has elapsed, then should increase for a time equal to the S period, after which it should increase no further since all the cells in metaphase after this time would have synthesized a full complement of DNA in the presence of label. The time between the first increase in the grain count over background and the time when the grain count saturates gives the length of the S period, and the intermediate grain counts give the manner in which DNA synthesis proceeds with time over the S period. Unfortunately, all the metaphases do not have the same number of grains over them at any time after addition of label, because there is variation in the length of the G_2 period, there is autoradiographic variation, and there is possible variation in DNA synthesis rates amongst cells. If, however, a distribution of the number of metaphases which have a grain count within a given interval is plotted for each time, the peak grain count values of these distributions should show the time dependence which would have been observed had there been no variation in the metaphase grain counts.

The slides of experiment A, which showed the smaller variation in the G_2 period, were selected for this experiment. Duplicate slides were used, one set being exposed for 15 days, the other for 8 days. Grains were counted over 50 metaphases generally, for each hour after addition of ^{3}HTDN, and a histogram of the number of metaphases within a given grain count interval plotted. Fig. 5 shows two such histograms for

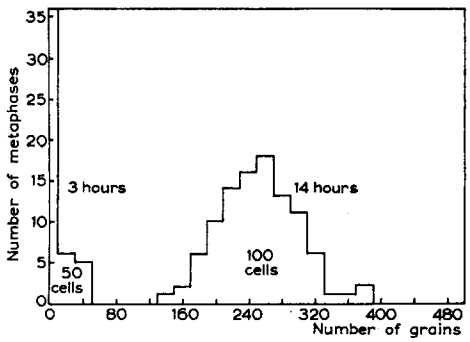
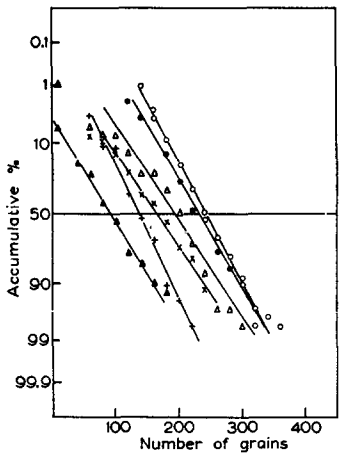


Fig. 5. Grain counts over individual metaphases; histograms of the number of metaphases within a given grain count interval for 3 h and for 14 h after addition of [^{3}H]thymidine.



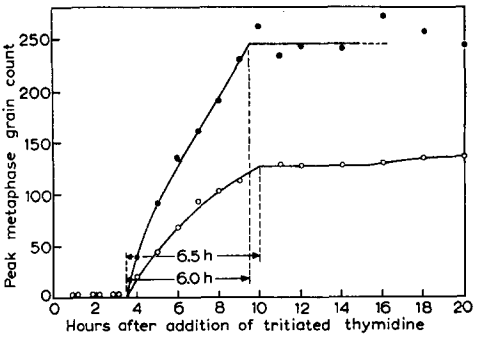


Fig. 7. Peak metaphase grain count values for various times after addition of [^{3}H]thymidine. Film exposure time for the upper curve was 15 days, lower curve, 8 days.

Fig. 6. The percentage of metaphases with a grain count up to and including the abscissa grain count values, plotted on probability paper. A Gaussian distribution appears as a straight line with its peak value at the 50% intercept. Six lines are given, corresponding to six times after addition of [^{3}H]thymidine: ▲, 5 h after addition of ^{3}HTDN; +, 6 h; ×, 7 h; △, 8 h; ●, 9 h; ○, 14 h.

3 and 14 h after addition of label. The 14-h distribution is a Gaussian, as were the distributions for all other times where all the metaphases were labelled.

The experimentally determined grain count distributions for each successive hourly sample were plotted on probability paper. Several of these are depicted in Fig. 6. When plotted on probability paper, a Gaussian distribution appears as a straight line, the peak value being given by the 50 % intercept of the line. The peak grain counts determined in this way for each hour after addition of ^{3}HTDN is plotted in Fig. 7. This method of determination of the peak grain count was not applied to the distributions for hours 1 to 4, since a Gaussian distribution would not be expected until all metaphases were labelled.

In Fig. 7, the upper curve represents peak grain counts as a function of time for a 15-day exposure, and the lower curve for an 8-day exposure of duplicate slides from the same experiment (A). Since grains are most conveniently counted when they number about 100 over a metaphase, the upper curve is more reliable for $t < 8$ h and the lower curve is more reliable for $t > 8$ h. It is seen that the metaphase grain count saturates in both cases 9.5 to 10 h after addition of ^{3}HTDN. The DNA synthetic period is therefore 6 to 6.5 h long.

From Fig. 7, it is seen that DNA synthesis is close to being linear with time over the *S* period. The curves are slightly concave which may indicate the DNA synthesis occurs at a slightly greater rate near the end of the *S* period than at the beginning, though the significance of the shapes of the curves is doubtful. From the lower curve, which gives a more reliable saturation plateau, it is seen that the peak metaphase grain count is virtually constant from 10 to 20 h, which is the generation time. The slight rise occurring from 16 to 20 h, which represents an increase of about 6 % in the total grain count, could be due either to a second period of DNA synthesis at a very reduced rate, or to the presence of a few cells in the population which have short enough generation times to have entered the *S* period a second time. In point of fact, since the standard deviation of the peak grain count values for the lower curve is

about 4 grains, the rise is not statistically significant. Determinations, to be described below, of the percentage of interphase cells which take up label, yield results that are in agreement with the single period hypothesis. Thus, the grain counts over metaphases as a function of time indicate that DNA synthesis in L-cells occurs at a near linear rate over a single 6.0 to 6.5-h period which ends 3 to 4 h before mitosis.

Percent labelled cells: The percent of labelled interphase nuclei measured each hour after addition of ^{3}HTDN should give an independent measure of the S period, and should be consistent with the values of G_1 and G_2 measured by the metaphase grain count method. The disadvantage of the percent labelled cells method is that it is very sensitive to any synchronization within the culture. In order to evaluate S, the degree of synchronization must be determined.

Asynchronous culture

We shall define an asynchronous culture as one in which the number of cells increases in a perfectly smooth exponential fashion with time, that is, one in which there is a constant fraction of cells undergoing division. For such a culture the frequency distribution of cells around the mitotic cycle (Fig. 2), or the number of cells in each portion of the cycle, is an exponential function[18] (see APPENDIX) with twice as many cells leaving mitosis as entering it, and the mitotic index is constant. It will be seen that a determination of the mitotic index as a function of time provides a sensitive indicator of the degree of synchronization present in a culture.

In experiment B, the mitotic index was found to be nearly constant with a value of about 1 % over a generation time of 20 h. It can thus be treated as an asynchronous culture. The length of time τ_m which the cells spend in mitosis for such a culture can be evaluated by applying eqn. (7) of the APPENDIX:

$$\tau_m = \frac{MT}{0.693}$$

where $M \times 100$ is the mitotic index and T is the generation time. This equation yields a value of τ_m of about 0.3 h. This is probably a minimum value for τ_m since slides that have not undergone preparation for autoradiography show mitotic indices of from 2 to 3 % *i.e.* a τ_m of about 0.7 h. However, since in this experiment the mitotic index obtained under constant conditions of slide preparation was found not to vary, it is valid to treat the culture as asynchronous.

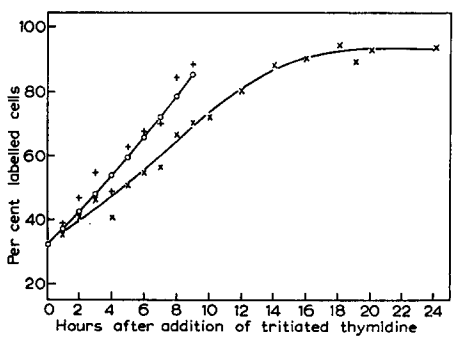


Fig. 8. The percentage of labelled cells for an asynchronous culture (B) at various times after addition of [^{3}H]thymidine. ×, experimental curve; O, the theoretical curve calculated from eqn. (14) using $S = 7.1$ h. For comparison, the corrected experimental points, +, are shown.

Determinations were made of the percentage of cells showing more than 5 grains above their nuclei ("percent labelled cells") for each hour after addition of ^{3}HTDN. Changes in the percentage of labelled cells could be due to flow of unlabelled cells into the S period and division of already labelled cells, yielding an increase, or to division of unlabelled cells, yielding a decrease. Since we wish to relate the percentage of labelled cells to the S period only, the experimental values obtained were corrected to what would have been observed had no multiplication taken place (see APPENDIX). A further correction was made for the fact that 5 % of the cells in the culture were unable to synthesize DNA. This is evident from the observation that the percent labelled cells curve levels off at 95 % after 16 h, as shown in Fig. 8. This correction is straightforward and the details will not be given. As discussed in the APPENDIX, further corrections may be avoided by using only those values for times less than G_1.

From each determination, a value of S was calculated by applying eqn. (15) derived in the APPENDIX:

$$S = \frac{1}{\alpha} \ln \left[L(t)_{\text{corr}} + e^{\alpha G_2} \right] - (G_2 + t), \qquad t < G_1$$

where $\alpha = \ln 2/T$, T is the generation time, and $L(t)_{\text{corr}} \times 100$ is the corrected percentage of labelled cells. ^{3}HTDN was added at $t = 0$.

The results are tabulated in Table I. The constancy of S serves as a test of the formulation.

TABLE I

EVALUATION OF THE S PERIOD

Time *h*	*Asynchronous culture (B)*		
	Experimental percentage labelled cells	*Corrected percentage labelled cells*	*Value of S (from eqn. (15))* *h*
1	36	39	7.2
2	42	47	7.6
3	47	55	7.9
4	41	49	6.0
5	51	63	7.3
6	55	68	7.1
7	57	71	6.5
8	67	85	7.6
			Mean: 7.1 h

From Table I, the average value found for S was 7.1 h. Using this average value for S in eqn. (14) of the APPENDIX the "theoretical" curve shown in Fig. 8 was constructed. Also shown are the corrected points and the uncorrected experimental curve.

Comparison of the curves shows that the results are consistent with the hypothesis that a single period of DNA synthesis exists for all cells, the period being about 7 h in duration and ending approx. 4 h before division.

Partially synchronized culture

If the mitotic index of a culture is not constant with time, the frequency distribution relating the number of cells within each portion of the cell cycle is not exponential. This can have a very pronounced effect upon quantities which depend upon the distribution, such as the percent labelled cells.

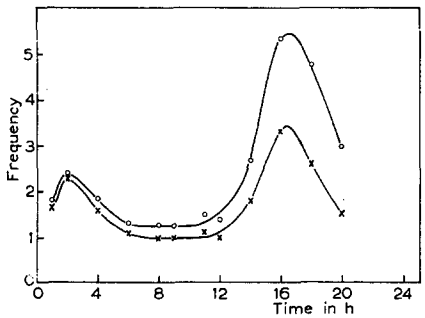


Fig. 9. Lower curve: the mitotic index at times after addition of [^{3}H]thymidine for a partially synchronized culture (A). Upper curve: the derived frequency distribution $n(t, o)$, giving the relative number of cells in each portion of the mitotic cycle for the same culture.

The mitotic index of experiment A was found to vary with time as shown in Fig. 9. The slides of this experiment were used to determine the S period by the metaphase grain count method, though it should be emphasized that the mitotic variation would have no effect upon this determination, since the treatment is not dependent on the distribution of cell numbers in the various portions of the mitotic cycle. Nevertheless, as a check on the grain count method, an independent estimate of the duration of the S period may be obtained from a count of the percent labelled cells, and this does depend strongly on the distribution of cell numbers throughout the mitotic cycle. An analysis of this dependence is outlined in the APPENDIX.

Assuming that all the cells move around the mitotic cycle at the same rate, the relative number of cells in each portion of the cycle at $t = o$ is given by $n(t,o)$, where t represents the time separating a particular cell from division. $n(t,o)$ is related to the measured mitotic index $M(t)$ according to eqn. (9) of the APPENDIX:

$$n(t,o) = \frac{1}{\tau_m} N_0 M(t) \exp \frac{1}{\tau_m} \int_o^t M(t)\mathrm{d}t$$

where N_0 is the total number of cells anywhere in the cycle at time $t = o$, and

$$\tau_m = \frac{1}{\ln 2} \int_o^T M(t)\mathrm{d}t$$

where T is the generation time.

The percentage of labelled cells was corrected to what would have been observed in Fig. 9, yield a value of 0.5 h for τ_m. The values derived for $n(t,o)$ using eqn. (9) are also plotted in Fig. 9.

The percentage of labelled cells were corrected to what would have been observed had no cell multiplication taken place. The procedure for this correction is given in the APPENDIX. Since the experimental $L(t)$ curve reached virtually 100 % after about 15 h, no correction was required to allow for cells unable to synthesize DNA. The corrected percent labelled cell values, as a function of time, $L(t)_{corr} \times 100$, can be predicted from $n(t,o)$ using eqn. (13) of the APPENDIX, by assuming a value for S:

$$L(t)_{corr} = \frac{\int_{G_2}^{G_2 + S + t} n(t,o)\mathrm{d}t}{N_0}, \qquad t < G_1$$

The value of S which gave best agreement between corrected experimental and

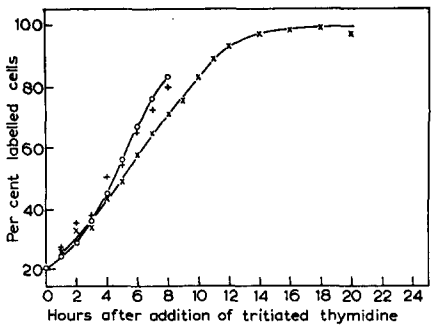


Fig. 10. The percentage of labelled cells for a partly synchronized culture (A) at various times after addition of [^{3}H]thymidine. ×, experimental curve; ○, the theoretical curve calculated from eqn. (13) using $S = 8.0$ h. For comparison, the corrected experimental points, +, are shown.

TABLE II

EVALUATION OF THE S PERIOD

Time *h*	*Partly synchronized culture (A)*		
	Experimental percentage labelled cells	*Corrected percentage labelled cells*	*Value of S (from eqn. (13))* *(h)*
1	26	27	8.3
2	33	36	8.8
3	34	38	8.2
4	44	51	8.4
5	49	55	7.8
6	58	65	7.8
7	65	73	7.6
8	71	80	7.4
			Mean: 8.0 h

theoretical $L(t)$ values, was determined for each hour, the integration being done graphically. These values are tabulated in Table II. The calculated values of $L(t)$ obtained using the mean value of S from Table II are plotted in Fig. 10, along with the corrected points and uncorrected experimental curve.

The mean value obtained for S was 8 h. This is somewhat larger than the value of 6.0 to 6.5 h obtained for metaphase grain counts performed on the same set of slides. This discrepancy may be due either to the assumptions inherent in the analysis, or to errors in the mitotic index values. It is quite difficult to obtain a reliable measure of the mitotic index, due to statistical errors and preparational inconsistencies.

DISCUSSION

The mitotic cycle found for L-strain mouse cells is similar to those reported by other workers for mammalian cells cultivated *in vitro*[4,7] in that they are characterized by a long post mitotic non-synthetic phase (G_1) involving approximately half of the total generation time, followed by a period of DNA synthesis (S) with a duration of about one-third of the generation time, followed in turn by a short premitotic non-synthetic period (G_2) which lasts for about one-fifth of the generation time. The division process itself is rapid, requiring less than an hour for completion.

The evidence also favours a linear rate of synthesis during the S period. The slight decrease in slope apparent in the curves of Fig. 7 would imply a decreased rate of DNA synthesis during the earlier stages of the period, since moving towards the right in Fig. 7 represents moving to earlier times in the mitotic cycle, *i.e.* away from mitosis.

The quantitative interpretation of much data relating to exponential cultures is only possible through an analysis similar to that given in the APPENDIX for the rate of accumulation of labelled cells. The procedure is complicated by the fact that the frequency distribution of cells around the cycle is exponential rather than constant. For example, suppose the percentage of labelled cells after a brief exposure to label for an asynchronous culture with a generation time of 20 h, was found to be 30 %. The length of the S period is not given by taking $0.30 \times 20 = 6$ h which assumes a constant distribution, but by eqn. (15), which yields $S = 6.7$ h, a difference of over 10 %. An exponential distribution, also, can be expected only when the mitotic index is constant with time. If it is not, the distribution must be evaluated (see APPENDIX), since cyclic quantities are very dependent upon it. For example, in one partially synchronized culture, the initial percent of labelled cells was found to be 20 %, which, using eqn. (15), gives $S = 4.7$ h. When the proper distribution was evaluated, S was calculated to be 7.3 h.

From the measured values of the rate, duration and location within the mitotic cycle of the S period, the average DNA content of a cell in relation to its premitotic and postmitotic DNA content can be calculated. This calculation is presented in the APPENDIX, and yields the result that the average content is 65 % of the premitotic content.

ACKNOWLEDGEMENT

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APPENDIX

Distribution of cell numbers at different portions of the mitotic cycle

Asynchronous case: In an asynchronous, exponentially multiplying culture, the cell concentration $N(t)$ as a function of time t is given by:

$$N(t) = N_0 e^{\alpha t} \tag{1}$$

Where N_0 is the initial concentration and $\alpha = 0.693/T$, T being the doubling time.

To locate the position of any given cell within the mitotic cycle of Fig. 2, a variable τ, where $0 < \tau < T$ is assigned, representing the time separating the cell from division. At $\tau = G_2 + S$, the cell begins DNA synthesis which lasts a time S; at $\tau = G_2$ the cell ceases DNA synthesis and enters the premitotic non-synthetic period of duration G_2; at $\tau = \tau_m$, the cell enters mitosis and at $\tau = 0$ the cell divides. Thus, the G_2 period is defined as the period from completion of synthesis to division.

Let $n(t, \tau)$ be the number of cells per unit time at time t flowing through a point on the cycle which precedes division by a time τ. At $\tau = 0$, the number of cells dividing in a time interval t to $t + dt$ is $dN(t)$, the number of new cells appearing:

$$n\,(t, 0)\,dt = dN(t) \tag{2}$$

From eqn. (1),

$$n\,(t, 0) = \alpha N_0 e^{\alpha t} = \alpha N(t) \tag{3}$$

Assuming a uniform rate of flow of cells around the cycle, a cell which is at position τ at a time t will have moved to $\tau = 0$ at time $t + \tau$, so that:

$$n\,(t+\tau,\,0) = n(t,\,\tau) \tag{4}$$

which yields, using eqns. (1) and (3),

$$n\,(t,\,\tau) = aN(t)\,e^{a\tau} \tag{5}$$

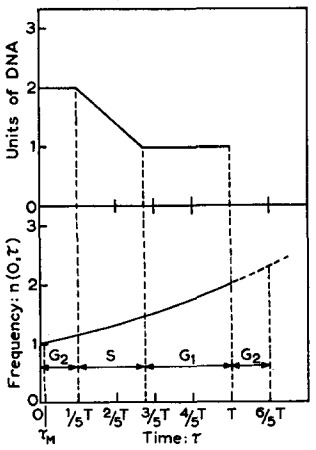

Fig. 11. Lower graph: the frequency distribution of an asynchronous culture giving the relative number of cells in each part of the mitotic cycle and showing the approximate position of the G_2, S and G_1 periods. Upper graph: The DNA content, in arbitrary units, of cells in the G_2, S and G_1 periods.

In Fig. 11, $n(t, \tau)$ is plotted for constant t. It is apparent that

$$N(t) = \int_{\tau=0}^{T} n(t,\,\tau)\,d\tau$$

which is the area between $\tau = 0$ and $\tau = T$ in Fig. 11.

Defining the mitotic index, $M(t) \times 100$, as the percent of cells in the interval $\tau = 0$ to $\tau = \tau_m$, we have:

$$M(t) = \frac{\int_0^{\tau_m} n(t,\,\tau)\,d\tau}{\int_0^{T} n(t,\,\tau)\,d\tau} \tag{6}$$

$$= e^{a\,\tau_m} - 1$$

$$\simeq a\,\tau_m,\ \tau_m \ll T \tag{7}$$

The mitotic index is constant with time.

Partially synchronous case: To evaluate $n(t, 0)$ for the case where the growth curve is not smoothly exponential, one makes use of eqn. (6):

$$M(t) = \frac{\int_0^{\tau_m} n\,(t,\,\tau)\,d\tau}{N(t)} \simeq \frac{n(t,\,0)\,\tau_m}{N(t)}, \qquad \tau_m \ll T$$

so that $$n(t,0) \simeq \frac{1}{\tau_m}\,N(t)\,M(t) \tag{8}$$

Using eqn. (2), which still applies, and integrating, the result is obtained:

$$n(t,\,0) = \frac{1}{\tau_m}\,N_0\,M(t)\,\exp\frac{1}{\tau_m}\int_0^t M(t)dt \tag{9}$$

From measured values of $M(t)$, $n(t, o)$ may be calculated. To evaluate τ_m, the following boundary conditions which arise from the assumption in eqn. (4) are applied:

$$M(t + T) = M(t)$$

$$n(t + T, o) = 2n(t, o)$$

From eqn. (9):

$$\tau_m = \frac{1}{\ln 2} \int_o^T M(t)dt \tag{10}$$

The percentage of cells taking up label

Asynchronous case: Referring to Fig. 11 and using eqn. (4), the percent of labelled cells $L(t) \times 100$ as a function of time t is given by:

$$L(t) = \frac{\int_{G_2}^{G_2 + S + t} n(t,o)dt}{N(t)}, \text{ for } t < G_2$$

$$= \frac{\int_{G_2}^{G_2 + S + t} n(t,o)dt + \int_{T + G_2}^{T + t} n(t, o)dt}{N(t)}, \text{ for } t > G_2 \tag{11}$$

where label is added at time $t = o$.

The second integral in eqn. (11) for $t > G_2$ arises from the fact that after time G_2 has elapsed, cells that have picked up label begin to divide, thus increasing the percentage of labelled cells. It is assumed in eqn. (11) that all cells have the same G_2 period. The theoretical treatment is improved by correcting for the division of labelled and unlabelled cells, taking into account the variation in G_2 times. The first labelled cells to arrive in division are spread out in time in the way depicted in Fig. 4 in the text. From the experimental data of Fig. 3 and the observed $L(t)$ values, the percentage of labelled cells which would have been observed had no cell division taken place, can be obtained. It has the form

$$L(t)_{corr} = \frac{1}{N_0}\left[L(t)_{exper}\, N(t) - \int_o^t P(t - \tau_m)\, \alpha N(t)dt\right] \tag{12}$$

Where $\alpha N(t)dt$ is the increase in cell number over a time interval dt, and $P(t - \tau_m)$ is the fraction of labelled cells entering division, taken from Fig. 3. Allowance is made for the fact that metaphase precedes division by time τ_m. The first term in the bracket represents the total number of labelled cells that are seen, and the second term represents the number of these that are due to division of already labelled cells.

After a time G_1 has elapsed, a further correction would be required to allow for cells which have divided after addition of label and which have not been in the S period in the presence of label. There is little point in extending the treatment for times larger than G_1, however, since the spread in S and G_1 times would have to be accurately known or the uncertainty introduced in making such a correction would be large.

Thus for $t < G_1$, eqn. (11) becomes:

$$L(t)_{corr} = \frac{\int_{G_2}^{G_2 + S + t} n(t,o)dt}{N_o} \tag{13}$$

Using eqn. (3), this gives:

$$L(t)_{\text{corr}} = e^{\alpha G_2}\left[e^{\alpha(s+t)} - 1\right], \qquad t < G_1 \tag{14}$$

From which one obtains:

$$S = \frac{1}{\alpha} \ln\left(L(t)_{\text{corr}} + e^{\alpha G_2}\right) - (G_2 + t) \tag{15}$$

For each corrected experimental $L(t)$ value, a value for S can be computed.

Partially synchronous case: Using the $n(t, 0)$ values obtained from eqn. (9), the experimental $L(t)$ values can be corrected for division by applying eqn. (12) substituting $n(t,0)$ for $\alpha N(t)$. Eqn. (13) still applies, and using $n(t,0)$ again, values of $L(t)_{\text{corr}}$ may be calculated for various values of S, and the S chosen which gives best agreement between calculated and experimental values. The integration is done graphically.

Calculation of average DNA content per cell for an asynchronous exponential culture

Assuming a linear synthesis of DNA over the S period and referring to Fig. 11, the total DNA in the culture at time t is given by the sum of three terms: first, a term for the cells in the G_2 period which all have a DNA content of 2 units (2D), second, a term for the cells in the G_1 period which have a DNA content of 1 unit (1D) and third, a term for those in the S period which have a DNA content ranging linearly from 1 unit to 2 units. The average DNA/cell is thus given by:

$$\frac{\text{Total DNA}}{N(t)} = \frac{1}{N(t)}\left\{\int_0^{G_2} 2\,\mathrm{D}n(t,\tau)\mathrm{d}\tau + \int_{G_2+S}^{T} \mathrm{D}n(t,\tau)\mathrm{d}\tau + \int_{G_2}^{G_2+S} \mathrm{D}\left[2 + \frac{G_2}{S} - \frac{\tau}{S}\right] n(t,\tau)\,\mathrm{d}\tau\right\} \tag{16}$$

The time (t) dependence naturally drops out of eqn. (16) so that the average DNA/cell is constant with time. If S is 7 h, G_2 4 h and T is 20 h, eqn. (16) gives the average DNA per cell to be 1.30 D or 0.65 the premitotic content.

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