RELATION OF PROTEIN SYNTHESIS TO THE DIVISION CYCLE IN MAMMALIAN CELL CULTURES

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ABSTRACT

Protein synthesis in suspension cultures of human cells in logarithmic growth was inhibited with puromycin or chloramphenicol, and the growth rate and mitotic index were measured as a function of time. The mitotic index remained constant for about 1 hour after addition of inhibitor; this indicates that any protein synthesis necessary for mitosis is completed before the beginning of prophase. For rates of protein synthesis equal to or greater than 0.3 that of untreated cells, the index decreased over a 5- to-7-hour period and then remained constant. The final value of the index relative to that of the uninhibited control was approximately equal to the relative rate of protein synthesis. The period from the end of DNA synthesis to mitosis (G_2) was increased by partial inhibition of protein synthesis. A mathematical model of the inhibition process has been formulated which predicts the shape of the mitotic index curves and the increase in the G_2 period. An interpretation of the model is that the rate-limiting step is the synthesis of an enzyme which catalyzes the formation of a compound necessary to initiate mitosis.

INTRODUCTION

The stages in the growth cycle of the cell which constitute mitosis occupy about $\frac{1}{25}$ of the time between divisions in mammalian cells in culture. Many of the necessary chemical steps such as DNA synthesis are complete before prophase begins. In this work we have investigated some aspects of the relation of protein synthesis to mitosis by inhibiting protein synthesis and measuring the mitotic index and rate of cell division as a function of time.

A number of specific proteins must be synthesized prior to division, but the mitotic spindle protein is probably the major component. In the sea urchin egg it accounts for 5 to 10 per cent of the total cell protein (1). It was expected that the primary effect of inhibition would be to prevent synthesis of the mitotic spindle protein. In earlier experiments with newt explant cultures inhibited by chloramphenicol (2), abnormal mitoses appeared in about 1 hour. The mitotic spindles were shorter than the average of the controls and in some cases consisted only of a pair of asters. To explain this effect, it was suggested that the cells entered mitosis without completing the synthesis of the spindle protein. Because newt cultures are not suitable for chemical analysis, we could not be certain that chloramphenicol acted specifically on protein synthesis. Mammalian cells provide a better system for chemical studies of mitosis since they can be maintained in logarithmic growth on a reasonably well defined medium.

The present report describes experiments on inhibition of human cells (strain K. B., reference 3) by chloramphenicol and puromycin. Puromycin proved to be both a more powerful and more

specific inhibitor of protein synthesis than chloramphenicol, and the abnormal spindles produced by the latter were not found in puromycin-treated cultures.

A mathematical analysis of the mitotic index decline after partial inhibition of protein synthesis is presented in terms of a number of simple models. The experimental curves can be fitted by a model in which the rate-limiting step is the synthesis of an enzyme which catalyzes the formation of a product necessary for mitosis. It should be stressed that although kinetic theories can be used to prove that a particular explanation is incorrect, the interpretation placed on a model which agrees with experimental results is not unique.

EXPERIMENTAL

Growth Conditions

A clonal derivative of the human carcinoma strain K.B. (3), originally obtained from Microbiological Associates,[1] was used in most experiments. This strain was eventually lost, but repetition of experiments at widely separated times on different populations of K.B. cells yielded essentially similar results. Some of the experiments were repeated with HeLa S-3 cells. The cultures were grown on Eagle's basal medium modified for suspension cultures by increasing the phosphate concentration tenfold, the amino acids and vitamins twofold, and omitting $CaCl_2$. The medium was supplemented with inositol 3×10^{-5} M, glutamine 4×10^{-3} M, penicillin 250,000 units/l, streptomycin 0.05 gm/l, and 5 per cent horse serum. All manipulations of the cells were performed in a room maintained at 36.8°C. The cells were grown in all-glass vessels and stirred with an unsupported Teflon-coated magnet. Stock cultures were grown in some cases in containers utilizing a magnet supported by a glass rod or by a chain and fishing swivel. All three methods were satisfactory at moderate stirring speeds but the unsupported magnet type was more convenient for experiments in which the culture had to be sampled frequently. The cultures were maintained in logarithmic growth at a density of between 10^5 and 2.5×10^5 cells per ml by daily feeding with an equal volume of pre-warmed growth medium.

Cell Counts

Counts were made with a Coulter electronic counter. A 2-ml aliquot of the culture was incubated for 5 minutes at 37°C with 1 ml of 5×10^{-3} M Versene in phosphate buffered saline, pH 7.4 (composition,—NaCl 140 mM, KCl 27 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM. This solution was used routinely for dilutions and will be referred to as phosphate-saline). The suspension was forced five times through a No. 18 needle, diluted with 10 ml of phosphate-saline, and counted immediately. The sample was counted three times to give a total count of about 5×10^4. Counts on duplicate samples were reproducible to about ± 2 per cent. Examination of samples in a hemocytometer revealed less than 1 per cent double cells and no clumps. The dispersion procedure did not decrease the hemocytometer count, and samples treated in this way could be plated with high efficiency. The Hela cells were not as easily converted to a single-cell suspension, and the counts were not better than ± 5 per cent in some instances.

Microscopy and Mitotic Index Measurements

Routine determinations of the mitotic index were made on small aliquots centrifuged for 1 minute at low speed to concentrate the cells. A drop was placed on a microscope slide and the cells were flattened under the coverslip by absorbing part of the suspending fluid with lens papers. Cells were examined by phase contrast microscopy. The mitotic index as here defined is the percentage of cells in the stages of mitosis from the appearance of definite chromosomes in prophase to a nearly complete cleavage furrow. Although earlier stages of prophase can be distinguished, they are more easily missed in routine counting. Cultures in logarithmic growth have a mitotic index of 2.5 to 3.0 per cent. For experiments in which a more accurate measure of the index was required, the cells were washed by gentle centrifugation with phosphate-saline and fixed for at least 1 hour in alcohol-acetic (3:1). A few drops of the cell suspension were air dried on a microscope slide coated with Meyer's albumin and stained with methyl green-pyronin according to the recipe of Pearse (4). At least 2000 cells were counted for each determination. Living cells were examined in a thin chamber formed between a coverslip and a microscope slide ringed with Apiezon L high vacuum grease. Cells entered mitosis for at least 2 hours in this chamber. Mitotic spindles were examined by polarized light using a modified Leitz petrographic microscope (2).

Determination of DNA, RNA, and Protein

Aliquots of the culture were washed three times by centrifugation in phosphate-saline buffer and then twice extracted for 5 minutes with ice-cold 0.2 N perchloric acid. For the determination of protein the residue was dissolved in normal sodium hydroxide by incubation overnight at 37°C and assayed by the method of Lowry (5) as modified by Eagle (6). Nucleic acids were extracted by heating the residue with 1 N perchloric acid for 20 minutes at 90°C. The DNA content of the extract was determined by

[1] Microbiological Associates, Bethesda, Maryland.

Burton's modification (7) of the diphenylamine reaction, and the RNA content by the orcinol method (8).

Syntheses of protein, DNA, and total nucleic acid were determined by incorporation of DL-valine-C^{14}, thymidine-C^{14}, and adenine-C^{14}, respectively. The labeled compound, dissolved in phosphate-saline, was added to the culture. Over a period of some hours, 3-ml aliquots were withdrawn and pipetted into 10 ml of ice-cold phosphate-saline. The cells were washed three times by centrifugation in phosphate-saline and filtered onto a 2.5 cm Millipore filter (pore size 0.45 microns). Acid-soluble material was extracted by twice allowing the sample to stand 5 minutes in cold 0.2 N perchloric acid. The filter was cemented to a planchet with rubber cement and air dried overnight. Finally, it was counted in a Nuclear Chicago counter. Control experiments showed that no correction was necessary for self absorption.

Cell Fractionation

A number of homogenizing methods were investigated, including grinding in a glass Potter homogenizer, passage through porous stainless steel plates of pore sizes 10 and 30 microns (9), and passage between close fitting steel plates. All of these methods proved to be unsatisfactory. A compromise between loss of nuclei and incomplete breakage of whole cells was finally adopted. The cells were washed with buffered saline and suspended in 0.24 M sucrose, 10^{-4} M $MgCl_2$, 0.01 M Tris buffer, pH 7.4. The homogenizer consisted of a stainless steel outer cylinder (inside diameter 3.7 cm) surrounded by an ice jacket and a cylindrical nylon piston (length 3.5 cm) threaded to a steel shaft which was fitted into the chuck of a drill press. The gap between piston and cylinder was 0.0025 cm. Essentially quantitative breakage of whole cells was obtained after 15 strokes. The yield of nuclei as determined by hemocytometer counts was about 60 per cent. This procedure was used for the preparation of nuclei. The homogenate was layered over 0.34 M sucrose-Tris buffer, pH 7.4, and centrifuged for 10 minutes at 700 *g*. The precipitate was resuspended and the layering step was repeated. To obtain cytoplasmic fractions the homogenization was stopped after eight strokes. Samples examined in a hemocytometer usually showed about 40 per cent unbroken cells with a count of whole cells plus nuclei equal to about 90 to 95 per cent of the original cell count. Nuclei and whole cells were removed by sedimentation through a layer of 0.34 M sucrose as before, and were discarded. The cytoplasm was divided into particulate and soluble fractions by sedimentation at 105,000 *g* for 2 hours.

Radioautography

Aliquots of the culture were washed twice with buffered-saline by centrifugation and a drop of the concentrated cell suspension was spread on a microscope slide coated with gelatin-chrome alum (3 per cent gelatin, 0.5 gm/l Cr K SO_4). After 1 minute acetic alcohol (1:3) was added drop-wise and the slide was allowed to dry. The slides were extracted for 20 minutes by immersion in 0.2 N perchloric acid at 4°C and then washed for 30 minutes in running tap water. Autographs were prepared by standard methods (10) using Kodak NTB-2 emulsion. The slides were stained through the emulsion with methyl-green pyronin.

RESULTS AND DISCUSSION

Inhibition by Chloramphenicol

A very high chloramphenicol concentration is required to inhibit growth of mammalian cells. Preliminary experiments showed that the growth rate decreased in the concentration range from 1 to 4 mM. At the highest concentration, collapse of the spindle was observed upon addition of the drug; after a few hours in the presence of inhibitor the culture did not return to logarithmic growth when resuspended on fresh medium. Below 3 mM no immediate effects on mitosis were observable by phase microscopy, and after exposures of 6 to 8 hours the metabolic changes could be reversed by resuspension of the culture in fresh medium. The culture returned to a normal growth rate in less than 12 hours.

Inhibition of protein and total nucleic acid synthesis was measured over the range from 0.8 to 4.2 mM, and the results are shown in Fig. 1. Examples of the experimental data used to obtain the points in Fig. 1 are plotted in Figs. 2 and 3. Rates of incorporation were constant for 6 to 8 hours after addition of chloramphenicol to any final concentration less than 3 mM. The relative rate is given by the ratio of the slopes of the incorporation curves of the inhibited and control cultures. At the higher concentrations, the inhibited cultures exhibited a slowly decreasing rate of protein synthesis. In such cases, the slope averaged over the first 3 to 4 hours was used. Since in most experiments the curves extrapolated back to the origin, maximum inhibition was established in much less than 30 minutes.

Cell counts and mitotic index measurements for a culture in which the rate of protein synthesis was reduced to 60 per cent of the control are shown in Fig. 4. The cell count in the controls increased at about 3 per cent per hour which is of the order of the experimental error. Thus, the time interval

during which the growth curve changed slope could not be determined accurately. A straight line fitted to the points for the inhibited culture intersected the control curve at values of from 2 to 3 hours. The mitotic index began to increase after 1½ hours and reached about twice its initial value about 3 hours after inhibition. The maximum was followed by a slow decrease so that by 6 to 8 hours the index had dropped to a new value which was maintained for a few hours.

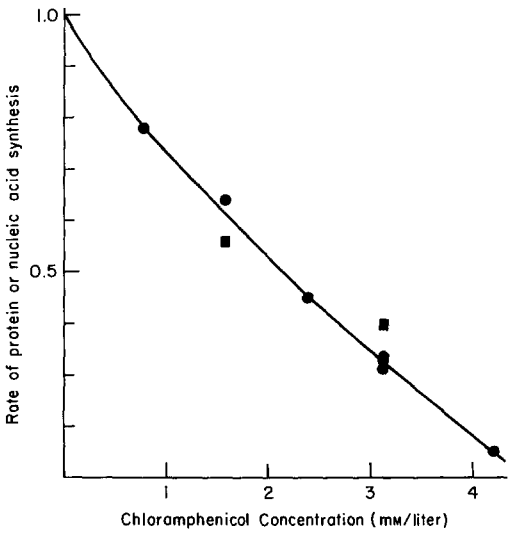

FIGURE 1 Relative rates of protein (●) and total nucleic acid (■) synthesis as a function of chloramphenicol concentration.

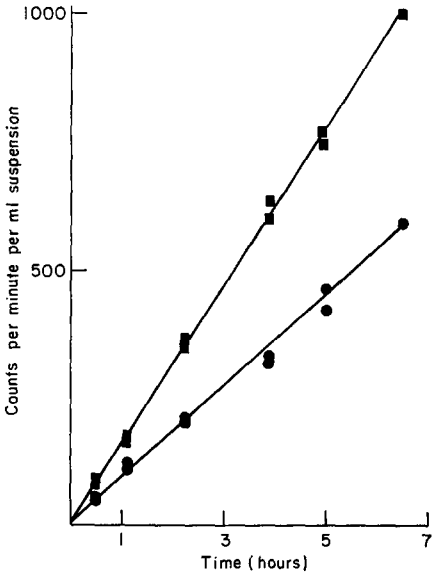

FIGURE 2 Incorporation of C^{14}-valine into cell protein, for chloramphenicol concentration of zero (■) and 1.6 mM (●). The paired points represent duplicate analyses. Chloramphenicol and C^{14}-valine added at zero time.

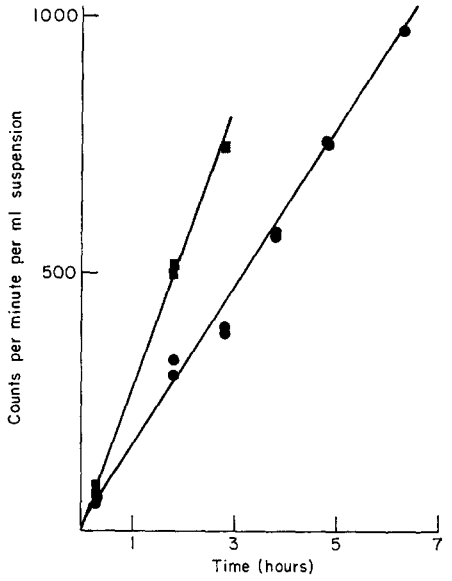

FIGURE 3 Incorporation of C^{14}-adenine into total nucleic acid for chloramphenicol concentrations of zero (■) and 1.6 mM (●). The paired points represent duplicate analyses.

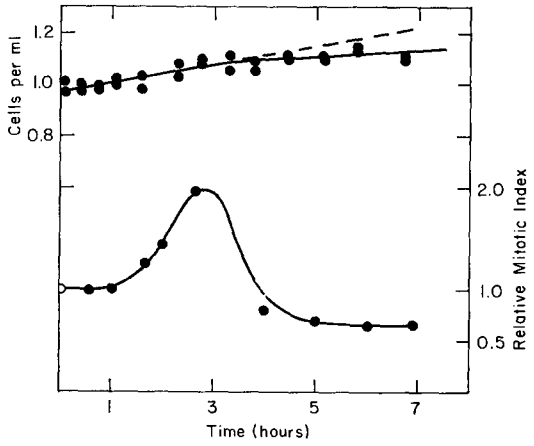

FIGURE 4 *Upper curve:* Cells per ml (arbitrary units) as a function of time after addition of chloramphenicol to a concentration of 1.6 mM. The points (●) and solid line refer to the inhibited culture. The dotted line refers to the control. The experimental points for the control were omitted to avoid confusion. *Lower curve:* Mitotic index relative to control.

The data presented in Figs. 2 to 4, all at a concentration of 1.6 mM, show the following correlation. Both nucleic acid and protein synthesis were reduced to about 60 per cent of the control value and the division rate and mitotic index were ultimately reduced by approximately the same amount. Thus chloramphenicol does not selectively inhibit protein synthesis. It also appears to partially block completion of mitosis, as evidenced by the transient increase in mitotic index. Cultures examined during this transient phase showed cells with a characteristic metaphase configuration (previously observed in newt cells), in which chromosomes were arranged in a rosette about a birefringent body, either a short spindle or pair of asters. Examination of living cells in thin slide preparations showed that the rosette metaphases did not go into anaphase but regressed to an interphase-like configuration in a few hours. The peak in the index was presumably caused by blocking of a fraction of the cells in metaphase. However, some cells with birefringent spindles and normal metaphase plates were present throughout the entire time interval and could be followed through division.

Inhibition by Puromycin

Protein and RNA Synthesis

Puromycin is known to be a powerful inhibitor of protein synthesis in animal cells (11, 12). The relative rates of protein and nucleic acid synthesis as a function of puromycin concentration are shown in Fig. 5. The concentration necessary to reduce protein synthesis to 20 per cent of the control rate was 4×10^{-6} M compared with 4×10^{-3} M for chloramphenicol. Examples of the experimental data are shown in Figs. 6 and 7. In contrast to the action of chloramphenicol, there was a lag of about 15 minutes before the inhibition of protein synthesis was fully established. There was also a gradual decrease in the rate of nucleic acid synthesis. The incorporation was almost entirely into RNA, since about 85 to 90 per cent of the radioactivity was extracted by incubation for 18 hours at 37°C in 0.3 M sodium hydroxide. The inhibition of DNA synthesis described in a later section (Conclusions) can account for at most a 10 per cent decrease in rate of total nucleic acid synthesis, so that there was certainly some inhibition of RNA synthesis. However, since the inhibition of protein synthesis is established in a very short time, while the rate of RNA synthesis decreases slowly, this may be a secondary effect resulting from the coupling of RNA and protein synthesis.

Growth Rate

The rate of increase in cell number was reduced by puromycin in a manner similar to chloram-

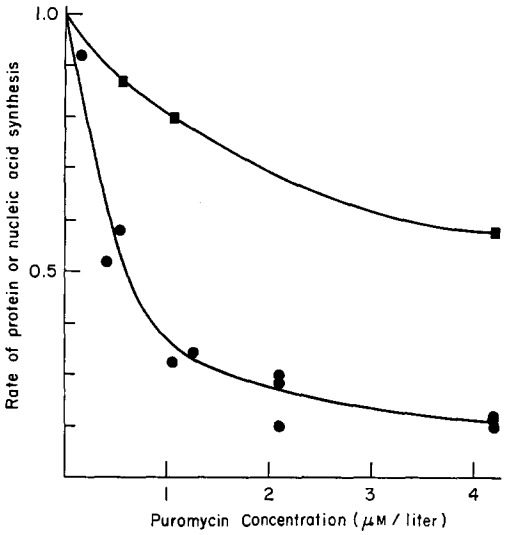

Figure 5 Relative rates of protein (●) and total nucleic acid synthesis (■) as a function of puromycin concentration.

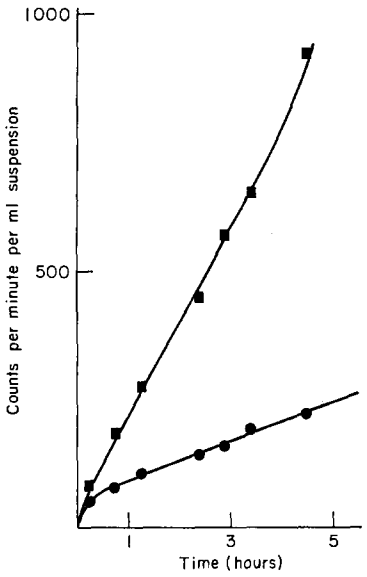

Figure 6 Incorporation of C^{14}-valine into cell protein for puromycin concentrations of zero (■) and 2.1 μM (●). Each point is the average of duplicate analyses.

phenicol. Growth curves for various levels of inhibition are shown in Fig. 8. After about 3 to 4 hours a constant growth rate was attained which was reduced in proportion to the degree of inhibition of protein synthesis. (The relative rate of protein synthesis will be referred to by the symbol α). The data are summarized in Table I. The doubling times were obtained from the slopes of the growth curves. (A plot for $\alpha = 0.21$ was not included in Fig. 8 since the points lie too close to the curve for $\alpha = 0.29$. Moreover, the data for

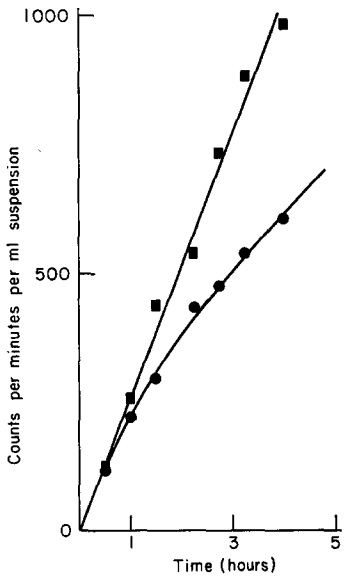

FIGURE 7 Incorporation of C^{14}-adenine into total nucleic acid for puromycin concentrations of zero (■) and 2.1 μM (●). Each point is the average of duplicate analyses.

$\alpha = 0.21$ are barely significant, since the total increase during the interval from 3 to 9 hours was only two to three times the experimental error.) In the third column of Table I the relative growth rates are given. It can be seen that they are approximately equal to the relative rates of protein synthesis, although the close agreement for $\alpha = 0.21$ is probably fortuitous.

As described in more detail in the next section, the growth rate can also be obtained by measuring the rate of accumulation of mitotic figures in the presence of colchicine. In the experiment illustrated in Fig. 9, colchicine was added to the cultures (final concentration 10^{-7} M) followed by puromycin 1.5 hours later. The time of addition of puromycin is taken to be zero time. The rate of accumulation of mitotic figures for $\alpha = 0.29$ (Curve *2*) decreased slowly for about 6 hours after addition of inhibitor and then remained approximately constant for the duration of the experiment. The final value of the relative growth rate, calculated as the ratio of the final slope of Curve *2* to the initial slope of Curve *1*, was 0.25. (The slope

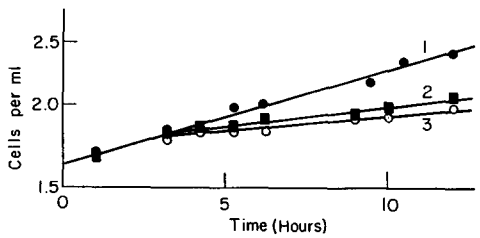

FIGURE 8 Cells per ml as a function of time after addition of puromycin. Curves *1*, *2*, and *3* are for relative rates of protein synthesis α of 1.0, 0.49, and 0.29, respectively.

TABLE I

Relation between the Relative Rate of Protein Synthesis after Puromycin Inhibition and the Growth Rate of the Culture

Relative rate of protein synthesis	Doubling time	Relative growth rate
(α)	*hrs*	
1	22	—
0.49	50	0.44
0.29	66	0.33
0.21	108	0.22

The doubling time was obtained from the slope of a plot of cell number versus time. Puromycin concentrations are zero, 0.75 μM, 1.5 μM, and 3 μM, respectively.

of the mitotic index versus time curve for a culture in logarithmic growth should increase with time. Over short time intervals the curvature is small so for convenience a straight line was fitted to the control points.) When the relative rate of protein synthesis was reduced to 0.21 (Curve *3*) the slope of the curve approached zero. After 8 hours reliable index counts could not be made because some of the cells contained pyknotic nuclei, indicating the onset of degeneration.

Resuspension of the culture in fresh medium after 10 hours' exposure to a concentration of

puromycin sufficient to reduce α to 0.2 did not lead to a resumption of growth. For values of α of 0.29 or greater the inhibition was reversible.

Mitotic Index

An interesting feature of the response of the culture to inhibition of protein synthesis was the slow decline of the mitotic index over a 5-to-7-hour period followed by a constant value for at least a few hours. Therefore, this aspect of inhibition was investigated in greater detail.

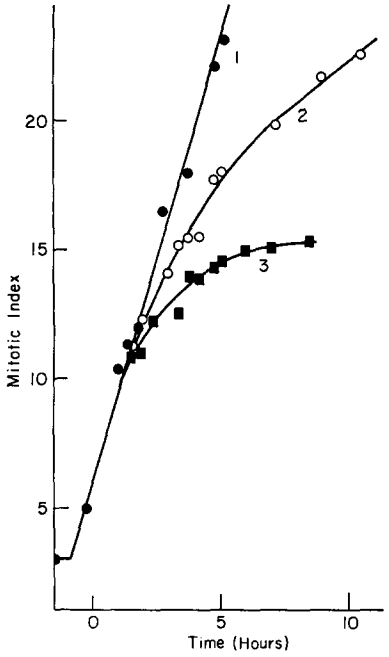

FIGURE 9 Mitotic index (per cent cells in mitosis) for control (Curve *1*) and puromycin inhibited cultures (Curves *2* and *3*). The rate of protein synthesis was reduced to 0.29 (curve *2*) and 0.21 (curve *3*) by addition of puromycin at time zero. Colchicine (final concentration 10^{-7}M) was added to cultures at -1.5 hours.

The mitotic index was determined on fixed and stained aliquots of the cultures as a function of time after addition of puromycin for various final levels of inhibition of protein synthesis. An experiment in which the relative rates of protein synthesis and the mitotic indices were measured at three inhibitor concentrations is illustrated in Fig. 10. It was found that a small fraction of the cells appeared abnormal in showing chromosome clumping at metaphase. Except for the longest exposure to the highest inhibitor concentration, the cells judged to be abnormal were less than 15 per cent of the mitotic cells counted. If some cells were blocked in mitosis the index curves would not be a true measure of the rate of entry into mitosis; therefore, index measurements were corrected for the fraction of apparently blocked cells. Three points should be stressed regarding the shape of the curves: (1) There is a lag of less than 1 hour, independent of the level of inhibition; (2) The index declines over a 5- to 6-hour period and the duration of the period is approximately independent of the level of inhibition; (3) The relative mitotic index eventually becomes constant and is approximately equal to the relative rate of protein synthesis. This aspect could also be inferred from the growth curves shown in Fig. 8. However, when the relative rate of protein synthesis was reduced to less than 25 per cent a constant index was usually not obtained. The solid curves in Fig. 10 were calculated from model II described in a later section (Kinetics of the Inhibition Process).

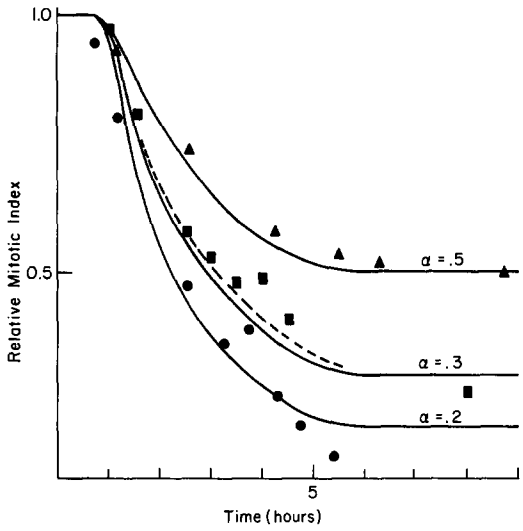

FIGURE 10 Relative mitotic index versus time for puromycin-treated cultures having relative rates of protein synthesis (α) of 0.2 (●), 0.3 (■), and 0.5 (▲). The broken curve represents the indices obtained from the accumulation of mitoses in the presence of colchicine (2.5×10^{-8} M) as described in the text. The solid curves were calculated from Model II using values of the parameter α of 0.2, 0.3, and 0.5. For details see text.

The slow decline in the mitotic index may be caused by an increase in the duration of mitosis.

This could arise either from a requirement for some protein synthesis during mitosis or from an effect of the inhibitor on the mitotic apparatus. Since the later effect was evident in the chloramphenicol experiments, this possibility had to be investigated.

A qualitative test was made by withdrawing aliquots of cells at various times after addition of puromycin and examining them in thin slide preparations. Cells in metaphase were selected for observation and the average time required to complete division was determined. In the majority of the cells the duration of mitosis fell within the range obtained for the controls; however, a small fraction of the cells did not divide in 2 hours and these cells could usually be distinguished by a clumping of the metaphase chromosomes. Since this configuration was also present in the controls, although to a lesser extent, the effect is caused partially by the handling of the cells in transferring from suspension to thin slide preparations. In each experiment the number of cells which could be followed was small, and observations were limited to the stages from metaphase to division since earlier stages were difficult to detect without undue flattening of the cells. Therefore, although these observations suggest that the duration of mitosis is largely unaffected by puromycin, they do not provide convincing quantitative evidence.

A more quantitative test for an increase in the duration of mitosis was made by blocking mitosis with colchicine. In experiments which will be reported in detail elsewhere it was shown that low concentrations of colchicine (*circa* 10^{-8} M) blocked mitosis at metaphase without affecting DNA, RNA, or protein synthesis or the rate of entry into mitosis. Colchicine was added to a final concentration of 2.5×10^{-8} M, and after 2.5 hours puromycin was added to reduce the rate of protein synthesis to 0.3 of the control. At this time the colchicine block was complete and no anaphases were present. (The accumulation of mitoses followed a curve which was essentially the same as curve *2*, Fig. 9). Since all cells which entered mitosis remained in metaphase, the slope of the curve is a measure of the rate of entry into mitosis. The relative mitotic index was, therefore, obtained by plotting the ratio of the slopes of the inhibited and control curves at successive times. The results are included in Fig. 10 (broken curve). From the manner of its derivation it is clear that the curve is subject to moderately large errors, particularly at short times, but it can be seen to agree well with the directly measured index curve. Therefore, the slow decline of the mitotic index cannot be a result of an increase in the duration of mitosis and thus measures a decreasing rate of entry into mitosis.

The index decay curves shown in Fig. 10 are representative of data obtained in a number of experiments at various rates of protein synthesis. However, when the relative rate was reduced to 0.3 or less, the mitotic index was very low. If 2500 cells are counted per point then the error expected from a Poisson distribution is still $\pm$ 20 per cent. In addition, any cells that are blocked in mitosis will raise the index appreciably. Therefore, for values of α less than 0.3 the uncertainty in the values of the mitotic indices may be too large to justify the conclusion that the index becomes constant for times longer than 6 hours after addition of inhibitor. The mitotic index for values of α of 0.29 and 0.21 was, therefore, determined from the accumulation of mitoses in the presence of colchicine. The data (Fig. 9) has been referred to in the previous section (Growth Rate).

When α was reduced to 0.29, cells continued to accumulate in mitosis for the duration of the experiment (Curve *2*). The slope of the curve from 6 to 10 hours was constant and yielded a relative index of 0.25. This agrees within experimental error with the value of 0.29 for α. However, when α was reduced to 0.20, the slope of the index curve was nearly zero after about seven hours in the presence of inhibitor (Curve *3*). After 8 hours, cell degeneration was present.

We, therefore, conclude that when protein synthesis is reduced to not less than 0.3 of the normal rate, cells are able to complete the mitotic cycle at at a reduced rate. After a transition period lasting about 6 hours, the rate is determined by the rate of protein synthesis. For higher levels of inhibition, the cells are unable to establish a new steady state of growth.

Radioautograph Experiments

After addition of puromycin a new constant rate of protein synthesis is established in about 15 minutes. Therefore, some explanation must be offered for the transition period of about 6 hours before a constant mitotic index is established. During this period, the slope of the growth curve presumably changes continuously but the experimental error in the determination of the

number of cells per ml is sufficiently large that the data can be fitted equally well by two straight lines (Fig. 8).

Although it may be reasonable to suggest that the slowly decreasing index provides information on the manner in which the final step in protein synthesis controls the rate of entry into mitosis, two other explanations will first be considered. (1) The slow decline may be a result of the variation in the time between the end of protein synthesis and the beginning of mitosis for different cells in the population. (2) A constant value for the rate of total protein synthesis after inhibition may not require that the rate be constant for a particular protein.

(1) A culture in logarithmic growth with a doubling time T_0 is made up of individual cells whose ages at division are distributed about T_0 as average value. (The age of a cell is defined as the time since cytokinesis of the mother cell.) The age distribution is assumed to be approximately normal. The standard deviation of the age at division is 0.2 T_0 for HeLa cells (13) and approximately 0.1 T_0 for human amnion cells (14) (*i.e.*, 68 per cent of the cells divide in the age interval $T_0 \pm$ the standard deviation). Thus for the HeLa cell, with $T_0 = 25$ hours, this interval is $\pm$ 5 hours.

It should be clear that the half width of this distribution does not in itself determine the decay time in our experiment. The significant quantity is the variation in the time interval between the completion of the step most sensitive to inhibition of protein synthesis and the initiation of mitosis. If the variation in age at division is introduced continuously, then the variation in the interval between the sensitive step and mitosis will be much less than 5 hours. Thus, if the average time for the completion of this step is 2 hours before mitosis, 96 per cent of the cells (two standard deviations) will complete this step in the period from 1 to 3 hours prior to mitosis. The variation may be even smaller since the data of Sisken and Kinosita (15) suggest that most of the variation in cell age at division is introduced in the G_1 period while in our case the sensitive step should occur either in G_2 or near the end of S. (G_1, G_2, and S have their usual meaning; S is the period of DNA synthesis, while G_1 and G_2 are the periods before DNA synthesis and from the end of DNA synthesis to the beginning of mitosis). Therefore, it appears unlikely that a distribution in the timing of the last protein requiring step could be responsible for the slow index decline.

The problem can be clarified by determining the effect of partial inhibition of protein synthesis on the duration of G_2. Under our experimental conditions, G_2 (defined as the time from the addition of tritium-labeled thymidine to the appearance of label in 50 per cent of the metaphase figures) was found to be approximately 3 hours (Fig. 11). The interval for the transition from 100 per cent unlabeled to 100 per cent labeled metaphases was about 2.5 hours. The decline in the mitotic index occupies the period from 1 to 7

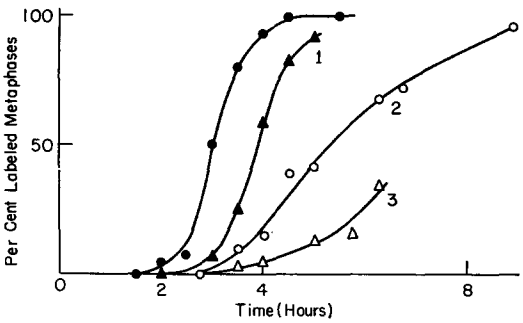

FIGURE 11 Time of appearance of labeled metaphases for various rates of protein synthesis. Puromycin was added at time zero to cultures 1, 2, and 3 to give relative rates of protein synthesis of 0.49, 0.29, and 0.21, respectively. Tritium-labeled thymidine (final concentration 0.25 μc/ml) was added to all cultures at time zero. The curve drawn through the filled circles refers to the control.

hours after the addition of puromycin. If this interval arises from the variation in the time between the completion of the last step involving protein synthesis and the beginning of mitosis, some of the cells must have completed protein synthesis before the end of DNA synthesis. Therefore, if labeled thymidine and puromycin are added together, labeled metaphases should start to appear at the same time as in the control although the rate of increase of labeled figures would be reduced.

The data plotted in Fig. 11 shows that the first appearance of labeled metaphases was delayed by inhibition of protein synthesis. The G_2 period was increased by approximately 0.9 and 2.3 hours for α equal to 0.5 and 0.3, respectively. At a relative rate of synthesis of 0.2 the appearance of labeled metaphases was further delayed, but the mitotic

index became too low for accurate counting. The experiments suggest that the last step in the pathway to division which involves protein synthesis is completed during the G_2 period. The distribution in the time interval from the end of this step to the beginning of mitosis does not appear to be very large since it does not overlap the timing of the end of DNA synthesis.

(2) CELL FRACTIONATION: The decreased rate of total cell protein synthesis is not necessarily a valid measure of the rate of one or a small number of proteins synthesized during a particular interval in the cycle. In less than ½ hour the over-all rate reached a constant value (Fig. 6). It may be asked whether there is some cellular compartment in which the rate of synthesis declines instead over a 7-hour period. Cells were fractionated into nuclei, particulate cytoplasm (microsomes plus mitochondria), and soluble cytoplasm. Incorporation of C^{14}-valine into all three fractions was inhibited by the maximum amount in less than 1 hour. Thus, there is no evidence from this experiment for a cellular fraction in which the rate of protein synthesis decays over an exceptionally long interval. The objection may still be raised that synthesis of specific protein (s) concerned with entry into cell division may be only a small fraction of total protein synthesis in unsynchronized cultures and, therefore, the cell may be able to overcome the inhibition without necessarily affecting the over-all rate. This explanation appears unlikely, particularly at high levels of inhibition. It could perhaps be tested by examining the effect of puromycin on enzyme induction, but no kinetic studies of induction are as yet available on mammalian cells.

CONCLUSIONS

The concentration of chloramphenicol necessary to inhibit protein synthesis in mammalian cell cultures is very high (10^{-3} M) and the relative rates of both RNA and protein synthesis are reduced to the same extent.

The difference between mammalian and bacterial cells lies in the sensitivity of protein synthesis, since a comparison of our results with the data of Kurland and Maaløe (16) shows that RNA synthesis is at least as sensitive to chloramphenicol inhibition in *E. coli* as in mammalian cells. In addition, the drug has a toxic effect on the mitotic spindle which does not seem to be related to its effect on RNA or protein synthesis since it occurs at low levels of inhibition. Earlier work on spindle formation in newt cells in the presence of chloramphenicol over the same concentration range demonstrated a similar toxic effect, namely, the production of rosette metaphases in which the chromosomes were distributed about a very short spindle or pair of asters (2). The hypothesis was advanced that this effect was caused by stopping protein synthesis before formation of spindle precursor protein was completed. The present results indicate that this explanation is incorrect, since a fraction of the cells continued to enter metaphase and anaphase with normal spindles for several hours after addition of chloramphenicol, while the mitotic index increased and then slowly declined to a low value. Chloramphenicol appears to produce two distinct effects on cell division: (1) a direct effect on the mitotic spindle for those cells which enter mitosis shortly after application of the drug; (2) a slowly decreasing rate of entry of cells into mitosis, caused by inhibition of protein synthesis.

In the puromycin experiments an increase in the mitotic index was not detected. This provides further evidence for the conclusion that the short spindles appearing shortly after application of chloramphenicol are not a direct result of the inhibition of protein synthesis. The occurrence of a lag before the index decreased and the fact that the duration of mitosis was not increased by the drug indicates that the synthesis of proteins necessary for mitosis is completed slightly before the end of interphase.

A protein or proteins which act as precursors for the mitotic apparatus must, therefore, be present in late interphase and early prophase. This conclusion might be expected on general grounds. If the spindle protein is 6 per cent of the total cell protein and if all of the protein-forming machinery were used for synthesis of this protein, the process would require about 2 hours. The formation of the spindle in late prophase is accomplished in about 15 minutes (17), and, therefore, it must be assembled from a pre-existing protein. Also, direct evidence for the presence in interphase cells of a protein precursor of the spindle has been obtained for sea urchin eggs (18).

The rate of entry into prophase is eventually determined by the rate of protein synthesis since, after the period of decreasing index, the relative mitotic index is equal to the relative rate of protein synthesis. The cells are able to establish a new

steady state growth rate if the rate of protein synthesis is not reduced to a value less than 0.3 of the normal value. For lower rates of synthesis, cell degeneration is evident after about 9 hours. Mammalian cells in logarithmic growth have a rate of protein turnover which may be as large as 20 per cent of the net rate (19). If turnover is not reduced in the same proportion as synthesis, at high levels of inhibition the cell may not be able to maintain a balance between synthesis and breakdown of protein.

In addition, at the highest level of inhibition the rate of DNA synthesis declined gradually to zero in 9 to 11 hours. This effect, which may be similar to the slow decline of DNA synthesis in a step-down experiment in bacteria (20), is under study and the details will be reported elsewhere. It cannot be the cause of the slowly decreasing mitotic index since, when DNA synthesis is directly blocked with 5′-fluorodeoxyuridine, mitosis continues for several hours at the normal rate (21).

The duration of the G_2 period was increased by partial inhibition of protein synthesis. This finding indicates that the last step requiring protein synthesis occurs during the G_2 period. The mitotic index begins to decrease about 1 hour after addition of inhibitor; thus, the protein-synthetic step is completed during the latter part of G_2, or about 2 hours after the end of DNA synthesis. The beginning of this step cannot be determined directly from these experiments. The theory proposed in the next section yields a value of 2.5 hours for the normal duration of this step.

The nature of the chemical processes occurring during G_2 is unknown. It has been shown by Yamada and Puck (22) that low doses of x-rays produce a reversible mitotic delay localized in the G_2 period. These authors have suggested that since chromosomal condensation occurs during this period, the primary radiation effect may be the production of chromosomal damage or interference with the chemically mediated condensation process. If the synthesis of structural proteins or enzymes is required for condensation, a similar explanation may be proposed for the mitotic delay found in our experiments. This interpretation might explain the observation that a small fraction of the cells entering mitosis 1 to 2 hours after addition of inhibitor show chromosomal fusion at metaphase.

Kinetics of the Inhibition Process

The slow decline of the mitotic index measures a slowly decreasing rate of entry into mitosis after partial inhibition of protein synthesis. As shown above, this period does not appear to be a result of a statistical distribution in the time relative to division at which the last step in protein synthesis is completed. The mechanism of inhibition might be better understood if the shape of the mitotic index curves could be calculated from some simple model of the inhibition process.

To this end, we have calculated the index versus time curves from a number of quantitative models. The details of the mathematical treatment are given in the Appendix. In this section we are concerned with the assumptions and the calculated results. In all models we assume that a cell must accumulate a given amount of a compound, either a protein or the product of an enzyme reaction before mitosis can be initiated. Although a number of such steps may occur, we assume that only one of these is rate limiting. If the doubling time of the culture is T_0, we assume that an age T can be defined for an individual cell, $0 \leq T \leq T_0$. (Thus we ignore the statistical distribution of ages discussed in the section on radioautography. If the distribution is such that most of the cells are within a 2-hour interval in age, then the curve calculated from the model will be spread out by an amount less than 2 hours).

Model I

We assume a protein must be synthesized during an age interval $T_2 - T_1$ which terminates before microscopically visible prophase. The various cell age parameters are shown in Fig. 13. The duration of mitosis ΔT_M is given by the relation $M = \lambda \Delta T_M$, where M is the mitotic index and $\lambda = 0.693/T_0$. The mitotic index for a doubling time of 24 hours was always in the range from 0.025 to 0.030, corresponding to a value for ΔT_M of 1 to 1¼ hours. ΔT_M was taken as 1 hour in the calculations.

CASE 1: Initially, we assume v, the rate of synthesis, to be a constant such that $v(T_2 - T_1) = 1$. After addition of inhibitor the rate is reduced to αv, but we assume the condition still holds that one unit of protein must be synthesized. Cells of age greater than T_2 will be unaffected by inhibition. The mitotic index begins to decrease in 0.75 hours, which fixes the value of T_2, namely $T_2 = T_0 - \Delta T_M - 0.75$.

Cells with ages in the interval $T_2 - T_1$ will be delayed and, therefore, divide at an age which increases for cells of decreasing age at the beginning of inhibition. During the period of index decrease, the mitotic index will not bear a simple relationship to the growth rate. Therefore, to calculate the index, the system must be referred to the logarithmic growth state at $t = 0$ (the time of addition of inhibitor). The cells in the mitotic interval ΔT_M at any time t are supplied from an age interval at $t = 0$ which shifts to smaller ages as t increases. Initially, the duration of this supply interval is equal to ΔT_M. The decrease in the mitotic index is determined by the decrease in this interval. Thus, the mitotic index as a function of time is obtained by calculating the duration of the interval supplying the cells in mitosis at successive times.

For this simple model in which v is constant, the mitotic index is given by Curve *1*, Fig. 14, for the case $\alpha = 0.3$. The index decreases from the initial to the final value α in the time ΔT_M. The shape of this theoretical curve clearly does not fit the experimental data (Fig. 10).

We next consider the case in which v is not a constant. This implies that the rate of synthesis of the required protein is controlled by the cell. We assume that the control mechanism operates by reducing the rate in proportion to the amount of protein already synthesized. It is necessary to specify the response of the control mechanism to partial inhibition of protein synthesis. Two cases are considered.

CASE 2: The cellular control mechanism acts independently of the added inhibitor. After addition of puromycin the rate continues to be reduced in proportion to the protein synthesized.

CASE 3: The cellular control mechanism acts to prevent the rate from exceeding a value determined by the amount of protein already present. After the rate is reduced by puromycin, cellular control is inactivated and protein is formed at a constant rate, until this rate becomes equal to the value permitted by the cell for the amount of protein present.

The mitotic index curves calculated for Case 2 are identical with those obtained from the simple model, v = constant (Curve 1, Fig. 14). For Case 3, the steady state index depends on the form of the function chosen for v, but the period of decreasing index is still equal to ΔT_M. The index curve, using a quadratic function for v, is given in Fig. 14, Curve *3*.

Therefore, including in the model a fairly general time dependence of the rate of synthesis of the limiting protein does not lead to agreement with experiment.

As an aid to finding a model which will successfully predict the experimental curves, it is of interest to determine why the simple model fails. A numerical example will serve to clarify the argument. Let the limiting protein be synthesized at a constant rate ending at age 22 hours, the generation time be 24 hours, the duration of mitosis be 1 hour, and the rate of protein synthesis be reduced to 0.5. Cells with ages less than 22 hours are delayed by an amount which increases as their age at the time of inhibition decreases. Cells of age 22 hours reach mitosis in 1 hour and complete mitosis in 2 hours. One hour after inhibition the cells in mitosis have been supplied by the cells in the age interval 22 to 23 hours. Cells of age 21.5 hours are delayed 0.5 hours and reach mitosis in 2 hours. Therefore, 2 hours after inhibition the cells in mitosis have been supplied by the cells in the age interval 21.5 to 22 hours. Similarly, at 3 hours the cells were supplied from the interval 21 to 21.5 hours. The number of cells in successive age intervals is proportional to the duration of the interval if the total interval is small compared with the doubling time. Therefore, the relative mitotic index decreases from 1 to 0.5 between 1 and 2 hours after inhibition and then remains constant.

Thus, an increasing mitotic delay does not necessarily lead to a changing index. If the delay is a linear function of $(T_2 - T)$, where T_2 is the age at the end of the limiting protein step and T is the age at inhibition, the mitotic index will be constant as soon as all of the cells in mitosis are supplied from cells which were younger than T_2 at the time of inhibition. This suggests that models be investigated in which the delay increases nonlinearly with decreasing age.

Model II

The condition is fulfilled by a mechanism in which the limiting protein is an enzyme. The product of the reaction catalyzed by the enzyme is assumed to be required in a definite amount before mitosis can be initiated. The following additional assumptions are made: the enzyme is synthesized at a constant rate during the age

interval $T_2 - T_1$; the rate of product formation is proportional to enzyme concentration; product is made during the interval $T_3 - T_1$, where $T_2 > T_2$. The interval $T_3 - T_2$ is equal to the time during which the cell is no longer sensitive to inhibition of protein synthesis. The mitotic index curve obtained from this model is shown in Fig. 14, Curve *2*, for α equal to 0.3. The shape of the curve depends markedly on the value of $T_2 - T_1$. It was chosen to obtain a fit to the experimental data for $\alpha = 0.2$ (Fig. 10). The curves calculated for $\alpha = 0.3$ and 0.5 agree reasonably well with the experimental points. The experimental results shown in Fig. 10 were not selected from available data on the basis of best fit to the theoretical curves, but represent an experiment in which the relative rates of protein synthesis and mitotic indices were measured at three different levels of inhibition.

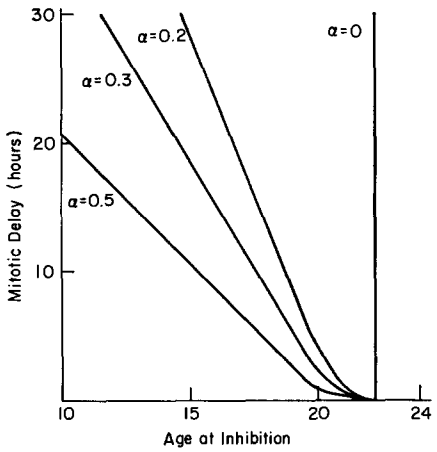

FIGURE 12 Delay of division versus age of the cell at time of inhibition for relative rates of protein syntheses α. Curves calculated for Model II. For details see text.

The model contains only one adjustable parameter, $T_2 - T_1$, which was assigned the value 2.5 hours. Therefore, the beginning of the sensitive step occurs at 19.75 hours. This corresponds approximately to the beginning of G_2.

Equation 9, given in the appendix, can also be used to predict the mitotic delay as a function of the age of the cell. In Fig. 12 the delay is plotted for $\alpha = 0$, 0.2, 0.3, and 0.5. The period of decreasing index arises from the non-linear region corresponding to the $T_2 - T_1$ interval. The delay in the appearance of labeled mitoses after partial inhibition of protein synthesis can be obtained directly from Fig. 12. In the radioautograph experiments the G_2 interval is measured with respect to metaphase, but it must be referred to division to predict the delay times. Also there will be a small correction for the time elapsed from the addition of labeled thymidine to the incorporation of sufficient radioactivity into DNA to give a detectable grain count. To calculate the delay, 0.75 and 1 hour were added to G_2. The predicted delay times in the appearance of 50 per cent labeled metaphases are 0.7 to 0.9 and 1.9 to 2.3 hours for $\alpha = 0.5$ and 0.3, respectively, while the experimental values were 0.9 and 2.3 hours. The shape of the per cent labeled mitoses versus time curves can also be obtained by adding the appropriate delay times to successive points on the control curve. The results shown in Table II, column 3, for a relative rate of protein synthesis of 0.3 were calculated for an uncertainty of 0.25 hours in the time of labeling of the control. The calculated labeling curve agrees as well as can be expected with the experimental results. The shape of the calculated labeling curve depends markedly on the value of the parameter $T_2 - T_1$ which was originally chosen to fit the mitotic index experiments. Therefore agreement with the labeling data provides additional support for the model. It should be noted that the labeling curve predicted by the simple linear model does not agree with the experimental data.

TABLE II

Time of Appearance of Labeled Metaphases after Partial Inhibition of Protein Synthesis

Per cent labeled metaphases	Time observed	Time calculated
	hrs.	*hrs.*
3	3.1	2.5–2.7
12	3.7	3.5–3.9
50	5.3	4.9–5.3
80	7.3	6.5–7.3
93	8.7	8.6–9.4

Percentage of labeled metaphase figures at various times after addition of tritium-labeled thymidine for a relative rate of protein synthesis of 0.3. The range of values given in Column 3 was calculated from Model II described in the text, assuming an uncertainty of 0.25 hours in the time of appearance of a given percentage of labeled figures in the control culture.

The effects of inhibition of protein synthesis on cell division have been studied in synchronized cultures of *Tetrahymena pyriformes* (23), and the results are in some respects similar to those reported here. A delay of division was obtained which increased with the concentration of *p*-fluorophenylalanine. There was no delay if the inhibitor was added at 65 minutes after the last heat shock for a culture which would normally undergo synchronous division at about 80 minutes. For comparison with mammalian cells, times must be expressed in units of the doubling time T_0. Thus the *Tetrahymena* cells become independent of protein synthesis at about 0.75 T_0 while for mammalian cells the corresponding value is 0.92 T_0.

An exposure to inhibitor for a short period, caused a "set-back." (The division time was greater than T_0 plus the duration of exposure to inhibitor). Moreover the set-back increased with the age of the cell up to the critical age at which the cell became independent of protein synthesis. It was postulated that there is a competition between synthesis and removal of proteins necessary for division which, under normal conditions, leads to an accumulation. When protein synthesis is inhibited, these proteins are removed and the cell requires a certain period of time to resynthesize them. Since this explanation is quite different from the one which we have proposed, it is important to determine whether the set-back could explain our results.

In the *Tetrahymena* experiments, the relative rate of protein synthesis was determined at a single inhibitor concentration only so that a direct comparison with mammalian cells is difficult to make. However, from the concentrations used in various experiments it can be inferred that a set-back occurs even for partial inhibition of protein synthesis. In our system, according to the set-back model cells of age greater than 22 hours should go through division without delay while all younger cells in the population should be set-back by an amount which increases with increasing age. Therefore after 2 or 3 hours, division should stop since the time necessary to reach division is increasing for all the cells in the population. This type of response was not found for $\alpha \geq 0.3$. Instead, cell division continued at a reduced rate for more than 12 hours after addition of inhibitor. Cultures resuspended on fresh medium after 6 hours returned to the normal rate of protein synthesis in about 1 hour and the normal division rate in 3 hours.

Whether or not a set-back can occur is determined by the rate of protein synthesis at which a balance is achieved between synthesis and removal of division proteins. In *Tetrahymena* cells, the balance appears to be reached for a relatively small reduction in the rate of protein synthesis. In mammalian cells, steady state growth is obtained for relative rates greater than 0.3. Therefore, for inhibition of protein synthesis by not more than 70 per cent the set-back model would not appear to be applicable to mammalian cells. However, at higher levels of inhibition the model which we have proposed is not valid since a steady state rate of division is not established.

APPENDIX

Kinetics of Mitotic Inhibition

Logarithmic growth is defined by the relation $N = N_0 e^{\lambda t}$, where $\lambda = \log_e 2/T_0$, T_0 is the doubling time, and N and N_0 are the number of cells per unit volume at time t and $t = 0$, respectively. We shall assume that a unique age T, $0 \leq T \leq T_0$ may be assigned to each individual cell, in the sense that all cells of the same age are carrying out the same step in the growth cycle. The statistical distribution of cell ages will not be considered. The age distribution in logarithmic growth is given by $dN_T = 2\lambda N_0 e^{-\lambda t}\, dT$, where dN_T is the number of cells of age T to $T + dT$ at a reference time $t = 0$.

The mitotic index M is the relative number of cells in the interval ΔT_M terminating at T_0, where ΔT_M is the duration of mitosis.

$$M = \int_{T_0 - \Delta T_M}^{T_0} dN_T \Big/ N_0 = e^{\lambda \Delta T_M} - 1 \fallingdotseq \lambda \Delta T_M .$$

The experimental error in the determination of M is usually much larger than the error introduced by approximating the index by $\lambda \Delta T_M$.

The index can be expressed in another way which is more suitable for the calculation to follow. At a time t in the future, the cells in mitosis will be drawn from the cells in the age interval $T - \Delta T_M$ to T at $t = 0$, where $T = T_0 - t$,

$$M = \int_{T - \Delta T_M}^{T} dN_T \Big/ N \qquad \text{(equation 1)}$$

$$N = N_0 e^{\lambda (T_0 - T)}$$

and we obtain $M = e^{\lambda \Delta T_M} - 1$, as before.

Model I

CASE 1: We assume that a process of protein synthesis occurring during the interval $T_2 - T_1$ must be completed before mitosis can begin. Let ΔT_C be the interval between T_2 and the first microscopically visible stage of prophase. The relation between the various values of T is illustrated in Fig. 13. The rate of synthesis v is assumed to be constant; $v(T_2 - T_1) = 1$; α = rate of protein synthesis after inhibition relative to the control. The short period before the inhibited rate becomes a constant will not be considered. The population is divided into three classes by the inhibitor:

1) Cells of age $T \geq T_2$ are not affected by the inhibitor and divide at age T_0.
2) Cells of age T, where $T_1 < T < T_2$, will require a longer time to complete synthesis of the limiting protein. These cells divide at an age greater than T_0 which increases as T decreases in the interval $T_2 - T_1$.
3) Cells of age less than T_1 will not be differentially affected by inhibition. These cells can be considered to belong to a population with doubling time T_0/α, since the rate of progress through the cell cycle will be reduced in proportion to the reduction in the rate of protein synthesis.

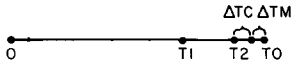

FIGURE 13 Relation of cell age parameters as defined in the text.

To obtain the mitotic index at any time t after inhibition, we note that at $t = 0$, the cells are in logarithmic growth and the index can be obtained from equation 1. It is necessary to determine the age interval at $t = 0$ which provides the cells in the mitotic interval at time t. Let this interval be $\Delta T_i = T'' - T'$. Cells of age T' at $t = 0$ divide at age T'_0. These cells reach mitosis at time t, such that $T' + t = T'_0 - \Delta T_M$. Cells of age T'' at $t = 0$ divide at age T''_0, such that $T'' + t = T''_0$. Therefore,

$$\Delta T_i = \Delta T_M + (T''_0 - T'_0) \quad \text{(equation 2)}.$$

$T'_0 = T' + x + \Delta T_C + \Delta T_M$ where x is the time required to complete the synthesis of the limiting protein.

$$v(T' - T_1) + \alpha v x = 1 = v(T_2 - T_1).$$

Therefore,

$$x = (T_2 - T')/\alpha \quad \text{and} \quad T'_0 = T_0 + (T_2 - T')(1 - \alpha)/\alpha. \quad \text{(equation 3)}.$$

A similar equation holds for T''.

It is necessary to consider the three classes of cells defined above:

1) If T' and T'' are both greater than T_2, $T'_0 = T''_0 = T_0$ and $\Delta T_i = \Delta T_M$.
2) If T' and T'' are both in the interval $T_2 - T_1$, then from equations 2 and 3 we obtain $\Delta T_i = \alpha \Delta T_M$.

If $T'' > T_2$ and $T' < T_2$, then $T''_0 = T_0$ while T'_0 is given by equation 3. Therefore,

$$\Delta T_i = \Delta T_M - (T_2 - T')(1 - \alpha)/\alpha.$$

After substitution for T' and rearrangement, we obtain

$$\Delta T_i = \alpha \Delta T_M + (1 - \alpha)(T_0 - T_2) - (1 - \alpha)t.$$

Since the first two terms on the right are constants, the interval decreases linearly with time from the value ΔT_M for $t = T_0 - T_2 - \Delta T_M$ to $\alpha \Delta T_M$ for $t = T_0 - T_2$. Therefore, the interval ΔT_i decreases linearly to the minimum value in a time ΔT_M.

3) If $T' < T_1$ or T' and T'' are less than T_1, our assumptions require $\Delta T_i = \alpha \Delta T_M$.

The mitotic index is given by equation 1.

$$M = \int_{T''-\Delta T_i}^{T''} dN_T \Big/ N = \frac{2N_0 e^{-\lambda T''} \lambda \Delta T_i}{N}$$

At a time t such that ΔT_i has decreased to the minimum value, $T'' = T_2$ and $t = T_0 - T''$. The number of cells present will differ by less than 2 per cent from the number attained in logarithmic growth. Therefore, N is given approximately by

$$N = N_0 e^{\lambda t} = 2N_0 e^{\lambda T''}, \quad \text{and} \quad M = \lambda \Delta T_i.$$

The relative mitotic index is $M_r = \Delta T_i / \Delta T_M$. Thus, the model predicts a linear decrease in M_r

from 1 to α in the time interval ΔT_M. The curve for $\alpha = 0.3$ is plotted in Fig. 14, Curve *1*.

Generalization of Model I

We next consider cases in which v is not constant throughout the period $T_2 - T_1$. We assume that the rate is reduced by a cellular control mechanism in proportion to the amount of protein synthesized. If the rate is taken to be proportional to one minus the amount present at age T, then v will be an exponentially decreasing function of T. However, it is more convenient to represent v by a power of $(T_2 - T)/(T_2 - T_1)$, since the

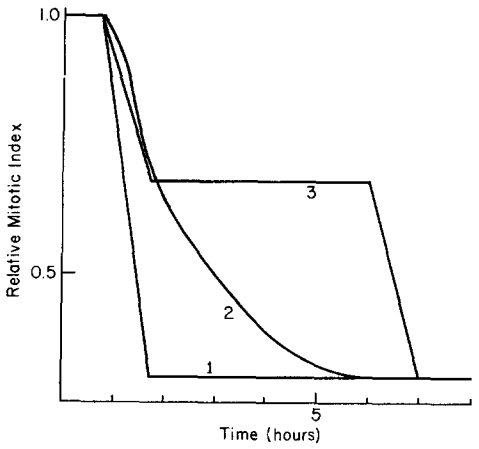

FIGURE 14 Relative mitotic index versus time curves calculated from Model I, case 1, or case 2 (*1*); Model I, case 3 (*3*), and Model II (*2*). The values T_2–$T_1 = 2.5$ hours, ΔT_c or T_3–$T_2 = 0.75$ hours, and $\alpha = 0.3$ were employed in all calculations.

exponential does not satisfy the upper boundary condition $v = 0$ for $T = T_2$. Let

$$v = v_0((T_2 - T)/(T_2 - T_1))^j.$$

Let A be the amount of product,

$$A = \int_{T_1}^{T} v\, dT.$$

Since $A = 1$ when $T = T_2$, we obtain

$$v_0 = (j + 1)/(T_2 - T_1)$$

and

$$v = (j + 1)(T_2 - T)^j/(T_2 - T_1)^{j+1} \quad \text{(equation 4)}$$

$$A = 1 - ((T_2 - T)/(T_2 - T_1))^{j+1} \quad \text{(equation 5)}$$

Since the rate of protein synthesis is under control by the cell, we must also specify the response of the control system to inhibition. Two types of behavior will be considered.

CASE 2: The rate after inhibition continues to decrease as product accumulates, but since the rate has been reduced by the inhibitor, the time interval of synthesis must be increased. The rate after inhibition is represented by

$$v = \alpha v_{T'}((T_2' - T)/(T_2' - T'))^j$$

where T_2' is the age at which synthesis is complete, T' is the age, and $v_{T'}$ is the rate at the time of addition of inhibitor.

CASE 3: The control system is such that for a given amount of product the rate cannot exceed the value specified by equation 4. Therefore, after inhibition the rate remains constant at $\alpha v_{T'}$ and product accumulates until the rate permitted for the amount of product present becomes equal to $\alpha v_{T'}$. The rate then decreases in the same manner as the control.

Predicted Decay Curves for Generalized Model I

CASE 2: The calculation is made by the same method used in Case 1.

A cell of age T' at inhibition completes the synthesis of the limiting protein in a time $T_2' - T'$ given by

$$1 - A_{(T')} = \int_{T'}^{T_2'} v\, dt.$$

After some manipulation we obtain

$$(T_2' - T') = (T_2 - T')/\alpha$$

This equation is identical with equation 3 for the linear model. Thus, for Case 2 the index curve is identical with that calculated for the linear model.

The problem can also be solved using a series expansion with arbitrary coefficients to represent v, and the resulting index curve is still identical with Case 1. Therefore, agreement cannot be obtained with the experimental results using the assumptions made for Case 2, even if an arbitrary function is used to represent v.

CASE 3: After inhibition the rate is constant until age T_a when the amount of product is equal

to the amount $A_{(T_b)}$, which would be formed by the uninhibited cell of age T_b such that

$$\alpha v_{T'} = v_{T_b} \qquad \text{(equation 6)}$$

Therefore,

$$T_a - T' = (A_{(T_b)} - A_{(T')})/\alpha v_{T'}. \qquad \text{(equation 7)}$$

The balance of the protein $1 - A_{(T_b)}$ is made in the interval $T_2 - T_b$.

$$T_0' = T' + (T_a - T') + (T_2 - T_c) + \Delta T_c + \Delta T_M \qquad \text{(equation 8)}$$

By substituting in equation 8 the values of $T_a - T'$ and $T_2 - T_b$ given by equations 6 and 7, we obtain after rearrangement

$$T_0' = T_0 + \left[\frac{1 + j(\alpha)^{\frac{j+1}{j}} - (j+1)\alpha}{(j+1)\alpha}\right](T_2 - T')$$

For cells of ages T' and T'' at inhibition, where both ages are in the interval $T_2 - T_1$, the relative mitotic index is given by

$$M_r = \frac{\Delta T_i}{\Delta T_M} = \frac{(j+1)\alpha}{1 + j(\alpha)^{\frac{j+1}{j}}}.$$

The complete curve for $\alpha = 0.3$ is plotted in Fig. 14, Curve *3*. The model predicts that the index will decay to the limiting value given above in a time ΔT_M. The limiting value is no longer equal to α unless $j = 0$.

Model II

We assume that the rate-limiting step is the synthesis of an enzyme. The product of a reaction involving the enzyme must reach unit concentration before mitosis can begin. Let the enzyme be made at a constant rate v in the interval $T_2 - T_1$. Let the product be made at a rate q proportional to the enzyme concentration in the interval $T_2 - T_1$. $T_3 - T_2$ is the interval during which the cell is insensitive to inhibition.

$$q = kE,$$

where E is the enzyme concentration. The interval of enzyme synthesis is short compared to the doubling time, so we can ignore the increase in cell volume during enzyme synthesis and $E = v(T - T_1)$. Therefore, $q = kv(T - T_1)$ and

$$A = \int_{T_1}^{T} q\, dT.$$

If $T \leq T_2$, $A = (kv/2)(T - T_1)^2$. During the interval $T_3 - T_2$, the rate of product synthesis is k; thus, if $T_2 \leq T \leq T_3$,

$$A = (kv/2)(T_2 - T_1)^2 + k(T - T_2).$$

When $T = T_3$, $A = 1$; therefore,

$$k = 2/(2T_3 - T_1 - T_2).$$

The age at division is calculated in the same manner as for Model I. For a cell of age T' at inhibition the product already present is

$$A_{(T')} = (kv/2)(T' - T_1)^2.$$

After addition of inhibitor, the time required to complete the synthesis of enzyme at the rate αv is $(T_2 - T')/\alpha$. The total amount of product present when enzyme synthesis is complete is given by the equation

$$A = (kv/2)(T' - T_1)^2 + (kv/\alpha)(T_2 - T') + (kv/2\alpha)(T_2 - T')^2.$$

The balance, $1 - A$ is made at maximum rate k in the time interval $(1 - A)/k$. Therefore,

$$T_0' = T' + (T_2 - T')/\alpha + (1 - A)/k + \Delta T_M. \qquad \text{(equation 9)}$$

If T' and T'' are in the interval $T_2 - T_1$, we obtain

$$\Delta T_i = \alpha \Delta T_M - (\alpha/k)(A'' - A'),$$

where A' and A'' are the amounts of product present at the end of enzyme synthesis in cells of ages T' and T'' at inhibition.

After substitution of $T'' = T' + \Delta T_i$, we obtain

$$\frac{(\alpha - 1)v}{2\alpha}(\Delta T_i)^2 + \frac{(\alpha - 1)v(T' - T_1)}{\alpha}(\Delta T_i) - \Delta T_M = 0$$

This equation, together with the relation $T' +$

$t = T'_0 - \Delta T_M$, can be solved for $\Delta T_i/\Delta T_M$ for any given t. The form of the curve depends on the value of $T_2 - T_1$. This interval was selected to be 2.5 hours by trial and error to obtain a fit to the experimental data for $\alpha = 0.2$. The complete curves for $\alpha = 0.2$, 0.3, and 0.5 are shown in Fig. 10.

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