LIFE CYCLE ANALYSIS OF MAMMALIAN CELLS

III. The Inhibition of Division in Chinese Hamster Cells by Puromycin and Actinomycin

R. A. TOBEY, D. F. PETERSEN, E. C. ANDERSON, *and* T. T. PUCK

From the Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico, and the Eleanor Roosevelt Institute for Cancer Research, Department of Biophysics, University of Colorado Medical Center, Denver

ABSTRACT Analysis of the effects of actinomycin and puromycin on the G_2 and mitotic parts of the life cycle in Chinese hamster ovary cells grown in suspension and synchronized by thymidine treatment has been carried out. Rates of division of partially synchronized cell populations were measured in the presence and absence of the drugs, and various controls were performed to test for absence of complex side effects. Actinomycin produces a block 1.9 hr before completion of division, while puromycin produces a block almost coinciding with the initiation of mitosis. Evidence is presented that the puromycin block may be a double one, inhibiting one kind of protein synthesis that virtually coincides with the beginning of mitosis and another that occurs about 8 min earlier. The data are interpreted in terms of the time interval between messenger formation and its associated protein synthesis in this region of the life cycle. The various events studied have been provisionally mapped in the G_2 and mitotic periods of the life cycle.

INTRODUCTION

A systematic investigation of the timing of specific biochemical events has been undertaken to define temporal markers in the life cycle of mammalian cells. The present study was designed to analyze the effect on cell division of inhibition of synthesis of protein and messenger RNA, respectively, during the G_2 and mitotic periods in Chinese hamster ovary (CHO) cells grown in suspension. Actinomycin D was employed to inhibit messenger RNA formation, and puromycin to inhibit protein synthesis. Previous investigators (for example, Taylor, 1963; Kishimoto and Lieberman, 1964) have studied the effects of such agents on cell division and demonstrated actions to occur in the G_2 region. The present paper localizes more precisely the points of action of these agents in the life cycle, and attempts to draw

specific inferences about the protein and messenger RNA biosynthetic processes which are involved.

A general method for determining the position in the cellular life cycle at which chemical agents exert their action has been devised by Puck and Steffen (1963) and involves scoring of the accumulation of cells in a recognizable stage of the life cycle (e.g. mitosis) behind a block produced by a specific inhibitory agent (e.g. colcemide). The present study utilizes a modified methodology which permits advantage to be taken of the rapid and precise scoring afforded by the electronic cell counter, as well as mitotic index measurements.

THEORY

In the accumulation method of life cycle analysis (Puck and Steffen, 1963) mitotic figures are scored by microscopic analysis at various time intervals after addition of colcemide, and the results are expressed as an accumulation function which is linear for a random culture but which deviates from linearity in predictable fashion for cultures in which inhibition has been introduced at any point in the life cycle (Puck, Sanders, and Petersen, 1964). In the present modification, colcemide is not used but the cells are allowed to go on to complete division, and samples are removed periodically for counting. Thus, the logarithm of the total cell number would be a linear function of time in a random culture. If at zero time an inhibitor is added which stops progress of the cells around their life cycle at a point t_1 hr before completion of mitosis, the logarithm of the cell number would increase linearly throughout the interval $t = 0$ to $t = t_1$, at which point the curve would flatten and the time of inhibition would be defined by the intersection of the growth curve and the horizontal line. If the agent merely changes the growth rate at the given point (t_1), the point is still identifiable by the intersection of the two lines, but in this case the second line will have a slope greater than zero but less than that of the first. Finally, if the agent halts cell progression at several different points, only the point nearest the completion of mitosis will, in general, be defined although special situations may arise as discussed later.

If partially synchronized cultures are employed, sharper delineation of the point of action of inhibitors is possible. It has been shown (Puck, 1964) that an excess of thymidine specifically and reversibly interrupts DNA synthesis of mammalian cells uniformly throughout the S period, so that all cells originally in S are held there while the remainder of the population accumulates at the G_1/S boundary. After such accumulation is completed, the block is released by removal of excess thymidine and the cells resume traverse of the cycle. Multiplication of the resulting two-component population is reinstated after a lag equal to the $(M + G_2)$ periods. The leading portion consists of cells originally trapped in S and shows an exponential growth rate equal to that of the random culture. This portion can be eliminated by application of a second thymidine block (Petersen and Anderson, 1964; Puck,

1964), but it is more convenient to use its appearance as an indicator of the approach to mitosis of the larger, more highly phased population and to add the inhibitor at times such that cell division ceases during this second phase. Cells of this phase, comprising about 50 to 60% of the initial population, would divide simultaneously if synchrony were perfect; however, dispersive effects broaken the wave front during the 6 to 7 hr required to reach division (Anderson and Petersen, 1964). The resultant rate of division is, therefore, not infinite but about 3 times greater than that of the random population, giving a corresponding increase in precision of timing.

An exponential accumulation function is not expected for this synchronized portion of the population, since the culture is not random and the shape of the division wave depends on the mechanism which disperses the synchrony. This dispersion is believed to result from random variations in generation times. (Engelberg, 1964; Anderson and Petersen, 1964). Kubitschek (1962) has shown that this variation can be described in terms of a normal distribution of growth rates leading to an asymmetric distribution of generation times (see also Quastler, 1963). Because of the brief duration of the synchrony wave in the present experiments, it is difficult to distinguish among alternative possibilities for the accumulation function, and we have found that normal distributions of rates and division times and an exponential accumulation all fit the data within experimental error and yield similar values for calculating inhibition times. In this paper, therefore, we use an exponential approximation.

MATERIALS AND METHODS

Partial Synchronization of Cells. Cloned Chinese hamster ovary (CHO) cells (Tjio and Puck, 1958) were grown in suspension culture in F-10 medium lacking calcium (Ham, 1963), supplemented with 10% calf and 5% fetal calf sera and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were synchronized by reversible inhibition of DNA synthesis with thymidine, a method allowing production of large quantities of partially synchronized CHO cells in the suspension culture (Petersen and Anderson, 1964). Thymidine was added to random cell cultures to a final concentration of 5 mM and, after incubation for a period somewhat longer than the sum of the postsynthetic (G_2), plus mitotic (M), plus presynthetic (G_1) portions of the life cycle [7.6 hr for cells growing randomly with a 12.4 hr generation time (Puck, Sanders, and Petersen, 1964)], the culture was released from thymidine blockade by resuspension in fresh growth medium. In certain experiments a second thymidine block was added following an incubation period of 5 hr [the synthetic (S) period plus 1 hr], and cells were released 9 hr later. This treatment eliminated the S portion of the division wave but did not change the slope of the main wave.

Total Cell Concentration. Aliquots of 2 ml were diluted with 8 ml of isotonic saline and counted immediately in an electronic particle counter (Kubitschek, 1958) using a 100 $\times$ 100 μ aperture. Total counts ranged from 0.3 to 2.0 $\times$ 10^6; therefore, the statistical errors were less than 0.6% in all cases.

Determination of RNA and Protein Syntheses. RNA and protein syntheses

were determined by measuring the incorporation of H^3-uridine and C^{14}-leucine into trichloroacetic acid (TCA)-precipitable material as described by Tobey and Campbell (1965).

Mitotic Index Determination. Aliquots of 5 ml of cell suspension were centrifuged at 1000 RPM for 5 min, and the cell pellet was resuspended by dropwise addition of 5 ml of cold 0.1 M sucrose with continuous agitation. After 3 min in an ice bath, the cell suspension was again centrifuged as above. Cold acetic acid-methanol (1:3) was added dropwise with continuous stirring to a final volume of 5 ml. The sample was then stored in an ice bath for 10 min. Following low speed centrifugation, the cell pellet was resuspended in 0.1 ml of the acetic acid-methanol solution, applied to a slide, air dried, and stained with Jenner-Giemsa stain (Paul, 1960). A total of 500 cells was scored for mitotic figures.

Chemicals. C^{14}-leucine (201 mc/mM) and H^3-uridine (4.4 c/mM) were purchased from Schwarz Bio Research Inc., Orangeburg, New York, puromycin dihydrochloride was obtained from Nutritional Biochemicals Company, Cleveland, Ohio, and actinomycin D was a gift from Dr. Horace Brown of Merck, Sharp and Dohme, West Point, Pennsylvania.

EXPERIMENTAL RESULTS

Behavior of Control Cultures. Measurement of growth curves of random control cultures showed the expected exponential increase in cell concentration with generation times varying between 12 and 16 hr for different cultures. In a typical case, least-squares analysis of 8 determinations of cell number carried out at hourly intervals gave a generation time (T) of 14.13 ± 0.29 hr, or a growth rate ($d \ln n/dt$) of 0.049 ±0.001 hr^{-1}. The average coefficient of variation of the points about the fitted line was 0.7% compared with 0.6 to 0.7% expected from counting statistics alone. This agreement establishes the precision of the cell counting method and achievement of a completely random population. Durations of individual components of the life cycle were usually calculated in terms of fractions of generation times for a particular experiment and were reduced to absolute terms by multiplying by the mean generation time cited above. After application of the thymidine block and the usual M + G_2 delay, the growth rate dropped to zero (i.e. to less than 1/100th of its previous value).

The duration of mitosis (M) under the conditions of these experiments was determined from the area under the mitotic index versus time curves for a division wave in the control populations and the total fraction of the population which divided. For 3 experiments the mean value of M/T was 0.040 ± 0.008, in reasonably satisfactory agreement with our previous determination (Puck, Sanders, and Petersen, 1964) of 0.065 ± 0.015. Table I summarizes the results of the determinations of the duration of the phases of the life cycle obtained from the growth curves of the various control cultures used in these experiments and lists for comparison the results of Puck, Sanders, and Petersen (1964) for a similar CHO stock measured by the colcemide accumulation method without thymidine block.

TABLE I

LIFE CYCLE ANALYSIS OF CONTROL CULTURES

The fraction of the total life cycle occupied by various component parts is shown and compared with the corresponding values obtained in an earlier study. The total generation time (T) varied from 12 to 16 hr in the present study with a mean of 14.1 hr, while the mean in the previous study was 12.4 hr. The standard deviation of each experimental measurement was less than 10% except for that of M, the smallest of the time intervals, which had an uncertainty of 20%.

	$(M + G_2 + S)/T$	$(M + G_2)/T$	M/T	G_2/T	S/T
Determinations of this paper	0.625*	0.275‡	0.040§	0.225||	0.350||
Previous determinations (Puck, Sanders, and Peterson, 1964)	0.623	0.291	0.065	0.226	0.332

* Measured as the time lag between the removal of the thymidine block and the midpoint of the second linear portion of the division wave.

‡ Measured as the time lag between the removal of the thymidine block and resumption of the beginning of the first portion of the division wave.

§ Measured by integration of the mitotic index versus time curve as explained in the text.

|| Obtained by subtraction of appropriate values in the preceding columns.

The general agreement suggests that the thymidine blocking procedure has not grossly distorted the timing of those parts of the life cycle here studied.[1] The scatter of the measurements in these experiments carried out on separate days is larger than that observed in duplicates performed simultaneously, the method always used in comparing puromycin- and actinomycin-treated cultures with controls. Day-to-day variations in the effective time of release from thymidine block, an operation involving centrifugation and resuspension with the attendant possibility of transient perturbation of the growth rate, appear to be unavoidable. However, these perturbations are negligible for the measurement of the short time intervals in the vicinity of mitosis, with which this paper deals.

Rate and Specificity of Action of Puromycin and Actinomycin. The fact that puromycin inhibits protein synthesis with little or no primary action on DNA-based messenger formation, and that actinomycin acts specifically in the converse fashion has been documented by various investigators in both mammalian and microbial systems (for example, Collins, 1965, Baltimore and Franklin, 1963,

[1] Further evidence suggesting that synchronization with thymidine has not grossly altered cell behavior in this part of the life cycle is obtained from the width of the parasynchronous wave as it passes division. When the data are fitted to a normal distribution function, the standard deviation is found to be 1.0 hr at 8 hr after release. If the dispersion is a continuing stochastic process of the "random walk" type, this corresponds to a standard deviation of 1.3 hr per generation time of 14 hr (calculated from a square root of time dependence), or 9.3%. Puck and Steffen (1963) have found 8.5% (1.85 hr in a generation time of 22 hr) for random HeLa cells, and we have reported 9 to 13% for CHO by a different method (Anderson and Petersen, 1964).

Franklin and Baltimore, 1962, Nathans, 1964, Allen and Zamecnik, 1962). Experiments were carried out to determine the minimum concentration of each reagent needed to produce its specific effect in CHO cells at a rate sufficiently rapid to permit accurate time resolution of the life cycle consequences of these biochemical actions. Use of minimum effective amounts of each drug decreases the probability of bringing into play additional side-reactions which might complicate the processes studied.

Experiment demonstrated that with 50 μg/ml of puromycin and 2.0 μg/ml of actinomycin, the rate of action produced was sufficiently rapid for the present purposes, extensive inhibition of the specific synthesis occurring within a few minutes in each case. Raising the concentration of either substance by as much as 4 fold, produced no change in the pattern of biosynthesis inhibition, as measured by incorporation of the appropriate radioisotopically labeled precursor into acid-insoluble material, or in the time course of inhibition of cell division. Results of typical experiments are shown in Fig. 1*A* and *B*, wherein the drugs were added to each culture 5 hr after release from thymidine block and the rates of synthesis were followed for at least 3 hr, the time span encompassing the synchrony wave. In each case there resulted a marked depression of the uptake of radioisotope corresponding to the primary biosynthetic target of each drug, followed by a slower and less extensive inhibition of incorporation of the second label. This behavior conforms to expectation, because RNA synthesis must ultimately be inhibited following a primary suppression of protein synthesis, and conversely, prevention of messenger RNA formation will cause subsequent halt of protein synthesis. It should be pointed out that the relatively high residual rate of C^{14}-leucine incorporation in the presence of puromycin is not an index of continued formation of functional protein at a level of approximately 20% of normal. Previous studies have shown that in the presence of puromycin, amino acids continue to be incorporated into acid-insoluble, incomplete polypeptides terminating in a puromycin residue (Allen and Zamecnik, 1962; Nathans, 1964). Moreover, in our experiments, cell division following puromycin administration dropped sharply to zero at a reproducible point in time after puromycin administration, and remained at zero for periods of observation ranging up to 20 hr. Hence, the residual production of acid-insoluble label cannot reflect a reduced rate of progress through the life cycle by the inhibited cells. We conclude therefore that the puromycin treatment described results in cessation of synthesis of functional protein required for cell division within a few minutes after its addition to the culture.

Reversibility of the puromycin effect on protein synthesis was demonstrated in two ways. First, the rate of C^{14}-leucine incorporation returned to normal immediately after removal of puromycin by centrifugation, washing, and resuspension in puromycin-free medium. Second, a synchronized culture was held in G_2 for several hours by addition of puromycin. When puromycin was removed by thorough wash-

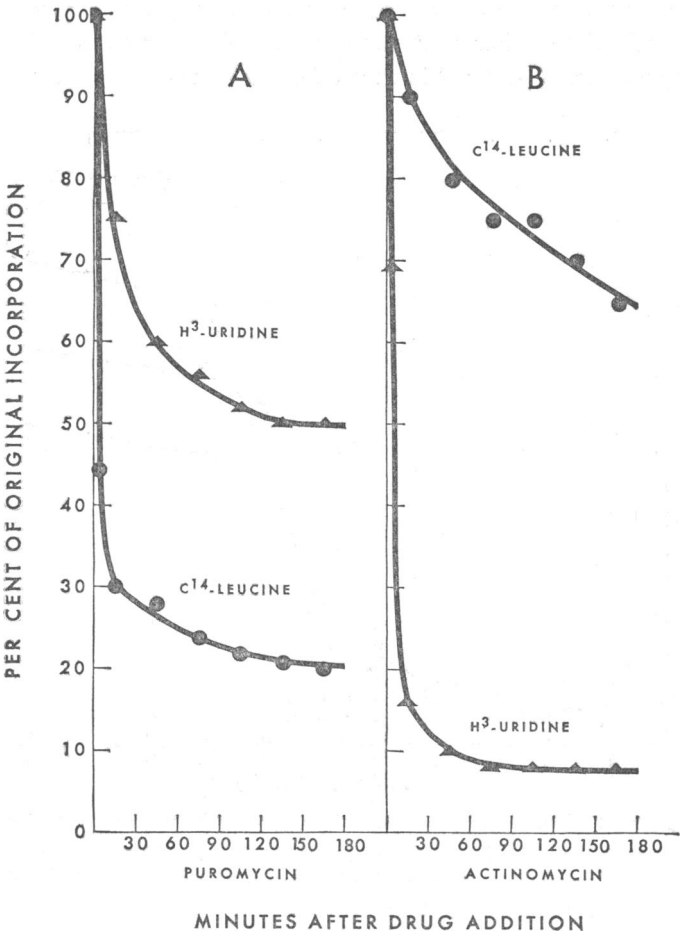

FIGURE 1 Effect of puromycin and actinomycin on RNA and protein synthesis. The rate of RNA synthesis was determined by measuring the amount of H^3-uridine incorporated into an aliquot of 100 μl of cell suspension during 5 and 30 min pulses. The rate of protein synthesis was derived from measurements of the amount of C^{14}-leucine incorporated over a period of either 5 or 30 min. Fig. 1*A* represents synthesis in cells to which puromycin was added at time 0. In Fig. 1*B* actinomycin was added at time 0. Concentrations of H^3-uridine and C^{14}-leucine during the labeling period were 1.0 and 0.1 μc/ml of cell suspension, respectively. Five minute pulses were employed in the period immediately after drug addition, followed by 30 min pulses for incubation times in the drug exceeding 30 min when the amount of label incorporated had grossly decreased. In each case, the average incorporation over the time interval used has been expressed as a per cent of the appropriate control.

ing, growth was resumed and the slope and amplitude of the resultant synchronous wave were equal to those of untreated cultures. These data afford evidence that puromycin in the concentration employed for these studies did not exert significant cytotoxic or other irreversible side effects.

Time Localization of the Action of Actinomycin and Puromycin. Actinomycin was added to cultures at varying intervals after release from thymidine block. As may be seen from Fig. 2 in which the log of the cell concentration is plotted

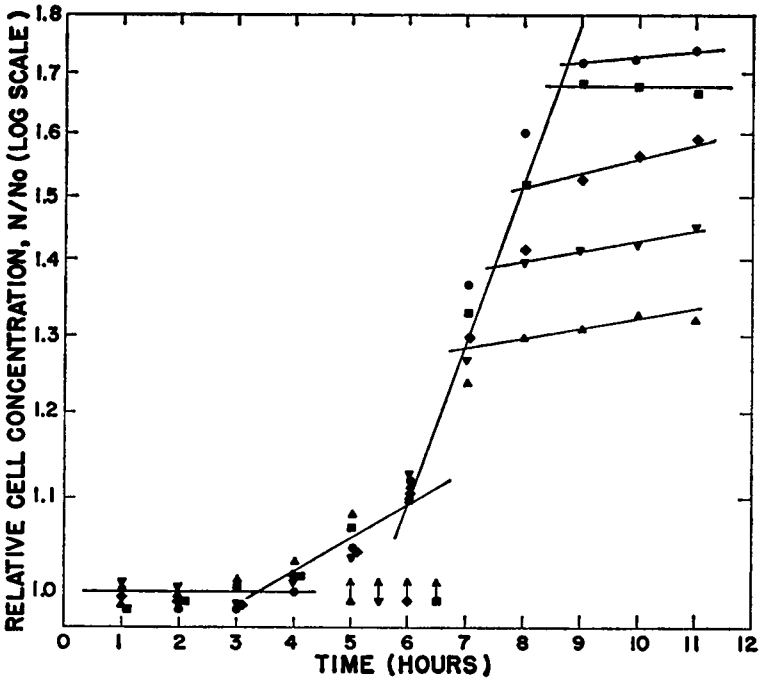

FIGURE 2 Effect of actinomycin D on division of partially synchronized cultures of CHO cells. The circles indicate experimental points in tests in which no drug was ever added. The other points represent experiments in which drug addition occurred at the times indicated by the corresponding arrows.

versus time after release from thymidine blockade, cell division always continued at its normal rate for a short time after drug addition and then ceased. The time of cessation of division can be determined with high precision and the lag time between addition of actinomycin and resulting inhibition of cell division calculated. Results of determinations in a series of experiments involving actinomycin-treated cells are summarized in Table II, in which the mean of the measured time between drug addition and division delay is 1.87 ± 0.08 hr. Experiments utilizing either single or double blockage with thymidine yielded the same results. The lag time between the addition of actinomycin and the cessation of cell division is interpreted

TABLE II

TIME OF ACTION OF ACTINOMYCIN AND PUROMYCIN ON DIVISION OF SYNCHRONIZED CHO CELLS

Inhibitor	Time of addition	Time of cessation of division	Apparent delay	Average delay ±SD (mean)
	hr	*hr*	*hr*	*hr*
Puromycin (50 $\mu g/ml$)	5.0	5.5	0.5	
	6.0	6.8	0.8	
	7.0	7.6	0.6	
	8.0	8.2	0.2	
	6.5	7.2	0.7	
	7.0	7.8	0.8	0.60 ± 0.09
Actinomycin D (2 $\mu g/ml$)	5.1	7.0	1.9	
	5.5	7.5	2.0	
	6.0	8.0	2.0	
	6.5	8.7	2.2	
	4.7	6.6	1.9	
	5.2	6.8	1.6	
	5.7	7.1	1.4	
	6.0	8.1	2.1	
	7.0	8.8	1.8	1.87 ± 0.08

as a measure of the time preceding cell division at which all the DNA-based RNA synthesis necessary for cell division has taken place. The time of this action is designated as point A of Fig. 4.

Results of similar experiments with puromycin are shown in Fig. 3. Previous investigators reported that puromycin prevents entry into mitosis of cells that have completed most of G_2 (Kishimoto and Lieberman, 1964). Our values for the delay between the time of puromycin addition and cessation of division summarized in Table II yielded a mean of 0.60 ± 0.09 hr. This period is indistinguishable from the duration of mitosis (M) within the accuracy of present timing measurements and locates a point P_D which virtually coincides with the beginning of M (Fig. 4).

As a further control, the simultaneous addition of both puromycin and actinomycin was tested in an effort to determine whether any synergistic effects would be revealed which would indicate side effects of the drugs that might prohibit a simple interpretation of their actions. The results (Table III) were equivalent to those of puromycin alone. Hence, the point P_D is provisionally interpreted as the last point in the life cycle at which protein synthesis necessary for cell division occurs. (see Conclusions).

The Effect of Puromycin Block on the Mitotic Index. The fate of the cells which have not reached P_D at the time of puromycin addition, and hence which

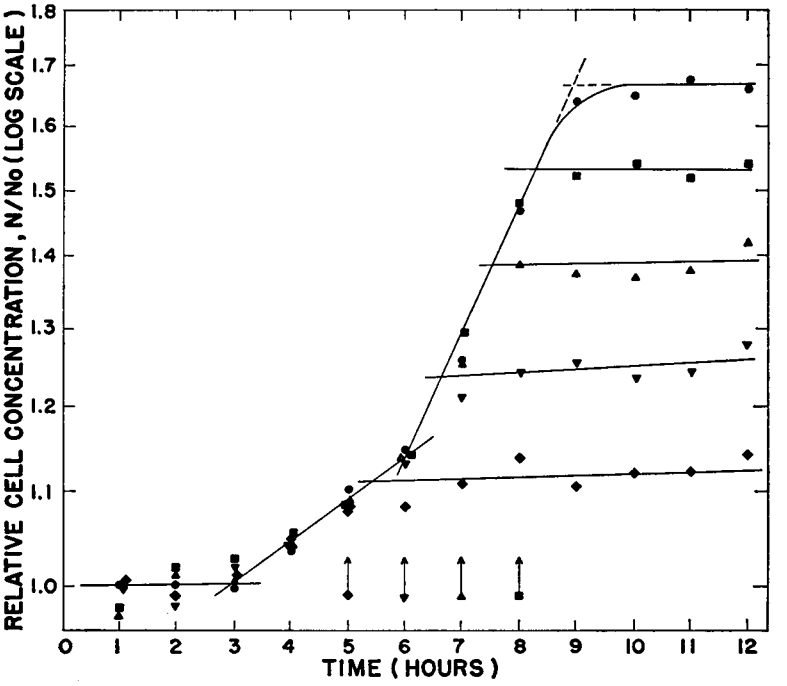

FIGURE 3 Effect of puromycin on division of partially synchronized cultures of CHO cells. The circles indicate experimental points in tests in which no drug was ever added. The other points represent experiments in which drug addition occurred at the time indicated by the corresponding arrows.

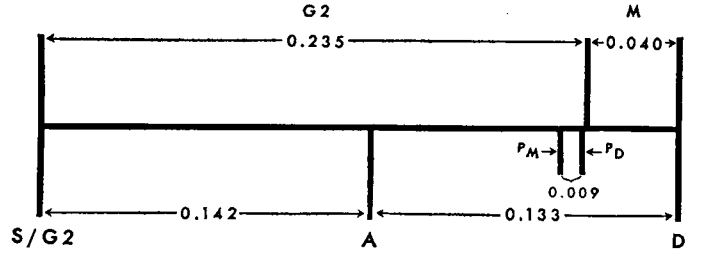

FIGURE 4 Provisional mapping of the metabolic events in the life cycle of CHO cells as here studied. The time units indicated are in fractions of the average generation time, which was 14.1 hr in the present experiments. S/G_2 = beginning of G_2; A = last point of actinomycin inhibition before cell division; P_D, P_M = last points of puromycin action in the life cycle. P_M represents a point of completion of protein synthesis required for initiation of visible mitosis. P_D represents the point of completion of protein synthesis essential for division. D = completion of cell division. The possibility that "division time" determined here with the particle counter may differ intrinsically from the end of mitosis as measured previously by as much as 0.1 to 0.15 hr is currently under investigation.

TABLE III

EFFECTS OF SIMULTANEOUS ADDITION OF ACTINOMYCIN PLUS PUROMYCIN AND OF PUROMYCIN ALONE ON DIVISION DELAY IN SYNCHRONIZED CHO CELLS

Time of drug addition	Cessation of division time		Apparent delay time	
	Puromycin only	Puromycin plus actinomycin	Puromycin only	Puromycin plus actinomycin
hr	*hr*	*hr*	*hr*	*hr*
4.7	5.3	5.6	0.6	0.9
5.7	6.4	6.5	0.7	0.8
6.7	7.4	7.5	0.7	0.8
Averages			0.67	0.83

do not divide cannot be determined from total cell count data alone. However, further elucidation of the effects of blocking of essential protein synthesis at P_D and at previous points, is possible by virtue of the fact that mitosis, the last period before division consists of a series of cytologically distinguishable phases. By observing the changes produced by puromycin addition on the value of the mitotic index and on the distribution of the mitotic cells among the various phases of mitosis, it is possible to draw inferences about the subsequent progression of cells in which failure of synthesis has occurred (*a*) of P_D protein alone and (*b*) of both P_D and previous proteins.

Puromycin was added to synchronized cultures of CHO cells at times ranging from 2 to 7 hr after release from thymidine block, and mitotic indices were determined at approximately hourly intervals thereafter up to 12 hr. In every case, the mitotic index dropped markedly within 30 to 60 min after addition of the drug, and then remained constant for 12 hr at a value approximately 0.23 of that which existed at the time of drug addition (Table IV). The data of the table indicate that the final constant level achieved by the mitotic index was always close to $\frac{1}{4}$ of the original value at the time of addition of puromycin, despite a $2\frac{1}{2}$ fold difference in this initial value between the experiments utilizing short and long periods after release from thymidine block.[2]

Analysis of the composition of the mitotic figures in the puromycin treated cultures described in Table IV throughout the 12 hr period of constancy of the mitotic index, revealed 100% of the mitoses to be metaphases. In contrast, the mitotic figures of the untreated cultures always demonstrated the presence of all the phases of mitosis, of which approximately $\frac{1}{3}$ were prophase and $\frac{1}{6}$ were anaphase plus telo-

[2] The constancy of the mitotic index throughout this period is taken as evidence of the absence of mitotic degeneration. It seems improbable that a slow leak-through of cells into mitosis and a mitotic degeneration without cell division would exactly compensate throughout a 12 hr period.

TABLE IV

DEMONSTRATION OF MITOTIC INDEX DROP TO A CONSTANT LEVEL AFTER ADDITION OF PUROMYCIN TO SYNCHRONIZED CULTURES

Approximately 8 samples were analyzed during the 12 hr period following puromycin addition, for each of the 3 experiments in which the period between release of thymidine block and puromycin addition was 2, 4, and 6 hr, respectively. The means and standard deviations are shown for each set of measurements.

A	B	C	
Time after release of thymidine block at which puromycin was added	Mitotic index at time of puromycin addition	Average value of mitotic index during 12 hr after puromycin addition	Ratio C/B
hr	%	%	
2	2.1 ± 0.7	0.53 ± 0.11	0.25 ± 0.1
4	4.8 ± 1.0	0.92 ± 0.15	0.19 ± 0.05
6	5.2 ± 1.0	1.35 ± 0.21	0.26 ± 0.06
Average			0.23 ± 0.04

phase, the remainder, constituting less than half of the total, being metaphase. Thus, treatment with puromycin swiftly causes the mitotic index of these cultures to fall to a constant value which, however, is greater than zero, and which is characterized by the presence only of metaphase.

CONCLUSIONS

The purpose of the series of studies of which this is a part is to map the position in the cell life cycle of the biochemical steps necessary for cell division; and to analyze the action of drugs and physical agents on these processes. The basic assumptions which underlie the conclusions of the present paper are that different subpopulations of the cell cultures are not caused to alter their relative order in the life cycle progression by the action of the agents studied; and that under the particular conditions employed which were designed to minimize complicating side effects of each drug, the effects on cell division of actinomycin and puromycin can be interpreted directly in terms of their known action in inhibiting synthesis of functional, DNA-based messenger RNA, and protein respectively. In addition, the interpretation of the action of puromycin on the distribution of mitotic cells among the various phases of mitosis involves the assumption that the drug has not caused a changed chromosomal morphology so that cells which are actually in prophase or anaphase-telophase take on the distinctly different appearance of metaphase; and that the constant value of the mitotic index achieved with 100% of the cells in metaphase is indeed a situation with trapped stationary cells rather than a fortuitous dynamic steady state.

While these assumptions appear reasonable and are supported in part by ex-

perimental tests described here, they are still not rigorously proved, so that the conclusions which will be drawn are provisional and subject to the uncertainty of the underlying assumptions. However, the model so furnished affords a clear and consistent picture of the present and of previous experimental observations, and appears to furnish a useful approach for study of the action of other agents. As additional such studies which are now in progress furnish similar analyses of the ordering of events in the cell life cycle through the use of a variety of physical and chemical agents, the consistency of the resulting conclusions will either confirm the validity of the present model or indicate where it must be modified.

On this basis, the action of actinomycin is interpreted to mean that in a total life cycle of 14.1 hr for CHO cells the last necessary messenger synthesis occurs 1.9 hr before completion of telophase, or 1.3 hr before initiation of mitosis.

The action of puromycin may be interpreted as follows: the cells which have completed synthesis of the P_D protein(s) at the time of puromycin addition are the mitotic cells. They will all have completed division and left mitosis in 0.60 hr. Those cells which have not completed P_D synthesis, but have synthesized all the preceeding proteins in the cycle, are unable to complete mitosis, but they need not stop at P_D; they might continue on into mitosis and undergo chromosomal condensation, stopping their traverse when the point is reached at which further progress is impossible without P_D protein(s). Such a difference between the point of action of a drug and the point of accumulation of affected cells has been described in the case of colcemide (Puck and Steffen, 1963). If the stopping point due to lack of P_D protein lies just before visible mitosis, the persistent mitotic index would be zero. If it lies in mitosis, the persistent mitotic index should be positive. Since the mitotic index falls only to 0.23 of its normal value, the stopping point due to lack of P_D protein lies in mitosis. Since the accumulated mitoses are distinct metaphases, the stopping point lies at metaphase, and is beyond the point of synthesis of P_D protein, which is at or very close to the beginning of prophase.

An additional inference is possible because of the fact that all of the accumulated mitoses are metaphases. Since no cells have stopped at prophase it may be concluded that while all cells lacking only P_D protein(s) can progress to metaphase, cells lacking both P_D and the next preceding essential protein(s) cannot even enter mitosis. Hence, this model proposes that a second point in time of protein synthesis blocked by puromycin, occurs at an earlier point, P_M. Normally at P_M protein synthesis occurs which permits entry of a cell into mitosis (Fig. 4). The number of cells trapped in mitosis would represent the cells which contain P_M but not P_D protein, and so are normally located between P_M and P_D. The duration of this interval can be calculated by use of the formula relating the fraction of a random cell population in any interval of the life cycle, to the duration of that interval (Puck and Steffen, 1963, equation 8 Appendix; Stanners and Till, 1960), and is found to be 0.009 of the life cycle or about 8 min, for this cell strain under these conditions.

The fact that this model calls for no protein synthesis essential for reproduction, to occur within the mitotic interval itself agrees with the findings of earlier investigators that no protein synthesis occurs during mitosis (Prescott and Bender, 1962; Konrad, 1963). It is of interest that the block in synthesis of P_D protein behaves like that produced by colcemide which has been shown to occur only at the beginning of mitosis, but to permit the affected cells to progress up to metaphase (Puck and Steffen, 1963). Since the spindle is a complex structure (Mazia, 1962) and colcemide is known to prevent normal spindle assembly, it may well be that colcemide and puromycin act to produce similar biological effects at this point. The difference between their behavior would then lie in the fact that colcemide specifically prevents only spindle assembly, so that mitotic figures accumulate in large numbers. However, puromycin also inhibits other necessary protein formation of which the nearest precedes mitosis by about 8 min and is essential for entrance into mitosis, so that only a small mitotic accumulation is obtained. Other studies on proteins needed for cell division are consistent with this model (Watanabe and Ikeda, 1965; Zeuthen, 1961).

An interpretation of the relationship between the actinomycin and puromycin points of action is also possible. Completion of the protein synthesis associated with the messenger-RNA formed at A in Fig. 4 might occur anywhere between that point and P_D, since A and D represent the last points before division of synthesis of necessary messenger and protein, respectively. Hence, the period between formation of this particular messenger and its associated protein synthesis is equal to or less than 1.3 hr. But if the puromycin blocking point, P_D, involves a protein unrelated to the messenger formed at A, the protein corresponding to P_D must have resulted from a messenger synthesized even earlier than A. Therefore, it may be concluded that if the same protein is involved in these two blocks at A and P_D, the period between completion of its messenger formation and its own synthesis is 1.3 hr. If completely different, essential proteins are involved, the interval between their messenger syntheses and their own formation must involve two different time periods: one greater than and one less than 1.3 hr.

The various events discussed here are plotted in a tentative map of the G_2 and mitotic periods (Fig. 4). This work is continuing.

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