PRIMITIVE ERYTHROPOIESIS IN EARLY CHICK EMBRYOGENESIS

I. Cell Cycle Kinetics and the Control of Cell Division

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ABSTRACT

The primitive line of embryonic chick blood cells develop as a relatively homogeneous cohort of cells. Using an analysis based on the continuous uptake of thymidine-^{3}H, we have established the generation time, G1, S, and G2 for progressively more mature generations of these immature erythroblasts. The data indicate that after the initiation of hemoglobin synthesis, the average cell will yield six generations of hemoglobin producing erythroblasts. The older generations of erythroblasts exhibit a longer generation time, G1, S, and G2 than the earlier generations of erythroblasts. Other methods of analysis corroborated these findings. One of these methods, an estimate of total erythrocyte productivity from the primitive stem cells (hematocytoblasts), led to the conclusion that the erythroblast cell lineage might be initiated as early as the sixth or seventh division following fertilization. In addition, primitive erythroblasts characterized by one set of cell cycle parameters, when grown in serum associated with erythroblasts of different parameters, showed no alteration in mitotic behavior. These results suggest the presence of programmed cell division not immediately cued by extracellular influence.

INTRODUCTION

The relationship between terminal differentiation, DNA synthesis, and cell division is a complex one (Holtzer, 1968; Chacko et al., 1969; Holtzer, 1970). While there is a mutual exclusivity between these two processes in many cell types, the notable exceptions argue strongly against a simple "on-off" type mechanism. One such exception is the developing erythroblast where the association of hemoglobin (Hb) with dividing cells has been noted by a number of investigators (Lucas and Jamroz, 1961; Grasso and Woodward, 1963, 1966; Thorell and Rawnick, 1966; Kovach et al., 1967; de la Chapelle et al., 1969). This report attempts to place in perspective the biological significance of this observation, and has used, as a measure, the number of generations the average chick primitive erythroblast gives rise to before entrance into the postmitotic state. In addition a number of observations have been made on the factors controlling the division processes in this system. Evidence is presented that the mitotic history of these cells is programmed i.e., independent of specific external cues, and that the program limits the mitotic potential of these cells to an average of six generations. Given the extensive proliferative capacity

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of the erythroid stem cells (hematocytoblasts) in most erythropoietic systems it appears that the restriction on mitotic activity of even the earliest erythroblasts is in some way coupled to the commencement of hemoglobin synthesis, and that the relationship between DNA synthesis and hemoglobin synthesis is not fundamentally different from that observed in cells synthesizing myosin, alpha-crystalline, or molecules associated with the terminal differentiation of nerve fibers (Ishikawa et al., 1968; Wessels, 1964; Eisenberg and Yamada, 1966; Sidman, 1968; Jacobson, 1968).

MATERIALS AND METHODS

In Vivo Studies

White Leghorn eggs were incubated at 37°C. Staging corresponded to that described by Hamilton (1952). For in vivo incubations, 5 ml of albumin was removed from the egg with a syringe. A 5 mm square was removed from the shell, and from 0.1 to 3 ml of solution, depending on the experiment, was introduced atop the embryo. The square was covered with tape and the embryo was incubated for varying lengths of time. Dosage: 3 ml of Colcemid (Ciba Pharmaceutical Co., Summit, N. J.) was used at 10^{-6}M. Lower concentrations resulted in the presence, over short exposures (2 hr), of anaphase and telophase figures. At this concentration, evidence of breakthrough from the block does not appear until the 6th or 7th hr. TdR-5-CH_3-^3H (New England Nuclear Corp., Boston, Mass., SA 0.036 mg/mCi), 5-uridine-^3H (New England Nuclear Corp., SA 0.0103 mg/mCi), and L-leucine-4,5-^3H (New England Nuclear Corp., SA 0.26 mg/mCi) were administered in 0.1 ml quantities at 250 μCi/ml. Halving the specific activity of thymidine had no effect on either the accumulation of labeled cells or the accumulation of labeled mitotic cells.

Radioautography

Blood cells were collected as described by Lucas and Jamroz (1961). Smears were made, air dried, and, in the case of TdR-^3H labeling, fixed in AFA (20 ETOH; 2 formaldehyde; 1 acetic acid). Uridine-^3H and leucine-^3H labeled slides were fixed in 10% trichloroacetic acid (TCA). All smears were subsequently washed in running tap water for 1 hr, dried at 40°C, and processed for radioautography by the methods described by Bischoff and Holtzer (1968). Longer exposures resulted in more grains per cell, but never in any increase in labeling index. After radioautography cells were stained with Wright's as described by Lucas and Jamroz (1961).

In Vitro Incubation

In vitro incubations were done in F10 (Grand Island Biological Co., Berkeley, Calif.) modified as described by Chacko et al. (1969). Preliminary analysis revealed linear uptake of precursors for protein, RNA, and DNA over at least a 4 hr period. All incubations discussed in this paper were for no longer than 0.5 hr. 5-methyl-TdR-^3H, uridine-^3H, and leucine-^3H were added at specific activities given above to a final concentration of 25 μCi/ml. Cells were then washed twice in F10, smeared, and processed as above for radioautography.

TdR-^3H Incorporation

4 or 5 day cells were incubated in F10 at specified external concentrations of thymidine and specific activities of TdR-^3H. Experiments were terminated by adding five volumes of distilled water at 0°C, followed by an equal volume of cold 20% TCA. Protein present in the incubation media served as carrier. The precipitate was washed three times in cold 10% TCA, dried under vacuum, dissolved in 1 ml of formic acid, and spotted onto glass fiber discs (Whatman, GF/A). These were dried and placed into scintillation vials. 6 ml of scintillation fluid (6 g 2,5-diphenyloxazole (PPO) and 0.15 g *p*-bis[2-(5-phenyloxazolyl)] benzene (POPOP) per liter of toluene) was added and the vials were counted at from 8 to 10% efficiency in a Beckman LS-200B Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, Calif.). Incorporation was determined as the difference between a sample taken at time zero and one taken, usually at 0.5 hr. As a control, cells incubated in the presence of 10^{-3}M hydroxyurea showed no incorporation whatsoever.

RESULTS

General Background

The morphological development of primitive erythroblasts derived from the blood islands of the developing chick embryo has been described by Lucas and Jamroz (1961). With respect to morphology these cells mature from their inception at 35 hr of incubation (O'Brien, 1961; Hell, 1964; Wilt, 1965) as a relatively homogeneous cohort. This is valid for Wright's, Giemsa's, and benzidine staining procedures (Lucas and Jamroz, 1961). The 2 day population consists predominantly of basophilic erythroblasts; the 3 day, of early and mid-polychromatophils; the 4th day, of late polychromatophils; and the 5th day, of immature, nucleated erythrocytes. By the end of the fifth day, the morphologically

distinguishable definitive line of cells makes its first appearance in the circulation. Preliminary radioautographic experiments with TdR-^{3}H demonstrated that by day 6, mitotic activity in the nucleated, primitive erythrocytes has ceased. Similar studies showed that uridine incorporation is reduced to less than 10% of the 5 day level, while leucine incorporation diminished much less markedly. This is similar to the pattern of macromolecular synthesis seen in other erythropoietic systems (Grasso and Woodard, 1965, 1966, 1967; Kovach et al., 1967; de la Chapelle et al., 1969). Our analysis will deal only with the primitive line of red cells.

Cell Cycle Analysis

Our first question concerns the number of generations a typical red cell gives rise to between 35 hr and 6 days. To answer this, the generation time of a typical erythroblast was established for five periods during development. From these data, it is possible to estimate the total number of divisions by fitting the known generation times at a given hour of incubation into the span of time between 35 hr and 6 days.

The primary technique is based on the continuous uptake of labeled thymidine as a function of time to estimate the generation times of each class of maturing erythroblast. Methods relying on pulse-labeling of cells were inappropriate for this system since tritiated thymidine is not rapidly metabolized by the embryo and dilution of the label by addition of cold thymidine might lead to altered cell cycle parameters. The analysis employed is a modification of one proposed by Stanners and Till (1960): under completely asynchronous conditions of growth, the distribution of cells in the mitotic cycle is exponential, skewed toward cells in early G1. Fig. 1 a illustrates an exponential distribution of cells (James, 1958; Stanners and Till, 1960; Mak, 1965). There are more cells per unit of time in G1 than in S or G2. Such a situation results from exponential growth of cells and from the fact that one cell in mitosis gives rise to two cells in G1. Thus, at a given time, t, the number of cells, $n(t, q)$, at time, q, from mitosis is given by:

$$n(t, q) = aN(t)e^{aq} \quad (1)$$

where $N(t)$ represents the total number of cells present at time, t, and a is a constant equal to $0.693/T_g$ where T_g is the generation time. Fig. 1 a is a plot of $n(t, q)$ versus q for a given t. In applying the equation, three factors must be considered: (*a*) the degree of synchrony, (*b*) the percentage of the population actually in the division cycle, and (*c*) the validity of the prediction that the distribution is exponential. For the present, it is assumed that the population is asynchronous, exponential, and uniformly active in division. Under these conditions, Stanners and Till (1960) have calculated a ratio, the labeling index, as a function of T_g (the generation time), TS (the time of the S period), TG2 (the time of the G2 period), and t (the time after addition of label):

$$L(t) = \frac{e^{aTG2}}{e^{at}} (e^{a(TS+t)} - 1) \; t \leq G2; \; a = \frac{0.693}{T_g} \quad (1\text{ a})$$

The restriction that "t" be less than TG2 applies so that further complications arising from the division of labeled cells need not be considered.

To obtain T_g ($= 0.693/a$) from equation 1, four variables must be determined. $L(t)$ and t are determined experimentally; TG2 + ½M is determined from the time it takes 50% of the mitotic cells to be labeled with TdR-^{3}H. For TS, we substitute its equivalent, $T_g - (T\text{G2} + T\text{G1} + M)$ where M is the time for mitosis. The quantity in parenthesis is determined from the continuous label experiment and is given by the time it takes for 100% of the cells to be labeled with TdR-^{3}H. Thus equation 1 a becomes:

$$L(t) = \frac{e^{0.693TG2/T_g}}{e^{0.693t/T_g}} \cdot \left[e^{\frac{0.693}{T_g}(T_g-(TG2+TG1+M)+t)} - 1 \right] \quad (2)$$

After appropriate substitution we solve for T_g in terms of the experimentally derived parameters. TS is then calculated from the difference between T_g and (TG2 + TG1 + M). TG_2, as mentioned, is derived from the TdR-^{3}H labeled mitosis curve; M, from the uridine-^{3}H labeled mitosis curve (as described in the Results); and TG1 as the difference between (TG1 + TG2 + M) and (TG2 + M).

Thymidine-^{3}H Labeling Kinetics

Five periods between 35 hr and 6 days were chosen (35–45 hr, 60–70 hr, 3–4 days, 4–5 days, and 5 days). For each of these periods the labeling

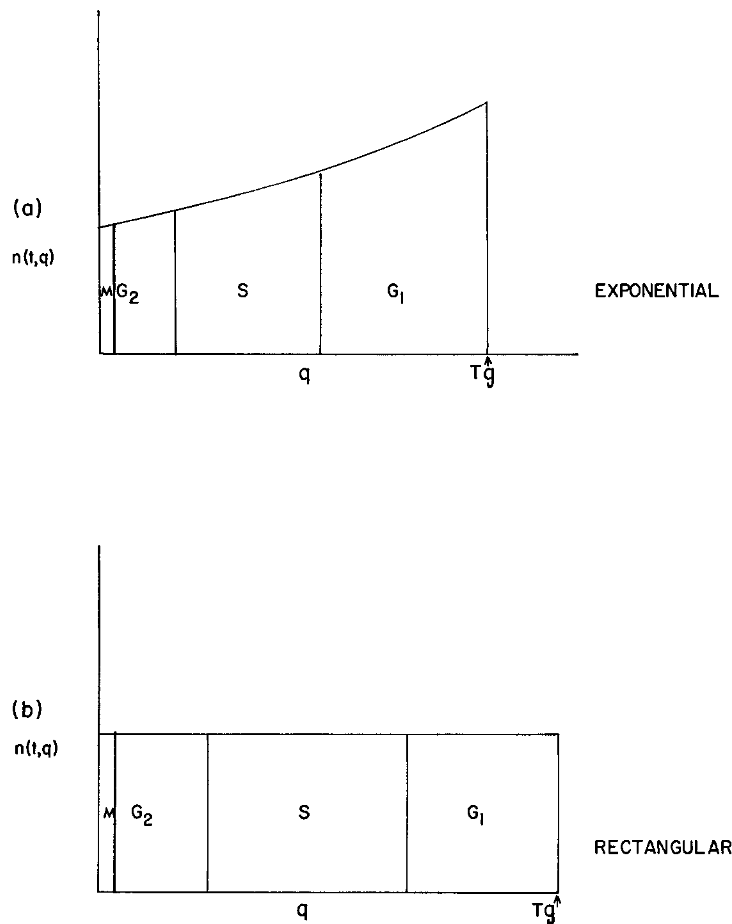

FIGURE 1 (a) An exponential distribution of cells. (b) A rectangular distribution of cells. $N(t, q)$ is the number of cells and q represents time from mitosis.

index under a continuous exposure to TdR-^{3}H was determined as a function of time. The span of time covered by these experiments occupies approximately 60% of the total proliferative period of the primitive red cells. Fig. 2 displays the results for all five periods. Each graph represents the collected data from two independent experiments. Each point represents the results from at least 1000 cells derived from a single egg. These graphs suggest: (*a*) the cell cycle is increasing as the cells mature. (*b*) the fact that each graph approaches 100% labeling at a fairly constant rate indicates that during these experiments the population is asynchronous; i.e., continuously distributed throughout the cell cycle. The latter observation reveals that the growth fraction (Mendelsohn, 1963) is, for all populations except the one present on 5 days, greater than 99%. For 5 day cells the plateau of the curve at 95% indicates that 5% of the cells left the division cycle when TdR-^{3}H was first introduced. (*c*) in several instances in Fig. 2 the slope of the curve changes at from 92 to 98%. This could indicate either cell death, the tail end of a distribution of cell cycles, or a separate, subpopulation of cells. These points shall be discussed later; however, it is emphasized that this deviation represents only a small percentage of cells in any given population examined. (*d*) lastly, $(TG2 + TG1 + M)$ is determined from the extrapolated time it would take for 100% of the cells to be labeled.

From the same experimental material, the graphs seen in Fig. 3 were generated. The percentage of TdR-^{3}H–labeled mitoses is plotted as a func-

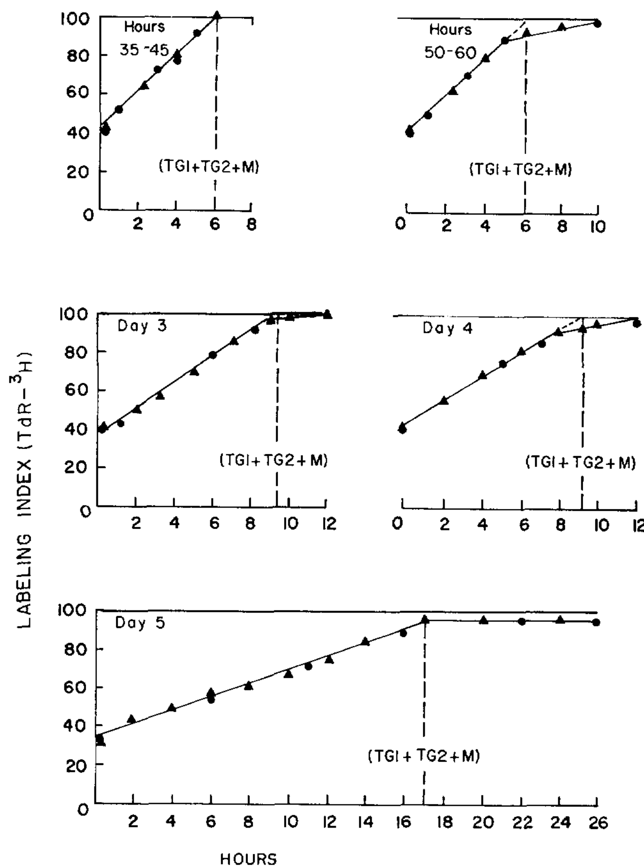

FIGURE 2 TdR-^{3}H labeling kinetics of progressively more mature generations of chick erythrocytes under a continuous exposure. 25 μCi of TdR-^{3}H was introduced into eggs between 35 hr of incubation and the 5th day of incubation. At subsequent intervals, an embryo was sacrificed, and the blood was collected and processed for radioautography as described in Materials and Methods. Circles and triangles distinguish two separate experiments. Each point represents data derived from one embryo. The extrapolated curve indicates the time used for ($TG1 + TG2 + M$). Zero time is the time when grains first appear in the radioautograms This is approximately 15 min after introduction of label.

tion of time after administration of label. For each of the five stages, the time for 50% of the mitotic cells to be labeled is given. This is the value used for the length of (G2 + ½M).

Using the values obtained above for ($TG1 + TG2 + M$) and for $TG2$, it is possible, as described in equation 2 to calculate, for a given labeling index, $L(t)$, and a given time, t, (Fig. 2), the generation times and their associated compartments (G1, G2, S) for each of the five periods investigated. The results of these calculations appear in Table I. Values for day 5 are corrected for 5% of the cells being out of the growth fraction. For each point on a given graph, a different T_g, $TG1$, $TG2$, and TS can be calculated. What appears in Table I represents an average derived from at least two points. Note that the length of the cell cycle increases as the cells develop, and that most of this increase occurs in G1 and S.

Support for the Thymidine-^{3}H Kinetics

CONFIRMATION OF T_g FOR 3 AND 4 DAY CELLS

It was desirable to confirm this method of analysis by an independent measure. We chose the method first described by Puck and Steffen (1963). Assuming exponentiality and asynchrony, they have derived, from the distribution function, $n(t, q)$ (see equation 1), an expression for the accumulation of cells in metaphase after addition of colce-

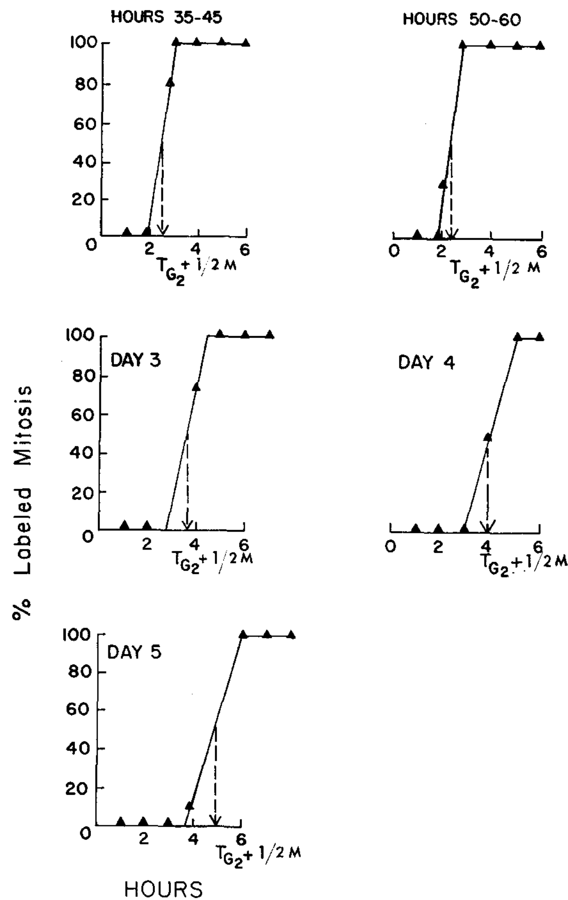

FIGURE 3 Estimation of G2 period for successive generations of cells. From the same radioautograms described in Fig. 2, the percentage of labeled mitoses was determined as a function of time after introduction of labeled TdR-^{3}H. The time for (G2 + ½ M) is taken as the time it takes for 50% of the mitotic cells to be labeled. Each point represents the data accumulated from one embryo and at least 200 mitotic cells.

mide. Their "collection function" relates the mitotic index, $N(M)$, to the generation time, T_g, and the time for mitosis, T_m, by the time, t, under colcemide:

$$\text{Log } (1 + N(M)) = \frac{0.301}{T_g} (T_m + t)$$

Fig. 4 is a plot of log (1 + $N(M)$) versus time under Colcemid for both 4 and 3 day red cells. The linearity observed validates the assumptions of asynchrony and exponentiality. The slope indicates a generation time of approximately 17 hr, a time which supports the thymidine uptake analysis for the 2 days.

For these conclusions to hold, it is necessary to exclude effects of Colcemid on compartments other than mitosis. The length of G2 was therefore measured for 4 day cells in the presence of Colcemid. No difference between these results and those seen in Fig. 3 were observed. Similarly, it is possible to calculate that the TdR-^{3}H labeling index of 4 day cells should increase (Fig. 1) from 40% to 47% after a 6 hr pretreatment with Colcemid. This did occur and the results therefore indicate that Colcemid does not effect passage through G1; moreover, the observed increase in labeling represents further confirmation that the population is exponential.

Table I

Lengths of the Cell Cycle Tg, G1, G2, and M for Five Stages during the Development of the Primitive Erythrocytes of Chick

Stage	G1	S	G2	M	Tg
	hr	*hr*	*hr*	*hr*	*hr*
45–55 hr	3.1	3.6	2.2	0.5*	9.6
60–70 hr	3.1	3.6	2.2	0.5*	9.6
3 day	5.8	7.2	3.6	0.5	17.1
4 day	5.6	7.2	3.8	0.5	17.1
5 day	11.8	11.9	4.8	0.5*	29.0

Starred values for M were inferred from measurements made only on 3 day and 4 day material. The values appearing in the Table were derived from the data given in Figs. 1 and 2 as described in the section of Materials and Methods dealing with the cell cycle analysis.

Predictions of the Analysis

The calculated cell cycle of 17 hr on both day 3 and day 4 places several limitations on the length of mitosis. The two are related via the mitotic index, $N(M)$, by the following equation (Stanners and Till, 1960):

$$N(M) = (0.693/T_g) \times (T_m) \qquad (3)$$

Using 17 hr for T_g and 0.027 ± 0.003 (five embryos) for the mitotic index derived from 5000 3 day or 4 day cells, we have calculated, as a time for mitosis, 0.66 hr. Similar calculations for 2 day and 5 day cells are consistent with this. To test this prediction we measured T_m directly.

Fig. 5 shows the percentage of mitotic cells labeled with uridine-^{3}H as a function of time after the first labeled cells appear. The time for 100% labeling is shown to be 45 min for 3 and 4 day embryos. Of this time, however, the first 15 min seems to represent a 15 min period before M during which no uridine-^{3}H is incorporated into mitotic cells, while interphase cells are heavily labeled. From this, we conclude that T_m is approximately 30 min and that, because of either permeability changes, pool size changes, or direct alteration of RNA metabolism, net uridine-^{3}H incorporation falls to less than 10% of interphase values 15 min before mitosis. These experiments also extend to the red blood cell the findings of many others (Prescott and Bender, 1962; Scharff and Robbins, 1966; Johnson and Holland, 1965) that uridine-^{3}H is not incorporated into RNA by cells in mitosis. Considering the rather large percentage error (resulting from the limited numbers of mitotic cells from a given embryo and not necessarily from variations between embryos) associated with the mitotic index, the measurement of 0.5 hr for T_m is in good agreement with the prediction of 0.66 hr from the cell cycle data.

A second prediction of the TdR-^{3}H uptake kinetics is that the G1 period of 3 day embryos is 5.8 hr. Fig. 6 shows that when embryos are treated with both TdR-^{3}H and Colcemid continuously, it takes approximately 6 hr for almost 100% of the cells to be *either* labeled with TdR-^{3}H or arrested in metaphase (or both). Since the data indicate that G1 is about twice as long as G2, we can conclude that, as measured directly, G1 is approximately 6 hr. When the data in Fig. 6 are plotted as a log function, a linear plot would be expected since the experiment occurs under conditions of constant cell number. There was some falling off

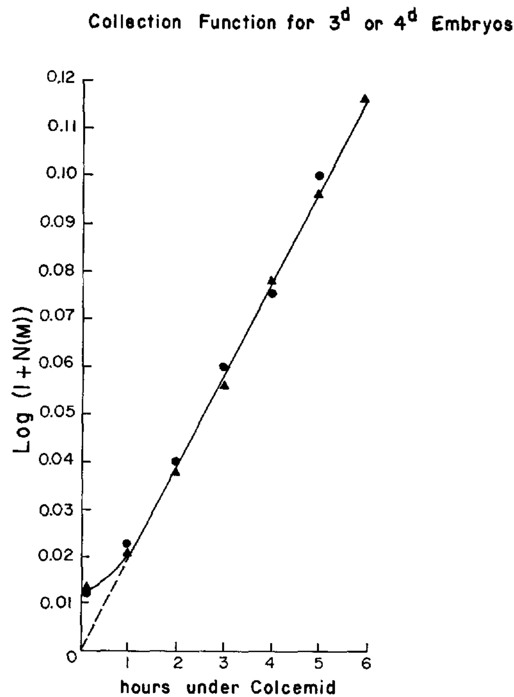

Figure 4 As described by Puck and Steffen (1963), the collection function is plotted against hours under colcemide. Colcemide (3 ml at 10^{-6} M) was introduced into 3 and 4 day embryos as described in Materials and Methods. The mitotic index was determined for the next 6 hr at hourly intervals. Triangles represent 3 day cells. Circles represent 4 day cells. In both cases, the slope is consistent with a generation time of 17 hr.

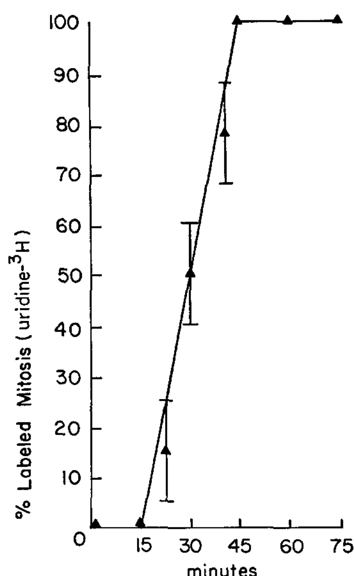

Figure 5 The length of the mitotic period is determined by following the progression of cells labeled with uridine-^{3}H through mitosis. The method depends on the fact that mitotic cells do not take up the label. Uridine-^{3}H was introduced into both 3 and 4 day embryos. Points were taken over the next 75 min. 200 mitotic cells derived from a single embryo were analyzed for each point. Graph shows a plot of percentage of labeled mitotic cells versus the time after the appearance of label in interphase cells. The first 15 min represent a period before mitosis during which label is present in interphase cells but absent in mitotic cells. The next 30 min corresponds to the length of mitosis.

from linearity. This indicates that 6 hr is probably somewhat longer than the actual G1 and that the measured lengthening is due either to cells escaping the Colcemid block or to a direct slowing effect of Colcemid on G1 cells. With respect to this, we have noted that telophase figures do occur, but only after long (6 hr) exposures to Colcemid.

Validation of the Assumptions

In using equation (2), we have made three assumptions: (*a*) an exponential distribution of cells, (*b*) 100% of the cells in the growth fraction (Mendelsohn, 1963), and (*c*) asynchrony of cells in the division cycle.

Exponentiality

Fig. 4 shows that the collection function is linear with time. Since this function applies to an exponential population of cells, its linearity, as a log plot, supports the original assumption that the distribution of cells is exponential.

Growth Fraction, Asynchrony, and Deviant Kinetics

Both the degree of asynchrony and the percentage of the population actually in the division cycle can be appreciated by considering deviations observed in the graphs of Fig. 2. These graphs reveal that from 2 to 8% of a respective population are unlabeled when extrapolation of the early kinetics would predict that they should be labeled. Extension of the labeling period usually reveals achievement of 100% labeling, but at a slower rate. Two explanations for this behavior are most likely.

The first, the possibility of cell death ("ineffective erythropoiesis" (Lajtha, 1960), probably accounts for some of the deviation observed. This is verified by noting that less than 1% of the cells from a given population fail to incorporate either uridine-^{3}H or leucine-^{3}H into RNA or protein, respectively. A similar fraction is stained with eosin (Watanabe and Okada, 1967). Since this is too small a fraction of the population a second hypothesis to explain the deviant kinetics might be proposed. There is not complete synchrony at the time of differentiation into Hb-producing cells. This allows generations of cells with different cycle times to coexist in the same circulation. As a con-

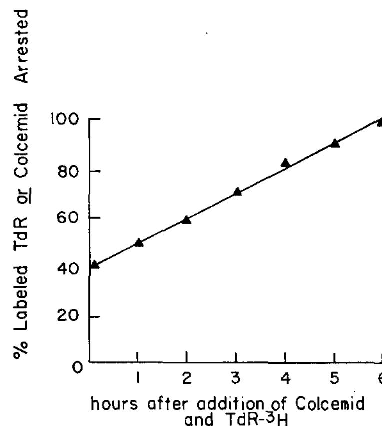

Figure 6 Both TdR-^{3}H (25 μCi) and colcemide (3 ml at 10^{-6} M) were introduced into 3 day embryos. Radioautograms were prepared at subsequent periods as indicated. The abscissa measures the union of labeled cells with arrested cells. The time for 100% accumulation is a measurement of the G1 period.

sequence, some period must exist during which both 4 day cells with their characteristic 17 hr cycle and 5 day cells with their 29 hr cycle share the same circulation.

The following observations confirm the suggestion that two generations coexist in the same circulation: In Table I the length of the S period for the predominant cell cycle on the 5th day is almost twice that seen on the 4th day. If the amount of DNA to be synthesized by these respective cell cycles is the same, then this indicates a rate for 4 day cells which is approximately twice that of 5 day cells. Assuming that differences in pool sizes can be overcome, it should be possible to distinguish the presence of two S periods (i.e., two cell cycles) in the same circulation by means of grain counts derived from radioautography with TdR-^{3}H. Fig. 7 displays the results obtained when "late day 4–early day 5" cells (late polychromatophils postulated to be in their fifth and sixth generations) were pulsed in vitro for 15 min with thymidine at high specific activity and high concentration (10.96 μg/ml). This high concentration is needed to swamp the endogenous thymidine flux, making internal pools less significant. Measurements were made on 500 cells. Two slides derived from the same material showed virtually identical curves. In addition, the average grain count per labeled cell was halved as the specific activity of label was halved. The distribution obtained is clearly bimodal, with relative modalities appearing at approximately the intervals expected on the basis of the lengths of the S periods for day 4 and for day 5.

To assure that the bimodality observed was a result of two populations and not of two different rates of DNA synthesis in the same S period, the same analysis was performed on day 5 cells. Fig. 8 shows that the distribution is unimodal as would be expected since by this time most of the cells should be in their last division cycle of 29 hr.

With respect to the interpretation of growth fraction and asynchrony, it is likely that the deviation in the predicted kinetics associated with the graphs of Fig. 2 can be explained on the basis of a subpopulation with cycle length longer than the norm. This subpopulation probably represents cells further along in their mitotic program. This is consistent with the cytophotometry data (Campbell et al., 1971) which also indicates the presence of several different cell cycles in the same circulation.

The Length of the S Period is Regulated Internally

That two generations of erythroblasts, each with its respective S period, occur in the same circulation is strong evidence for internal regulation of the DNA synthetic period. To extend the analysis, we have measured, using an isotope dilution technique (Warner and Rich, 1964; Adams, 1969), the rate of DNA synthesis and the internal TTP pools of cells under various conditions.

The Synthesis of DNA in F10: 4 Day Versus 5 Day Cells

First consider the behavior of 4 day and 5 day cells in F10. Previous analysis has shown that the uptake of TdR-^{3}H is linear for at least 4 hr. This linearity, but not the slope, is independent of the age of the cells and is interpreted as indicating rapid achievement of a steady state (within 10 min) between internal TTP and external TdR-^{3}H. Table II shows the calculated rates of DNA synthesis and pool sizes for 4 and 5 day cells in F10. As described by Adams (1969), these have been derived from measuring TdR-^{3}H uptake at two concentrations of external thymidine. Note that the relative rate of DNA synthesis on day 4 is almost twice twice that for day 5. This is consistent with the in vivo behavior of these cells where (Table I) the length of the S period on day 5 is slightly less than twice that on day 4.

The TTP Pool and the Control of DNA Synthesis

The internal TTP pool (Table II) decreases by a factor of two as cells pass from the 4th to the 5th day, suggesting that the lengthening of the S period might be due to a decreased availability of TdR. This was not the case, since increasing the concentration of thymidine in the incubation medium did not increase the relative rate of DNA synthesis as calculated with the isotope dilution method at *three* concentrations of external thymidine. This does not, however, eliminate the possibility that thymidine kinase is limiting.

The following experiment corroborated the estimate of the TTP pool: 10^{-5} M FUdR rapidly inhibits thymidylate synthetase in these cells (Weintraub and Holtzer, unpublished observations). Assuming this enzyme to be the predominant source of endogenous thymidylate and applying the iso-

Table II

Measurement of Internal TTP Pools of Relative Rate of DNA Synthesis (Arbitrary Units) for 4 and 5 Day Erythrocytes

Day of cells	External TdR concentration	Specific activity of external TdR	CPM in DNA	Calculated relative rate of DNA synthesis per cell	Calculated internal TTP pool
	(μg/ml)	*(μCi/μg)*			*μg/ml*
4	(a) 10.96	0.455	7330	18,300	1.52
	(b) 20.96	0.240	4050		
5	(a) 10.96	0.455	4145	9780	0.71
	(b) 20.96	0.240	2235		

Cells were incubated for 1 hr in F10 at external thymidine concentrations and specific activities as indicated. For each day the same number of cells were exposed to thymidine at two concentrations. Total TCA-precipitable counts were determined as described in Materials and Methods. The internal TTP flux and the relative rate of DNA synthesis were derived from two equations in two unknowns for each of the 2 days. An example of the calculations is shown in the legend to Table III. Radioautography revealed that the labeling index for 4 day cells was 40%, and for 5 day cells, 38%.

tope dilution technique with and without FUdR, it is possible as shown in Table III to generate two equations in two unknowns, solving for the internal pool. For both days, the results compare well with those obtained in Table II.

DNA Synthesis in Other Environments

To test whether some factor in the serum regulates the rate of DNA synthesis, 4 day cells were grown in either 4 day serum, 5 day serum, or F10. Converse experiments with 5 day cells were also done. The results are shown in Table IV. The external TdR concentration was kept at 10.96 μg/ml, a concentration (Table II) which tends to swamp the internal TdR flux, and thus, reflect the true rate of synthesis. In no case could significant deviations from the control be detected. 5 day serum does not inhibit DNA synthesis in 4 day cells, and 4 day serum does not stimulate DNA synthesis in 5 day cells. It is still possible that a rapidly turning over serum substance is operating, or that local interactions affect the red cell mitotic behavior.

To test whether serum was determining the rate at which cells entered S, 4 day cells were grown in 4 day serum and 5 day serum. TdR-^{3}H was added and after 5 hr radioautographs were made. The labeling index in both cases was the same (71%), and comparable to that seen in vivo (74%). 5 day serum does not affect the rate at which 4 day cells move through G1 and into S for at least 5 hr.

Discussion

A Model for Erythrokinetics in the Chick

Over 99% of primitive red blood cells in the developing chick do not display mitotic activity in vivo beyond the 6th day of incubation. Though withdrawn from the mitotic cycle (see, however, Bolund et al., 1969), they synthesize Hb well into the 7th day. That erythropoietic activity continues throughout the lifetime of all higher organisms demonstrates the extensive mitotic activity of the erythroid *stem* cells. Clearly, overt differentiation, as characterized by production of large quantities of Hb, coincides with a marked restriction on the number of progeny from a given erythroblast. In an attempt to evaluate this relationship kinetics of TdR-^{3}H uptake was employed using a modification of the equations derived by Stanners and Till (1960). The results of this analysis were validated by the use of independent criteria and were found, in all cases, to correspond well within a 10% deviation.

Fig. 9 incorporates these results into a model for the population dynamics of this system. The first line is the time parameter; the second line shows

TABLE III

Measurement of Pool Size and Rate of DNA Synthesis Using FUdR

	CPM in DNA	External TdR	Specific activity	Calculations
		(μg/ml)	*(μCi/μg)*	
Control cells (a)	19,700	0.9	5/0.9	TTP Pool (μg/ml)
FUdR at 10^{-4} M (b)	3830	2.85	$\frac{1}{2.85}$	1.85

4 day cells were incubated in F10 with and without FUdR at 10^{-4} M for 0.5 hr. Conditions of external thymidine and specific activity of labeled thymidine are as indicated. Using the isotope dilution method with the assumption that FUdR completely stops the internal flux of thymidine, both the magnitude of this flux and the rate of DNA synthesis, independent of pool sizes, can be calculated. The equations are:

$$(SA)\ (r) = CPM$$
$$(a): (5/[0.9 + x])\ (r) = 19{,}700$$
$$(b): \left(\frac{1}{2.85}\right)(r) = 3830$$

where r is the relative rate of DNA synthesis, x is the internal TTP flux (μg/ml), and SA is the specific activity of external TdR-^{3}H.

TABLE IV

TdR-^{3}H Uptake of 4 Day Cells and 5 Day Cells in Three Different Conditions

Medium/Cells	4 day cells	5 day cells
	cpm	*cpm*
4 day serum	11,890	7142
5 day serum	11,867	7039
F10	10,982	7015

4 and 5 day cells were washed in balanced salt solution three times. They were then incubated for 0.5 hr in either 4 day serum, 5 day serum, or F10. Thymidine was added to a concentration of 10.96 μg/ml to overcome any alteration in internal pools as a consequence of the different environments. Previous analysis showed less than 0.2 μg/ml of thymidine in either 4 day serum, 5 day serum, or the serum used in the preparation of F10. TdR-^{3}H was added to 5 μCi/ml, and TCA-precipitable cpm determined.

the experimentally derived generation times associated with the period of time during which the respective measurements were made. The third line indicates how these generation times might fit into the period between 35 hr, when Hb first appears, and the beginning of the 5th day, when the morphologically more mature cells fail to incorporate TdR-^{3}H. The associated compartments of each cycle are also included. The fourth line shows when the *last* cells to differentiate into primitive red cells might mature. They enter the population of circulating and dividing erythroblasts at 60 hr of incubation. This is corroborated by two sources: (*a*) morphological evidence showing the absence of the earliest erythroblasts during this period. (*b*) cytophotometric data (Campbell et al., 1971) revealing the absence, at 70 hr, of mitotic cells having the lowest quantities of Hb, presumably indicating that no cells entered the population one cell cycle (10 hr) earlier.

An important prediction of the model requires that several cell cycles coexist. The data in Figs. 7 and 8 confirm this. Further support comes from the in vitro studies which show that the rate of DNA synthesis, in vitro, for 4 and 5 day cells is comparable to the in vivo rate under several different conditions. Additional evidence comes from the cytophotometric results (Campbell et al., 1971) which indicate the presence of several classes of mitotic cells in the same circulation. Each class is distinguished by its unique quantity of Hb.

Further support for this model comes from estimates of cell number during development. Table V shows the number of red cells per embryo for each of the 4 days considered. Between the 2nd

TABLE V

The Increase in Cell Number with Increasing Age of the Embryo

Day	Moles Hb per cell $\times 10^{-16}$	Moles Hb per embryo $\times 10^{-8}$	Cell number per embryo $\times 10^{-8}$	Relative cell number
Late day 2	4.65	0.13	0.028	1
Early day 3	8.35	0.77	0.093	3.3
4	11.2	3.1	0.36	12.8
5	14.0	11.0	0.8	28.6

Hb was measured as described by Levere and Granick (1967). Cells were counted at two dilutions in a hemocytometer and averaged. Cell number per embryo was determined by dividing Hb per embryo by Hb per cell. The relative cell number was obtained by dividing the cell number per embryo for each of the given days by the cell number given for the 2nd day. The 28.6-fold increase is consistent with the five divisions expected between late day 2 and day 5.

and 5th day an increase of about thirty-fold is seen. This is approximately what would be expected on the basis of the model proposed in Fig. 9. Given 10^8 as the total productivity from the primitive line, it is possible to calculate, assuming six divisions of red cells and one of the parent hematocytoblast, that at the time when Hb first appears in the embryo (35 hr) there are 8×10^5 precursor cells present. If this figure is distributed over 30 hr (the time over which erythroblasts enter the population), then the average input is 5.2×10^4 red cells per hr. Wilt (1965) has given 60,000 as the number of cells present in the unincubated embryo. In addition, Emanuelson (1965) has estimated that the generation time for the average embryonic cell during this period is 7.4 hr. Assuming no cell death, this leads to a prediction of 2×10^6 cells at 35 hr of incubation. This would mean that approximately one out of 100 cells in the 35 hr embryo is a hematocytoblast. This figure is consistent with the notion that determination of red cells occurs very early after fertilization. In the extreme case, if one assumes no selective multiplication of one cell type for the first 35 hr of incubation and also a clonal derivation of primitive erythroid cells, it is possible to estimate that determination had, in some sense, occurred after the sixth or seventh division of the fertilized egg.

On the Passage Through the Cell Cycle

With respect to the relationship between red cell development and cell division, it should be emphasized that: (*a*) Cells synthesizing Hb divide. This indicates that developing erythroblasts possess the organizational and structural apparatus for the entire division process. (*b*) The cell cycle and its parameters change as the maturing cells cycle from one generation to the next; thus, there must be mechanisms for altering the length of these periods. (*c*) Although division occurs in the immature cells, it is limited; hence, the necessity for a mechanism to turn off division. The following discussion deals with the second and third points and asks only if these changes are programmed or not. "Program" is defined as *activity* independent of *specific* and *concurrent* external cues.

On the Alteration of the Cell Cycle Times and the Limitation of Mitotic Activity

At least two different cell cycles exist in the same circulation. This implies that environmental factors are not both necessary and sufficient for *maintaining* cell cycle length. It does not necessarily exclude all environmental influences specifically directed at regulating mitotic behavior. Conceivably, at a certain point during maturation the interaction between a given cell and a specific external cue results in either of the following: (*a*) A competent cell responds immediately; thus, a cell in the S period characteristic of a 17 hr cell cycle would be induced by this hypothetical factor to enter the S period characteristic of the 29 hr cell cycle. The cell thus reacts in a "step-down" fashion. (*b*) A competent cell is redirected by an external cue; however, the *expression* of this redirection is not manifest immediately; indeed, as discussed below, expression must lag until the cell completes at least its current cell cycle. Note that this last possibility corresponds with our definition of programming.

Consider the first possibility, i.e. that the cell responds immediately to a circulating substance, and ask at what point in the mitotic cycle might the cell express its susceptibility? Clearly, the point is not in G2 or *M* since the differences in length of these intervals from generation to generation are too small to account for the differences that are observed in generation times (Table I). S is also excluded by the same reasoning and also by the additional evidence that two S periods exist in the

same circulation (Fig. 7 and Fig. 8) and that 4 day cells synthesize DNA at the same rate independent of the serum in which they are incubated (Table IV). Finally, G1 is unlikely on the basis of the radioautographic evidence showing that 4 day cells are not altered for at least 5 hr in their progression through G1 and into S, even when challenged to do so in 5 day serum. This would argue that the 4 day G1 is not altered in 5 day serum. Also, the cytophotometric data (Campbell et al., 1971) show the presence of several generations of metaphase erythroblasts in the same circulation. Each such class can presumably give rise to a unique class of G1 cells. It is unlikely that these cells respond immediately to a specific environmental substance that lengthens their cell cycle.

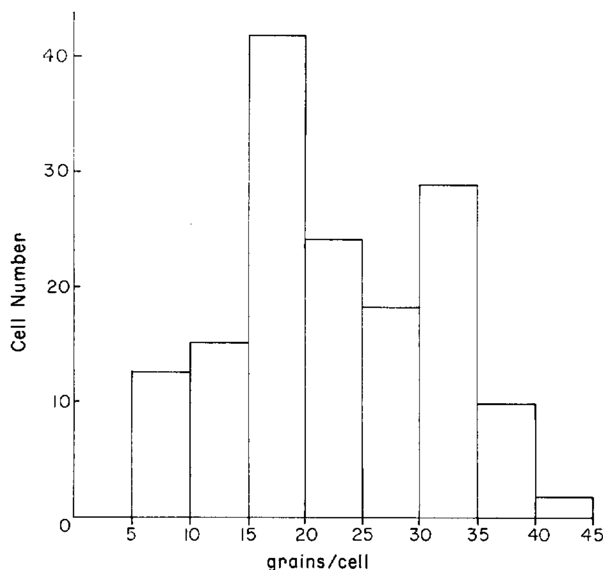

Figure 7 Late day 4 cells were pulsed with TdR-^{3}H (5 μCi/10.96 μg) for 15 min. Radioautograms were prepared. Cells were evaluated for grain counts and grouped into compartments distinguished by increments of 5 g per cell. Background was essentiall zero. 500 cells were evaluated.

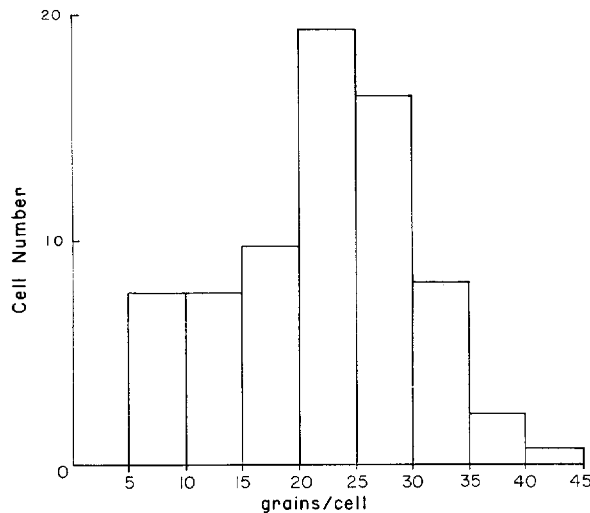

Figure 8 5 day cells were prepared as described in the legend to Fig. 7. 150 cells were evaluated for grain counts.

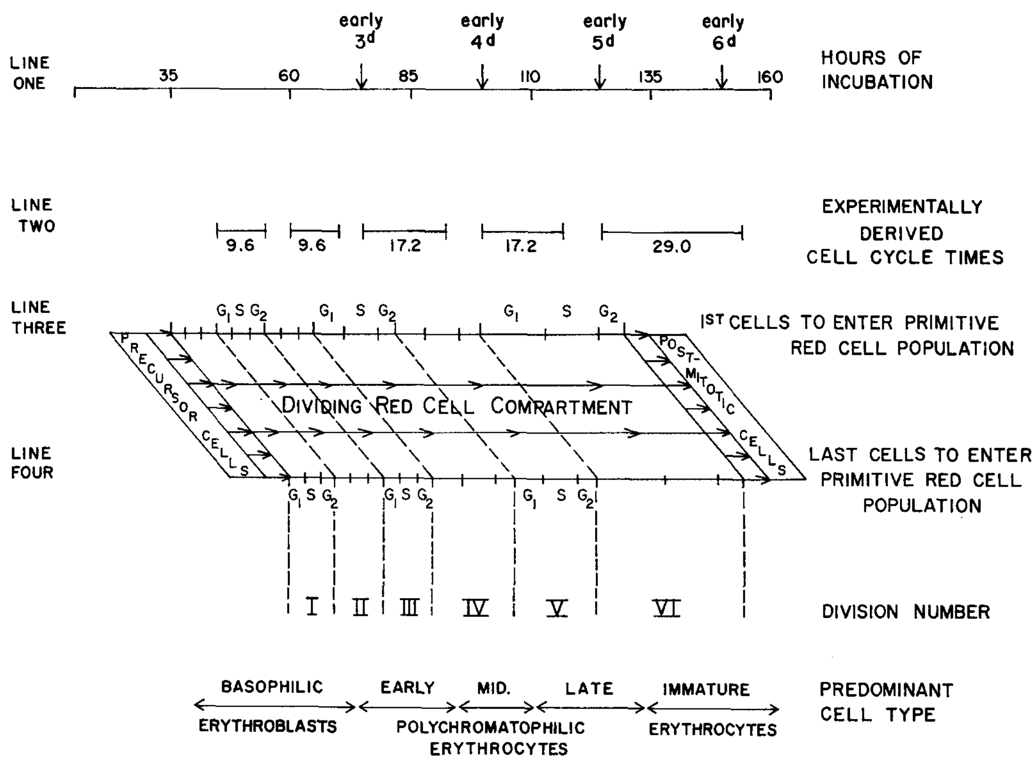

FIGURE 9 A model for primitive erythropoiesis in the chick. Cells are seen to enter and leave the dividing population over a 25 hr period. Each cell undergoes the same sequence of six divisions. Depending on the exact nature of the input function (the function describing the rate at which red cells differentiate between 35 hr and 60 hr) and the amount of spread that develops for each cell cycle, a cross-section of the red cell compartment gives the relative number of cells from each cell cycle present in the population at that time.

How then might some factor influence a given cell to follow some future mitotic behavior after the present cycle is completed? Two of the more likely schemes describing these interactions are illustrated in Fig. 10. Their most general characteristics can be used to explain either the changes occurring in the cell cycle or the limitation of cell cycle activity to six divisions: (*a*) Before dividing, the hematocytoblast is programmed such that its progeny of red cells become the progenitors for only six generations of Hb-producing cells. Such a program might involve the parcelling out to daughter erythroblasts of a given quantity of some substance, necessary for division yet beyond the synthetic capacity of the cell. The substance, halved with each division, eventually reaches nonfunctioning concentrations. Similar types of arguments could account for variations in the cell cycle parameters. Alternatively, it is possible that some negative regulator(s) is synthesized constantly throughout each generation. When its concentration reaches a specific level, the cell can no longer divide. (*b*) The second scheme is a stochastic one. Here the same precursor cell is primed, not for six, but for three divisions, each of 10 hr. At some point during these first three generations the cell becomes "competent" for redirection by a circulating factor, which can reprogram the competent cell for another two divisions, this time of 17 hr each. This same sequence of different external factors influencing only those cells which have reached a specific competence can continue until the postmitotic state is finally reached. Indeed, this stochastic interplay between competent cells and their environment may be a basic component of many differentiating processes (Holtzer and Matheson, 1970).

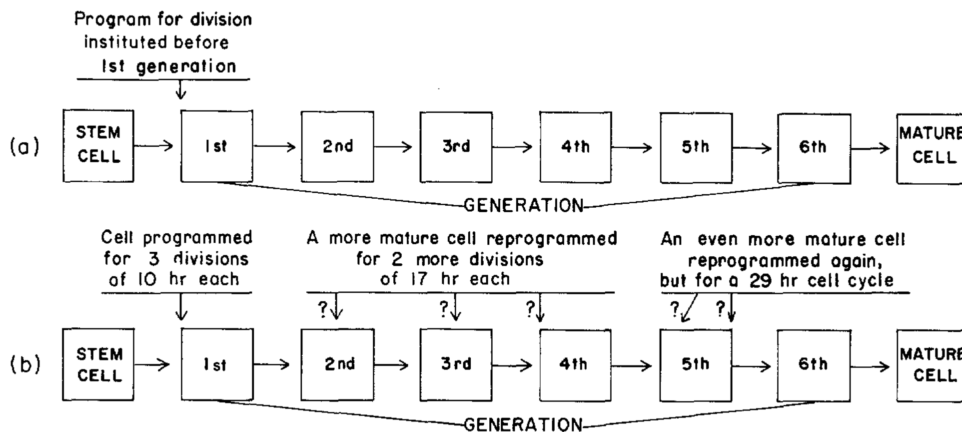

FIGURE 10 Three ways in which the observed mitotic activity might be programmed. In (b) external cues are operating during the reprogramming process, while in (a) they must do so before overt differentiation takes place.

Division and Erythropoiesis

The evidence presented here indicates that primitive red cells of chick undergo an average of six divisions. Tarbutt (1967, 1968) has arrived at a similar figure for rat erythrocytes, and Saint-Marie (1966) has indicated the same for humans. Clearly, division in the red cell line (and probably, in other blood cells) represents an amplification process, enabling a 128-fold increase in the output from a single stem cell. Put in another way, this amplification process, given the requirement placed on the erythropoietic organs for a specific number of mature red cells per hour, allows erythropoietic stem cells the opportunity to divide fewer times during the lifetime of the organism. As an example, if the ratio of stem cells to "mature erythrocytes produced per hour" is 3:1, then it can be calculated that stem cells need to divide some 40 times more frequently, if division is not occurring in maturing red cells.

Coupled with the evidence linking mutational and other deleterious effects (e.g., nondisjunctions) to cell-cycle–associated activities, it is proposed that division occurs in the Hb-synthesizing cell so as to limit the number of division associated mistakes in the stem cell line. Thus, although the total number of such mistakes per erythropoietic compartment (stem cells plus overt erythroid cells) does not vary, the burden of these events has been placed upon cells destined to die, i.e. the red cells, as opposed to cells that must last the life of the animal.

Erythrogenesis and Myogenesis

At least two roles for DNA synthesis have been postulated for cell differentiation (Holtzer, 1970a, b): (*a*) An obligatory requirement for DNA synthesis to alter the genetic program during the evolution of a cell lineage (e.g. between hematocytoblast and first generation erythroblast, between presumptive myoblast and myoblast, between somitic mesenchyme cell and chondroblast). These replication-dependent events occur during "quantal" cell cycles, but not during "proliferative" cell cycles, and (*b*) the cessation of DNA synthesis as a precondition for translating certain terminal luxury molecules (Holtzer and Abbott, 1968). Accordingly, it is interesting to compare DNA synthesis and the translation of hemoglobin in these erythroblasts with DNA synthesis and the translation for myosin in skeletal and cardiac myoblasts. Skeletal myoblasts synthesizing myosin, actin, and tropomyosin do not synthesize DNA and do not enter mitosis (Okazaki and Holtzer, 1965). However Rumyantsev and co-workers (1968), Sasaki, et al. (1968) and Manasek (1968, 1969) observed some cardiac myoblasts in metaphase, and Goode and Holtzer (cited in Holtzer, 1970b) and Weinstein and Hay (1970) observed uptake of TdR-^{3}H into cardiac myoblasts. That some cardiac myoblasts yield a progeny of several generations may be similar to the six generations of primitive erythroblasts. The fact that erythroblasts undergo a modest number of proliferative cell cycles following the

quantal cell cycle of the hematocytoblast should not obscure the main biological principal that normal cells synthesizing hemoglobin do not yield the numbers of progeny produced by erythroid stem cells in bone marrow. Erythroblasts and cardiac myoblasts, like skeletal myoblasts, function under stringent restraints of DNA synthesis. If DNA synthesis depends on depleting DNA polymerase from erythroblasts, then cardiac myoblasts might differ from skeletal myoblasts only in rate of turnover of the polymerase. These differences between erythroblasts and cardiac myoblasts, on one hand, and skeletal myoblasts, on the other, could represent an adaptation to the requirements of early embryogenesis; sizeable numbers of functional red blood cells and contracting heart cells must be produced quickly. This reasoning might also apply to the limited numbers of cell cycles that active antibody-producing cells undergo (Szeinberg, 1968).

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Note Added in Proof: Recently, Hagopian and Ingram (manuscript in preparation) have measured (*a*) the cell cycle times during the first few divisions, (*b*) the number of primitive red cells present in the circulation during the first 5 days of development, and (*c*) the number of precursor cells present at 35 hr. All of their results are virtually identical to those presented here.

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