PRIMITIVE ERYTHROPOIESIS IN EARLY CHICK EMBRYOGENESIS

II. Correlation between Hemoglobin Synthesis and the Mitotic History

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ABSTRACT

Primitive erythroblasts in the circulating blood of the chick embryo continue to divide while synthesizing hemoglobin (Hb). Hb measurements on successive generations of erythroblasts show that there is a progressive increase in the Hb content of both interphase and metaphase cells. Furthermore, for any given embryo the Hb content of metaphase cells is always significantly greater than that of interphase cells. The distribution of Hb values for metaphase cells suggests that there are six Hb classes corresponding to the number of cell cycles in the proliferative phase. The location of erythroblasts in the cell cycle was determined by combining Feulgen cytophotometry with thymidine radioautography on the same cells. Measurements of the Hb content for erythroblasts in different compartments of the cell cycle (G1, S, G2, and M) show a progressive increase through the cycle. Thus, the amount of Hb per cell is a function of the number of cell divisions since the initiation of Hb synthesis and, to a lesser degree, the stage of the cell cycle. Earlier generations of erythroblasts synthesize Hb at a faster rate than the terminal generation. Several models have been proposed to explain these findings.

INTRODUCTION

Erythropoiesis in the chick embryo involves successive and morphologically distinct populations. The primitive erythroblasts are the only cell type present until the 5th day of incubation, at which time the definitive erythroblasts appear. The development of primitive erythroblasts follows a well defined and predictable progression, with a close correlation between the day of incubation and the predominant morphological stage of development (Dawson, 1936; Sandreuter, 1951; Romanoff, 1960; Lucas and Jamroz, 1961).

After 35 hr of incubation, hemoglobin (Hb) is first detected in groups of basophilic erythroblasts localized initially in the posterior region of the area opaca (Slonimski, 1927; Wilt, 1962; Hell, 1964; Granick and Levere, 1965). While these erythroblasts synthesize Hb, they continue to divide. Their mitotic history has been described by Weintraub et al. (1971) who showed that the majority of these Hb synthesizing cells function as progenitors for six successive generations of erythroblasts.

Microspectrophotometric techniques have been used to determine the total amount of Hb in single unstained cells (Thorell, 1947; DeCarvalho, 1951; Grasso et al., 1963). As the specific absorption co-

efficient of Hb is very high in the Soret region, measurements may be made even in cells with relatively small amounts of Hb. In the present study the relative changes in the amount of Hb per cell that occur during development have been measured with an integrating cytophotometer, CYDAC (Bostrom and Holcomb, 1963; Mayall and Mendelsohn, 1970 a, 1970 b).

Since interphase and metaphase cells are readily distinguishable microscopically, Hb measurements have been made on cells from both stages during successive days of incubation. Furthermore, Hb measurements have been made on cells in different stages of the cell cycle (G1, S, G2, and M). Their location in the cell cycle has been determined by combining Feulgen cytophotometry with thymidine radioautography on the same cells. Similarly the synthesis of Hb has been followed after these erythroblasts undergo their final division. Consequently, the rate of Hb synthesis during the terminal cell cycle has been compared with that observed in the subsequent postmitotic progeny.

MATERIALS AND METHODS

White Leghorn eggs were incubated at 37°C. The 1st day of incubation was considered as day 0, and subsequent days of incubation were therefore numbered appropriately. Air dried smears were made from small volumes of blood collected from the blood islands and one of the main branches of the chorio-allantoic vessels from day 2 to day 10 embryos.

Cytophotometry

Absorbance measurements were made with the mechanical scanner of the CYDAC system (Bostrom and Holcomb, 1963; Mayall and Mendelsohn, 1970 a, 1970 b) as an integrating cytophotometer. Each measurement is expressed in $E_\lambda A$ units per cell ($E_\lambda \times A$), where: $E_\lambda = k\ cd$, E_λ = absorbance, K_λ = absorption coefficient, c = concentration, d = thickness, A = the area of the cell, and λ = wavelength of light defined by the interference filter used.

Absorbance measurements were made on cells from unstained smears and smears stained with benzidine (Ralph, 1941). The benzidine staining reaction was used to facilitate the recognition of the most immature stages in the development of primitive erythroblasts. Fig. 1 shows the absorption curves for both Hb and Hb stained with benzidine. Interference filters were used to measure the different chromophores. Filter 433 was used to measure unstained Hb, and Filter 566 was used to measure Hb stained with benzidine. The transmission spectrum for each filter is shown in Fig. 1. The transmissivity of each filter describes a narrow bandwidth, with a maximum transmission corresponding nominally to the filter number. In fact the maximum transmission for Filter 433 is 445 mμ, and similarly for Filter 566 is 590 mμ. In both cases measurements made with these filters represent approximately 30% of the maximum absorbance for each chromophore. Filter 433 was chosen for measurements on unstained Hb to minimize any effect that might be attributable to the presence of free porphyrin (see Thorell, 1950). Hb stained with benzidine and measured with Filter 566 demonstrates a stoichiometric relationship with measurements on unstained Hb with use of Filter 433 (Campbell, 1970). There is a fourfold increase in the sensitivity for the stained material. Under these conditions each measurement is proportional to the mass or amount of Hb per cell.

Estimates for the amount of DNA per cell were determined from cells that have undergone a modified Feulgen staining reaction (Mayall and Mendelsohn, 1970). These measurements were made using Filter 566.

Radioautography

To determine which cells in the circulating blood were synthesizing DNA, 50 μCi thymidine-^{3}H (6.7 Ci/mmole) in 0.5 cc of sterile water were added to each embryo for 1 hr. The subsequent smears were fixed in alcohol: formalin: acetic acid (20: 2 :1) and washed for 1 hr, then dried. The slides were dipped in Kodak NTB3 emulsion and exposed for 1–4 wk, after which they were developed (Bischoff and Holtzer, 1968).

For in vitro studies 20 μCi thymidine-^{3}H (6.7 Ci/mmole) in 0.2 cc F10 (Grand Island Biological Co., Berkeley, Calif.) was incubated with 0.1 cc blood for ½ hr. Radioautography for these smears was carried out as above.

Determination of Hb Values for Different Stages of the Cell Cycle

Smears were made from day 4 embryo blood that had been pulse labeled with thymidine-^{3}H in vitro. An appropriate area was selected and mapped photographically. Hb measurements were made on individual erythroblasts. The slide was then stained for Feulgen cytophotometry and estimates of the DNA were determined for each cell previously measured for its Hb content. Subsequent radioautography indicated which cells were in S when tritiated thymidine was added. G1 and G2 cells are readily distinguishable on the basis of their DNA values. After each cell was identified according to

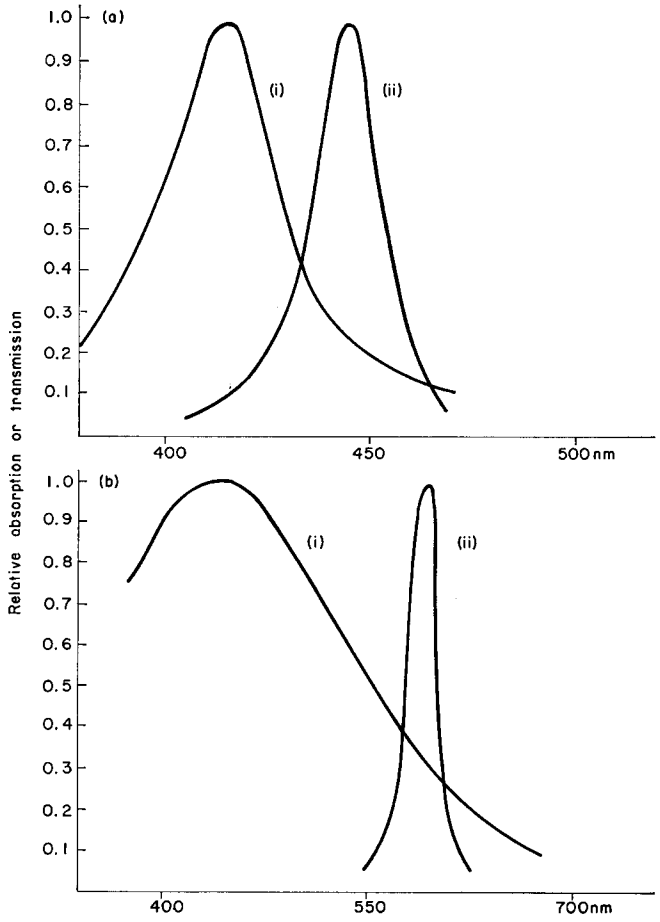

FIGURE 1 a (i), relative absorption spectrum from primitive erythroid cells (unstained); (ii), relative transmission spectrum for Filter 433.

FIGURE 1 b (i), relative absorption spectrum from primitive erythroid cells stained with benzidine; (ii), relative transmission spectrum for Filter 566.

its location in the cell cycle, the average Hb value for each stage of the cell cycle was determined.

Colcemid

The mitotic index for primitive erythroblasts is approximately 2–3% depending on the age of the embryo. To increase the number of metaphase cells, 3 cc 10^{-6}M Colcemid (Ciba Pharmaceutical Co., Summit, N. J.) were added to each embryo for 4 hr. Hb measurements on normal metaphase and Colcemid metaphase-arrested cells show no significant difference. This leads to two conclusions: (*a*) there is no detectable synthesis of Hb during mitotic arrest, and (*b*) Hb measurements on metaphase-arrested cells can be treated as if they had been made on normal metaphase cells. Consequently, in this paper, Hb measurements have been made on Colcemid arrested cells, and are referred to as *metaphase* values.

Terminology

The replicating erythropoietic cells in the circulating blood for the first 5 days constitute successive generations of the "primitive erythroblast" series (proliferative phase). After day 6, there are no further new generations, although the postmitotic primitive erythrocyte series may persist in the circulating blood until hatching (nonproliferative phase). In the following discussion *erythroblasts* are cells in the mitotic cycle which are concurrently synthesizing their characteristic amount of Hb. *Erythrocytes* are postmitotic cells that are either still

synthesizing or have finished synthesizing their characteristic amount of Hb. *Hematocytoblasts* are the immediate mother cells of the first generation of erythroblasts; currently there is no evidence that hematocytoblasts translate for Hb.

According to terminology introduced elsewhere (Holtzer and Abbott, 1968; Holtzer, 1970) the divisions of hematocytoblasts that yield erythroblasts would be "quantal" cell cycles, whereas the divisions of the Hb-synthesizing erythroblasts that yield the next generation of erythroblasts are "proliferative" cell cycles.

RESULTS

Hb Measurements on Primitive Erythroblasts and Erythrocytes

To determine the relative changes in the amount of Hb per cell during development, measurements were made on primitive erythroblasts and erythrocytes on successive days of incubation. The results of these measurements have been combined in Fig. 2 with an outline of the developmental history of the primitive series. This outline is expressed in terms of (*a*) the predominant cell type and (*b*) the divisional state of the cells (proliferative versus nonproliferative). There is a progressive increase in the average amount of Hb per cell until day 9, at which time there is no further increase and the cells have become mature erythrocytes. Following the transition from the proliferative to the nonproliferative phase (day 5–day 6), Hb synthesis continues for the next 3 or 4 days. However there is a decrease in the rate of Hb synthesis during this nonproliferative phase.

In summary (*a*) there is a progressive increase in the average amount of Hb per cell until day 9, and (*b*) there is a decrease in the rate of Hb synthesis after the final division.

Primitive Erythroblasts

In this section the relationship between the position of primitive erythroblasts in the succession of the cell cycles and their Hb content will be examined. Accordingly, Hb measurements were made on cells in the same stage of the cell cycle (i.e., mitosis) on successive days of development, and also on cells in different stages of the cell cycle (G1, S, and G2), during the same day of development. In the first series, measurements were made on both interphase and metaphase cells. In the second series each cell was located in G1, S, and G2 with thymidine radioautography. The average Hb value for each stage of the cell cycle was then determined (see Materials and Methods).

Interphase and Metaphase Cells

The average Hb content for interphase and metaphase cells was plotted against the day of in-

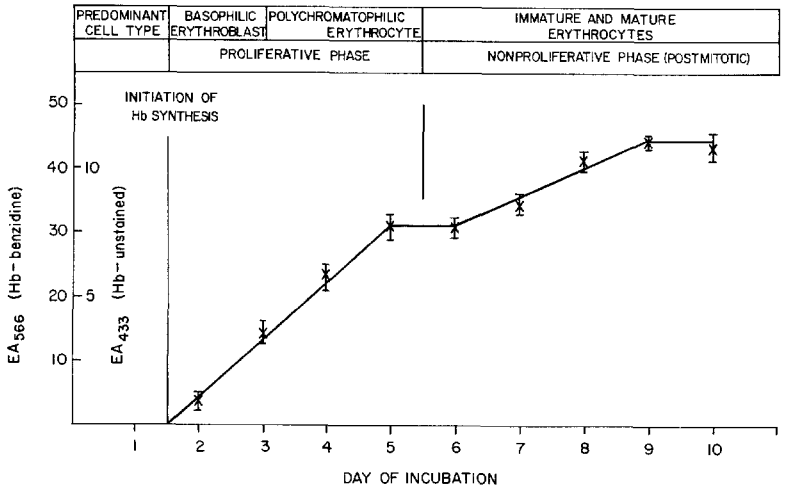

FIGURE 2 Hb measurements from small populations of primitive erythroid cells on successive days of incubation after the initiation of Hb synthesis. Each point represents the average value for at least fifty measurements. The Hb values are expressed either as EA_{433} units (unstained) or EA_{566} units (stained with benzidine). The measurements have been correlated with the developmental history of the predominant cell type and whether the cells are dividing or not.

cubation in Fig. 3. There is a progressive increase in the amount of Hb for both interphase and metaphase cells. Moreover, the Hb content of metaphase cells is always greater than the corresponding average content in interphase cells from the same embryo. "t" tests were made to determine the significance of these differences and the results are given in Table I. These tests show that for every day the average amount of Hb per metaphase cell is always significantly greater than the average amount of Hb per interphase cell.

Metaphase Cells and Hb Classes

Measurements made only on metaphase cells from day 2 to day 5 are plotted as frequency histograms in Fig. 4. The results are interpreted as follows: (*a*) there are two peaks for early day 2 erythroblasts. (*b*) by the end of day 2 the first two peaks remain, but a new peak has emerged. (*c*) by early day 3, the majority of the cells are represented by a third and fourth peak, but there may be a few cells corresponding to the lowest peak. (*d*) in late day 3 embryos, the majority of the cells are in a fourth peak. There is some correspondence of the lower values with the third peak in the earlier material. In addition, there are some cells present in a fifth peak, which correspond to the Hb values from day 4 embryos. (*e*) All the Hb values for day 4 and 5 embryos are in single but distinct peaks. This analysis of the Hb content of metaphase cells suggests that there are six different peaks or metaphase classes. The appearance and disappearance of these peaks is consistent with a progression of successive cell cycles or generations that occur during the proliferative phase.

As each peak represents a metaphase class, then

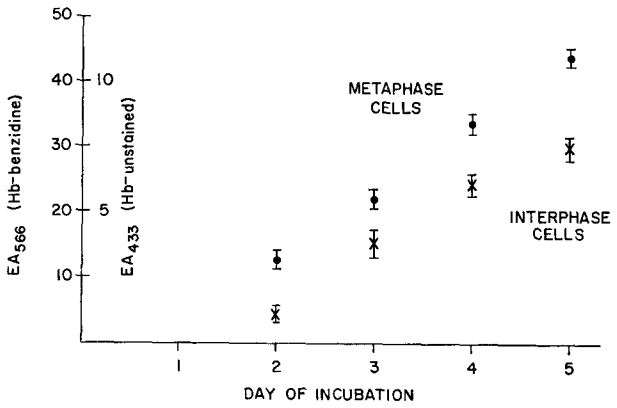

Figure 3 Hb measurements on interphase and metaphase cells from day 2 to day 5 (the proliferative phase). Each point represents the average value for at least fifty measurements. The Hb values are expressed either as EA_{433} units (unstained) or EA_{566} units (stained with benzidine). The relationship between EA_{433} units or EA_{566} units is described in Materials and Methods.

Table I

Hb Measurements Made on Interphase and Metaphase Cells from Day 2 to Day 5 Embryos. Comparison of the Mean Values

	Interphase $\bar{X} \pm SD_{\bar{X}}$	Metaphase $\bar{X} \pm SD_{\bar{X}}$	t tests	
			df.	t value
Day 2	1.2 ± 0.05	3.0 ± 0.10	98	17.3*
Day 3	3.9 ± 0.14	5.5 ± 0.16	98	7.6*
Day 4	6.1 ± 0.14	8.4 ± 0.13	98	12.2*
Day 5	7.6 ± 0.26	11.4 ± 0.19	98	11.2*

Measurements are expressed as EA_{433}.
Each set of measurements was made on 50 cells.
* Highly significant for two tailed t test.

the proliferative phase of primitive erythrocyte development may be characterized by a total of six metaphase classes, i.e., six cell cycles. This is in accord with the results of the cell cycle kinetics (Weintraub et al., 1971) which indicates that the majority of erythroblasts emerging on day 2 will yield, on the average, six generations of red blood cells. This suggests that each mitosis can be

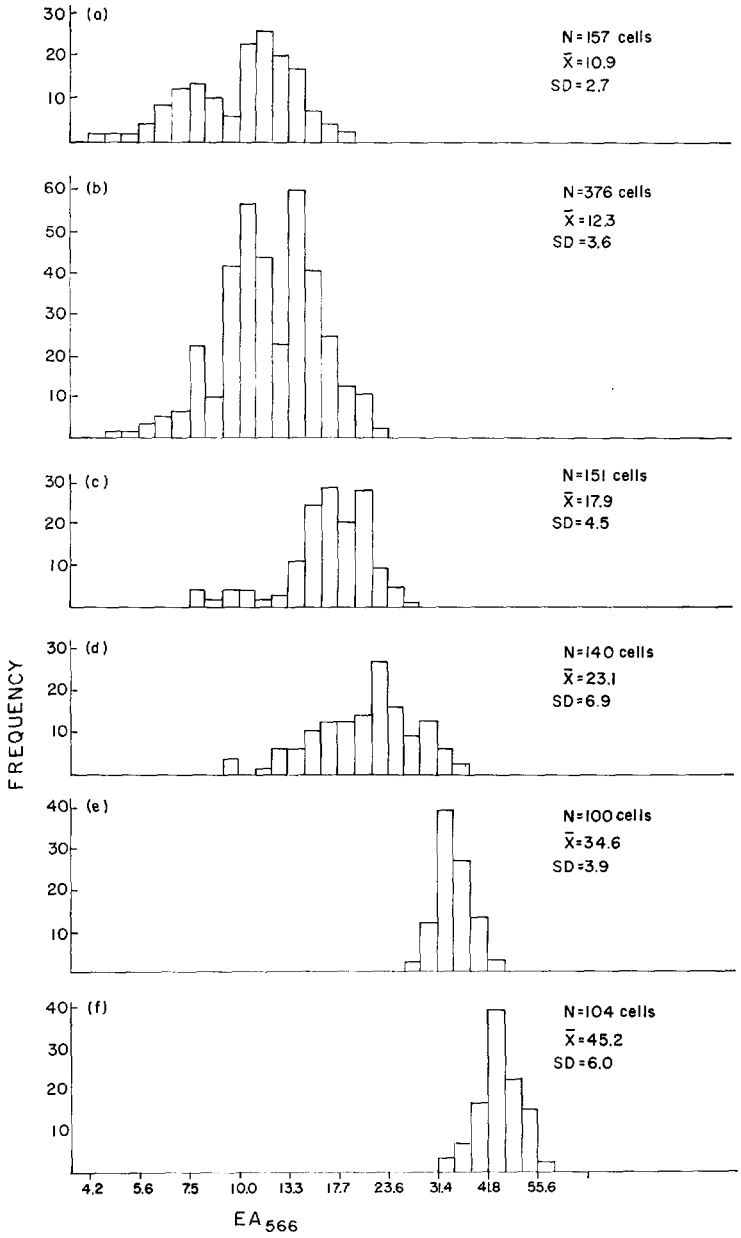

FIGURE 4 Frequency histograms for Hb measurements on metaphase cells from day 2 to day 5. The measurements are expressed as EA_{566}. Each interval represents a 10% increase in the Hb values. These measurements have been made on at least two embryos for each histogram. (*a*), day 2 embryo (early); (*b*), day 2 embryo (late); (*c*), day 3 embryo (early); (*d*), day 3 embryo (late); (*e*), day 4 embryo; (*f*), day 5 embryo.

uniquely classified on the basis of the total amount of Hb per cell synthesized during the previous cell cycle.

The cell cycle data have been summarized in Fig. 5. This summary can be used to predict what cell cycles will be present at different times during development. These predictions can then be correlated with the cytophotometric data. Since the first two cell cycles are 10 hr long, representatives from the first two cell cycles should be present in the circulating blood of early day 2 embryos (20 hr after the first appearance of Hb). The cytophotometric data support this prediction. Similarly, on the basis of the kinetic data, primitive cells from late day 2 embryos should be in the first three cell cycles. Again the cytophotometric data are in agreement with this prediction. By day 3, no cells should be present in the earliest cycles. This prediction is confirmed both by the cytophotometric data and by the cytological observation that there are no basophilic first generation erythroblasts present at this stage. On day 4 and 5, the lengths of the respective cell cycles are 17 and 29 hr (Fig. 5) and one would therefore predict that the majority of these cells come from one cell cycle. The cytophotometric data for both day 4 and day 5 suggest that there is only one metaphase class present. Thus, the cytophotometric data are in agreement with the predictions from the kinetic data.

Both sets of data support the concept that the primitive erythron in the chick embryo comprises a discrete population of cells that progress through six generations. There is one wave of entry of cells into the circulation from the hematocytoblast compartment. This wave is of approximately 30 hr duration, and there is no further influx from the primitive hematocytoblasts. Hence at any specified time the profile of the population is determined by the distribution of these cells in their successive generations. This situation is very different from the definitive series.

Different Stages of the Cell Cycle

So far the emphasis has been placed on comparing the Hb values from the same compartment (mitosis) of different cell cycles. In this section, Hb values for different compartments of the cell cycle will be compared. It has been demonstrated that erythroblasts at the beginning of either day 4 or day 5 from embryos will be predominantly from one cell cycle. As described in Materials and Methods, measurements on the same individual cells were made to determine both their relative Hb and DNA content. Subsequent radioautography combined with the DNA estimates showed each cell to be identified according to its location in the cell cycle.

The results of these measurements are illustrated in Fig. 6, in which the Hb and DNA values for each cell are plotted against each other. Each cell has also been identified according to its location in the cell cycle. There is some overlap between the Hb values for G1 and S cells and similarly for S and G2 cells, but the values for G1 and G2 are quite discrete. The spread of the values is greatest for S cells, and is reflected in the coefficients of variation for the Hb values shown in Table II. The interpretation of these data depends on (*a*) the relative lengths of the cell cycle compartments, and (*b*) the rate of Hb synthesis for each compartment.

The average Hb value for each compartment of the cell cycle has been determined in Table II. Although there is a progressive increase in the average Hb value from G1 through S to G2, there is little increase between the G2 value and the average metaphase value. The differences between G1 and S, and between S and G2 are significant ($P < 0.001$, two tailed t test). However the difference between G2 and M is not significant ($0.2 > P > 0.1$, two tailed t test). The plot of Hb and DNA values (Fig. 6), when considered in conjunction with the results of the t tests, indicates that Hb synthesis occurs throughout S. In contrast, there appears to be relatively little Hb synthesis during G2. It is not, as yet, possible to characterize in greater detail how much Hb is synthesized during G1. Nevertheless these data support the general concept that there are changing rates of Hb synthesis during the cell cycle.

In summary, the relative changes in the Hb content during a succession of erythroblast gener-

Table II

Hb Measurements Made on Cells in Different Stages of the Cell Cycle from Day 4 Embryos

	$X \pm SD_{\bar{x}}$	Coeff. Var.
		(%)
G1 (60 cells)	4.9 ± 0.08	11.4
S (80 cells)	6.2 ± 0.11	15.7
G2 (10 cells)	8.3 ± 0.26	10.3
M (50 cells)	8.9 ± 0.15	11.8

Measurements are expressed as EA_{433} units.

ations have been measured. By focusing on the same compartment of the cell cycle throughout the proliferative phase, it has been shown that each mitosis may be uniquely classified on the basis of its Hb value. There is a progressive increase in the Hb values for each generation and the number of cell cycles described in the kinetic analysis. Similarly, within each cell cycle there is a progressive increase in the amount of Hb per cell. Moreover there is some indication that the rate of Hb synthesis varies through the cell cycle.

Primitive Erythrocytes

After the primitive erythroblasts have passed through the succession of cell cycles in the proliferative phase, they undergo their final division and enter a nonproliferative phase, which terminates in cell death sometime during the next 14 days. After the final division, the primitive erythrocytes continue to synthesize Hb for 3 or 4 days (Fig. 2). Associated with this increase in the amount of Hb per cell over the 3–4 day period there is a decrease in the spread of the Hb values. This is reflected in the decrease of the coefficient of variation from 25–30% on day 6 to 10–15% on day 10. The initial spread can be explained by the fact that the primitive cells withdraw from the cell cycle over a period of 25 hr (Weintraub et al., 1971), whereas by day 9 each cell has reached the same mature state.

The following experiment was designed to demonstrate that the Hb content of each cell at any point during the postmitotic period will reflect the time since that cell underwent its final division. Tritiated thymidine was added to late day 5 embryos, at which time there are two populations of cells: one having already finished synthesizing DNA, the other still synthesizing DNA. It is the latter cells that will incorporate tritiated thymidine. Smears were made from different embryos on each of the subsequent days from day 6 to day 10. By combining Hb measurements with radioautography, it is possible to determine the average Hb value of these two populations. There is a marked difference between the mean Hb values from the two populations on successive days as seen in Fig. 7. This difference persists until about day 9, by which time both populations have attained the same value. The slope of each line shows that the rate of Hb increase in both populations is the same. The displacement of the two slopes indicates the average difference in the developmental time of the two populations. This displacement is consistent with the observation that the primitive erythrocytes withdraw from the cell cycle over a 25 hr period.

From this experiment the rate of increase in the amount of Hb per cell during the nonproliferative phase may be estimated and compared to the rate of increase during the last cell cycle. Approximately 7.0 EA_{433} units of Hb are synthesized during the terminal cell cycle, which is 29 hr long. Therefore the rate of increase in this cycle is 0.24 EA_{433} units per hr. The slope delineating the rate of increase of the nonproiliferatve cells is 0.06 EA_{433} units per hr. There is a fourfold difference between the two rates. This observation indicates that there is a marked decrease in the rate of Hb synthesis after the terminal division.

In summary, there is a progressive increase in the amount of Hb per cell after the terminal division. Most cells attain the same mature value, synthesizing Hb at the same rate. However, there is a decrease in the rate of Hb synthesis during the nonproliferative phase as compared with the terminal generation of the proliferative phase.

DISCUSSION

Analyses of mass, total protein, and RNA have been correlated with different compartments of the cell cycle (Caspersson, 1950; Prescott, 1955; Hanawalt et al., 1961; Killander and Zetterberg, 1965 a; Maaløe and Kjeldgaard, 1966; Seed, 1966; Pfeiffer and Tolmach, 1968). In general these studies have stressed the cyclic nature and the constant values for mass, total protein, RNA, and DNA that are maintained in cells that yield large numbers of cell generations (see, however, Fox and Pardee, 1970). More than one model of the cell cycle (Mazia, 1962; Zetterberg and Killander, 1965 b) postulates that the movement from one compartment of the cell cycle to another is coupled in some degree with the synthesis of a critical quantity of a given molecule (e.g. DNA, mitotic protein, etc.). In contrast to these studies, our investigation is concerned with a system that undergoes a specified and limited number of divisions and is one in which it is possible to follow the synthesis of a single cell specific macromolecule, hemoglobin, from generation to generation. Our aim was to follow the synthesis of Hb during this succession of limited generations and further to determine whether the synthesis of Hb and passage through the cell cycle were interrelated. Our results sug-

gest that the amount of Hb per cell correlates primarily with the number of cell cycles that each cell has undergone since the initiation of Hb synthesis at approximately 35 hr of incubation. The amount of Hb also correlates, to a lesser degree, with the stage of the cell cycle. Furthermore, daughter cells of each successive mitosis accumulate more Hb than the parental cells. Thus it is clear that although there are fluctuations in the Hb content of these replicating cells, they are not cyclical in nature. Any model concerning the differentiation of the primitive erythrocyte must therefore explain this correlation between the mitotic history and Hb synthesis.

Earlier studies have measured the average Hb content (O'Connor, 1952; Rychter et al., 1955), and the concentration of Hb per cell (Thorell and Raunich, 1966) on successive days of chick embryonic development. Cytophotometric studies in other erythropoietic systems have shown that after the initiation of Hb synthesis there is an inverse relationship between the Hb content and the RNA content per cell (Thorell, 1947; Grasso et al., 1963, 1966, and 1967). These investigations also correlated the amount of Hb per cell with the progression of the morphological stages through development. In this study we have not attempted to do this for two reasons. The different compartments of the cell cycle, particularly mitosis, represent relatively small time intervals in comparison with those for the different morphological stages of development. Hence by correlating the amount of Hb per cell with the mitotic history, we have increased the resolution for monitoring developmental changes that occur during erythropoiesis. Thus, for example, changes in Hb content over periods as short as 1 hr may be followed. Secondly, conventional criteria for identifying the different morphological stages of development are based on such parameters as cell and nuclear size or basophilic staining. The relationship between the cell cycle and such criteria are not well defined (Tarbutt, 1967; Wickramsinghe et al., 1968; Killman, 1970). Accordingly, our effort (see Fig. 5) to equate basophilic erythroblast, early, mid, and late polychromatophilic erythrocyte, etc., with the

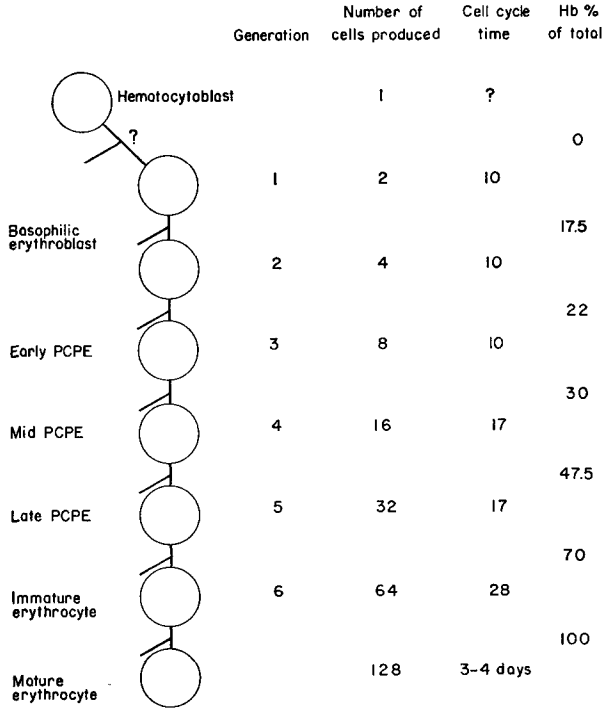

FIGURE 5 Schematic diagram of primitive erythroid development expressed in terms of: (*a*) morphological stages of development; (*b*) the number of generations; (*c*) The cell cycle time for each generation; (*d*) Hb value for each mitosis expressed as a percentage of the Hb content of the mature primitive erythrocyte. These data summarize the findings of Weintraub et al. 1971.

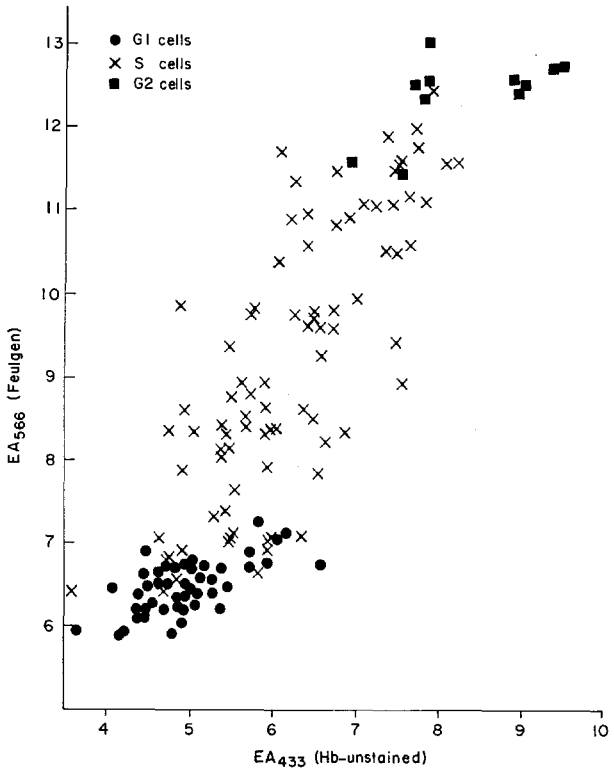

FIGURE 6 A plot of Hb measurements made on primitive erythroblasts from a day 4 embryo and of DNA measurements made on the same cells. The Hb measurements are expressed as EA_{433} units. The DNA measurements are expressed as EA_{566} units. The location of each cell in different compartments of the cell cycle is described in the text.

successive generations of primitive "erythroblasts" is at best only a first approximation.

A summary of the changes in the amount of Hb per cell throughout the successive generations of primitive erythroblasts and during the maturation of the primitive erythrocytes is given in Fig. 8. The Hb content for each mitosis has been derived from the model values of the frequency histograms. The Hb values for the progeny of each division are assumed to be half the parental value. The relative changes in Hb content during these generations have been plotted against the developmental time for each generation. Each slope is an estimate of the average rate of Hb synthesis during a specific interval of time. As these slopes represent rates of net Hb synthesis, they cannot be considered direct measures of Hb synthesis until there is some information to determine whether there is any turnover of Hb in the primitive erythroid cells. In Fig. 8, the average rate of Hb synthesis appears to be fairly constant during the proliferative phase. If this is so, then the amount of Hb synthesized during each cell cycle is dependent on the length of that cycle. However, it is difficult to evaluate the significance of that constant rate of Hb synthesis, particularly in light of the fact that the rate of Hb synthesis may vary within different compartments of the cell cycle.

Nevertheless, there is a fourfold decrease in the rate of Hb synthesis after the terminal division. Whether this decrease is directly coupled to or only fortuitously associated with the final division is still unclear. As in other erythropoietic systems (Grasso et al., 1963, 1966, and 1967), RNA synthesis reaches a minimal level about the same time as the final division (unpublished observations), hence the availability of some species of RNA may become rate limiting after the final division (Marks and Kovach, 1966). If, after the cessation of RNA synthesis, some species of RNA does become rate limiting, then one would predict a decreasing rate of accumulation in the amount of Hb per cell dur-

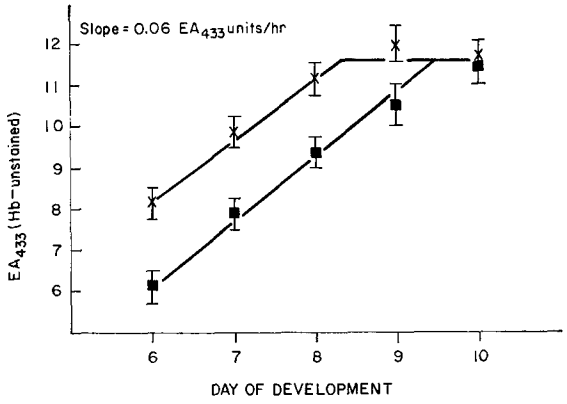

FIGURE 7 Hb measurements on primitive erythrocytes from day 6 to day 10 embryos. Each point represents the average value for at least twenty measurements. The average Hb content is expressed as EA_{433} units. Unlabeled cells, (-X-X-); labeled cells, (-■-■-). For further details see the text.

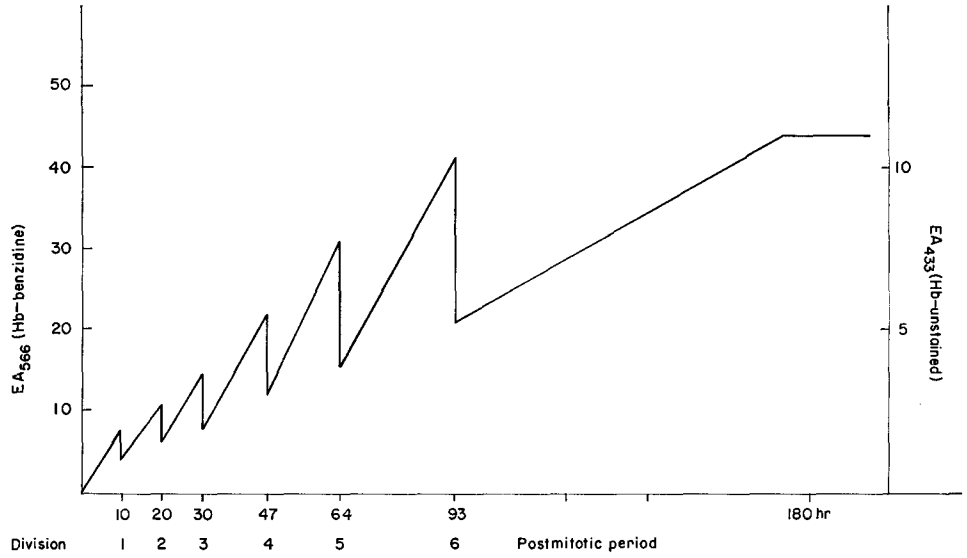

FIGURE 8 Summary of the relative changes in the Hb content during the successive generations of primitive erythrocytes and the postmitotic period in the primitive erythrocytes.

ing the nonproliferative phase concomitant with the degradation of RNA. Since this is not apparent (see Fig. 7), either the data do not allow us at present to detect such subtle changes or the situation is clearly more complex.

Previously, Tooze and Davies (1963) have suggested that the high Hb content associated with chromatin of the mature erythrocyte may influence both the condensation of the chromatin and the synthesis of Hb. Subsequently, Small and Davies (1970) have reexamined the situation and produced evidence indicating that there is relatively little Hb associated with the chromatin in the mature nucleated red blood cell. From this, they argue that it is less likely that Hb will influence either of these processes. Our own observations support this present contention. In Fig. 5, each successive mitosis is expressed as a percentage of the final mature value. The Hb value for the terminal mitosis is the same as the final Hb value for the mature cell. Since the relative size differences are minimal, there will be little difference in the concentration of Hb for both cells. These results argue against the negative control of Hb per se on

its own synthesis. At present it is not possible to determine whether the amount of Hb per cell will influence other events such as DNA synthesis or mitosis (see, however, Stohlmann, 1964).

Models for integrating the correlation between Hb synthesis and the mitotic history of primitive erythroblasts must distinguish between the following: (*a*) the two processes are completely independent. They appear to be dependent only because their time tables are in step. Thus the correlation is a reflection of a precise internal clock for each process. Such a model would predict that selective interference with one process should not affect the other. (*b*) the two processes are interrelated either because they share a common metabolic pathway or because genetic influences direct this correlation. This model would predict that interference with one process would eventually affect the other. Such a mechanism has been postulated to explain ineffective erythropoiesis in various kinds of anemias (Wichramasinghe et al., 1968, 1969 a, 1969 b). At present there is insufficient evidence to choose between these two models. By manipulating one process and monitoring the concomitant events in the other process, the cause and effect relationship between the amount of Hb per cell and the mitotic history of that cell could be determined.

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