

# DEVELOPMENTAL CHANGES OF ERYTHROPOIESIS IN CULTURED CHICK BLASTODERMS

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## ABSTRACT

The erythropoietic area of very early chick embryos was cultured as a tissue for up to nine days to study the changes in red cell type and hemoglobin type, the cell cycle time, the cell population kinetics, and the DNA synthetase activity of these cells. It was found that the area vasculosa without the participation of the embryo proper contained the information and the timing mechanism required to produce not only the early primitive erythroid cell population, but also in due course, the later definitive cell type, each with its appropriate hemoglobin types. Also the precursors of the definitive cell type are active in DNA synthesis and therefore are probably in cycle very early in the culture period.

## INTRODUCTION

Numerous reports in the literature describe the sequential appearance in early chick embryos of morphologically distinct erythrocytes and of electrophoretically different hemoglobins (review by Wilt, 1967). The first red cell population to appear and mature is called the "primitive" red cell series, distinguished by its much larger volume, among other morphological criteria. The cell series does not persist. It is replaced around day 7 of incubation by the "definitive" red cell series which is evidently self-perpetuating and which remains throughout the rest of embryonic and adult life. Definitive red cells are readily distinguished by their smaller, oval shape and other morphological criteria. Different hemoglobins are associated with these different red cell populations. A recent thorough study in this laboratory (Bruns, 1971) relates quantitatively the change in red cell type and the change in electrophoretically characterized hemoglobin type. The latter changes in our nomenclature involve the disappearance of two early hemoglobins—the

major primitive (P)<sup>1</sup> hemoglobin and the minor embryonic (E) hemoglobin—and the appearance of two hemoglobins characteristic both of the later embryo and of the adult—the major adult (A) hemoglobin and the minor definitive (D) hemoglobin. Analogous phenomena have been studied in other animal systems, notably in the fetal mouse (see, for example, Fantoni et al., 1968; Chapelle et al., 1969) and in the tadpole (see, for example, Moss and Ingram, 1968).

It is known that the potentially erythropoietic tissues of the very early chick embryo—the area vasculosa—can be maintained in tissue culture for a number of days under conditions which allow erythropoiesis to continue (Murray, 1932; Settle, 1954; O'Brien, 1960; Spratt and Haas, 1960 *a* and *b*; Hell, 1964; Levere and Granick, 1967; Wilt, 1967). The effects of inhibitors of DNA, RNA, and

<sup>1</sup> *Abbreviations:* A, major adult hemoglobin; D, minor definitive hemoglobin; E, minor embryonic hemoglobin; P, major primitive hemoglobin.

protein synthesis on the commencement of hemoglobin production in these systems have been studied (O'Brien, 1960; Wilt, 1967; Hell, 1964; Levere and Granick, 1967). These experiments suggest that stable hemoglobin messages are present from the "seven somite" stage (approximately 36 hr of incubation) onwards; probably these are messages for the earliest hemoglobin types only. Furthermore, according to Wilt (1962), globin chains are already present before complete hemoglobin can be detected.

The present experiments were undertaken to see whether the extraembryonic area vasculosa from very early embryos contained the information to produce in organ culture not only the early, but also the late hemoglobin and red cell types without the participation of the embryo proper.

## MATERIALS AND METHODS

### *Blastoderm Cultures*

Fertilized eggs of the White Leghorn breed were obtained from Thompson's Farm, Andover, Mass. The following approximate incubation periods were used: 12 hr for embryos at the primitive streak stage, 24 hr for the head fold stage, 36 hr for the six to nine somite stage. Blastoderms were prepared in sterile chick Ringer solution, essentially as described by Levere and Granick (1967), using sterile conditions. The amount of yolk clinging to the blastoderms varied and could not be controlled. Blastoderms at the head fold or six to nine somite stage were usually, but not always, de-embryonated by removing most of the area pellucida with fine scissors. The entire de-embryonated blastoderm was cultured with the dorsal side up. In all experiments reported in this paper the tissue was supported on cellulose acetate discs ( $1\frac{5}{16}$  inches diameter, 0.45  $\mu$  pore size, Schleicher & Schuell, Inc., Keene, N. H., or Matheson-Higgins Co., Cambridge, Mass.) which rested on the inner well of sterile plastic organ culture dishes (Falcon No. 3010, Falcon Plastics, Div. of Bioquest, Oxnard, Calif.). The cellulose acetate discs were sterilized under UV light and soaked in sterile chick Ringer solution overnight before use, to remove detergent in the filter. Medium 199 with Hanks' salts, minimal essential medium with Hanks' salts and nutrient mixture F12 were purchased from Grand Island Biological Co., Grand Island, N. Y. The culture medium (medium 199:egg white [3:1]; approximately 1.2 ml) filled the center well of the culture dish up to the support disc. The outer well was moistened with sterile chick Ringer solution. The cultures were incubated at 37°C in air at 100% humidity and changed to fresh medium

every other day; it was found in early experiments that 5% CO<sub>2</sub> in the air decreased the yield of hemoglobin considerably.

### *Electrophoresis of Hemoglobin*

Red cells were harvested by tearing the tissue in a drop of chick Ringer solution at room temperature. The cells would usually pour out, but sometimes the tissue had to be teased to release the red cells. A suspension of such cells in ice-cold chick Ringer's was freed from most of the remaining yolk by sedimenting the cells during a gentle centrifugation (half-speed in a cold clinical table centrifuge for 5 min). When required, cells were counted in a hemocytometer. The button of cells from one to five blastoderms was suspended in 0.5–1.0 ml of cold chick Ringer's and the cells were sedimented again. The packed cells were lysed routinely in 25–50  $\mu$ l of water, left to stand at room temperature for 10 min, then mixed vigorously with 5  $\mu$ l of water, left to stand at room temperature for 10 min, then mixed vigorously with 5  $\mu$ l toluene and 10  $\mu$ l carbon tetrachloride, and centrifuged. The clear supernatant was used at once for hemoglobin estimation by the *o*-dianisidine-H<sub>2</sub>O<sub>2</sub> procedure (Hell, 1964) and for electrophoresis after conversion to the methemoglobin form with ferricyanide. In some later experiments the cultures were changed to F12:egg white medium (1:1) and incubated for 1 hr before harvesting. This procedure makes the harvesting of red cells from the tissue much easier and relatively free from tissue debris. Analytical polyacrylamide gel electrophoresis was performed according to Ornstein (1964) and Davis (1964) as modified by Moss and Ingram (1968), using their pH systems in Canalco model No. 66 apparatus (Canalco Inc., Rockville, Md.), a Buchler Polyanalyst (Buchler Instruments, Inc., Fort Lee, N. J.), or similar homemade equipment. All buffers contained 100 mg/liter KCN to convert any remaining methemoglobin into its cyanide complex. Sample application and benzidine staining after electrophoresis were carried out as described by Moss and Ingram (1968). The intensities of the bands were determined in a Gilford 2420 scanner (Gilford Instrument Labs, Inc., Oberlin, Ohio).

The following radioactive nucleotides were used: (New England Nuclear, Boston, Mass.) amino acid-<sup>14</sup>C mixture, catalogue No. 459-172; deoxyadenosine-<sup>3</sup>H (21.8 Ci/mmol) and thymidine-<sup>3</sup>H (6.7 Ci/mmol).

### *Radioautography*

Slides of cells for radioautography (Rogers, 1967) were fixed in methanol for 5 min and air-dried. The cells were stained for hemoglobin with *o*-dianisidine

DAY 8.5 HEMOGLOBIN ELECTROPHORESIS

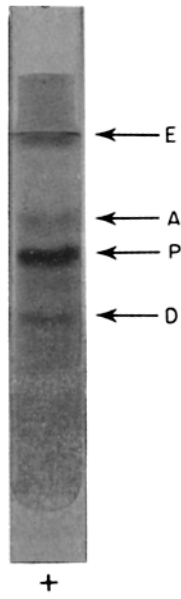


FIGURE 1 a

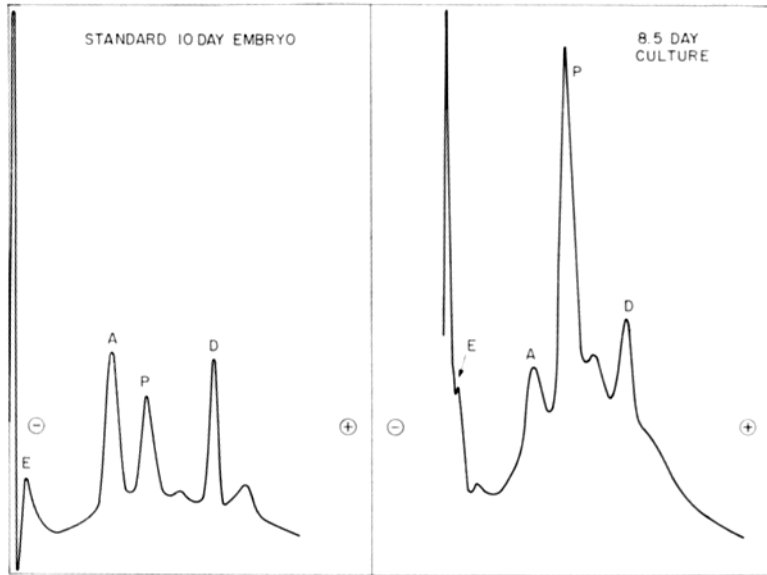


FIGURE 1 b

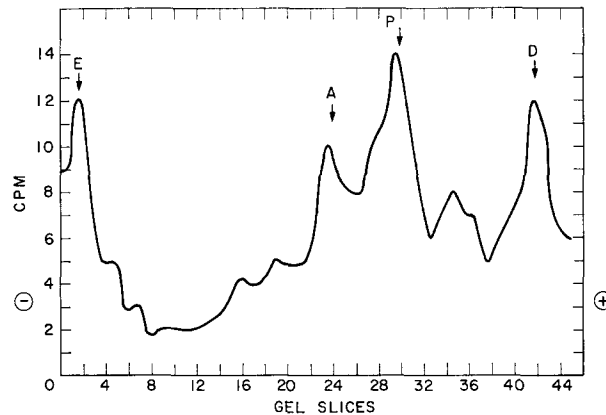


FIGURE 1 c

FIGURE 1 Appearance of hemoglobins A and D in cultured blastoderms. De-embryonated blastoderms from five embryos with six to nine somites were cultured as described in Methods. On the 6th day of culture (total age 7.5 days), the replacement medium contained in addition  $10 \mu\text{Ci/ml}$  of mixed uniformly-labeled amino acids- $\text{C}^{14}$ . After 24 hr, the red cells were harvested, washed three times in buffered saline and lysed as usual with  $30 \mu\text{l}$  of water. About one-third of this hemoglobin was examined by gel electrophoresis and stained with amido black. The photograph (a) and the scan at  $260 \text{ m}\mu$  (b) are shown. The gel was frozen and sliced. Each slice was counted in Bray's solution in an Ansitron scintillation spectrometer (c). A weak band with radioactivity is seen between hemoglobins P and D. This is a breakdown product from hemoglobin A produced on overnight storage of the hemoglobin solution in the refrigerator.

(O'Brien, 1960). Extraction of excess labeled precursors, coating with Kodak NTB 3 emulsion, exposure, and development were done as described by Prescott (1964). The developed radioautograms were stained with Wright-Giemsa's stains.

### Cell Morphology and Mitotic Index Measurements

Cells were harvested from segments of blastoderm in buffered saline containing 1% bovine serum albumin. Three successive time points were derived from one blastoderm, because it was found possible to detach a segment with a sterile platinum loop in such a way that the remaining tissue remained intact and that there was no leakage of erythroid cells. Slides were prepared with these cell suspensions on the cytocentrifuge (Shandon-Elliott, [Shandon Scientific Co., Inc., Sewickley, Pa.] 10 min at 600-700 rpm). After drying, the slides were stained with benzidine and counterstained with May-Grünwald's followed by Giemsa's.

## RESULTS

### Hemoglobin Synthesis

The culture of the de-embryonated six to nine somite embryo, described in Methods, produces after 1 day and on subsequent days a tissue which is a network of sinuses and capillaries full of

maturing red blood cells. This leads readily to a suspension of red cells which could be examined morphologically or labeled with radioactive precursors to be counted or submitted to radioautography; furthermore, solutions of hemoglobin could be obtained by lysis of the cells.

The hemoglobin electrophoretic pattern for the first few days of culture contains only P and E hemoglobins, as does the intact embryo in vivo of corresponding age. After 6 days of culture (total age 7.5 days), however, the electrophoretic pattern begins to show A and D hemoglobins also, thus indicating that differentiation has occurred towards the latter hemoglobin types. This resembles the situation *in ovo*.

When such a culture is examined after 7 days (total age 8.5 days), a hemoglobin pattern with distinct hemoglobins A and D is seen (Fig. 1). Radioactive amino acids are incorporated during the last 24 hr into all four hemoglobins, A and D as well as P and E. This suggests that the appearance of the late hemoglobins A and D is the result of *de novo* synthesis of these proteins, particularly as the specific radioactivity of the new hemoglobins is more than twice that of hemoglobin P. The ratio of D to A made is greater than 1, which contrasts with the situation in the adult hen when the ratio is 0.15:0.2. The apparent asynchrony in

the quantities of these hemoglobins when they first appear was also noted *in ovo* by Bruns (1971) and has as yet no explanation.

It must be pointed out, however, that total synthesis of hemoglobin in these cultures is only about 2% of that found *in ovo* and that accumulation of hemoglobin has ceased by 7 days in culture. On the other hand, our cultured blastoderms do not expand, as is the case *in ovo*, so that our yield per square centimeter of area vasculosa corresponds to the situation *in ovo* (see Discussion and Table I).

### Erythrocyte Production

Harvesting of red cells from cultured de-embryonated blastoderms is not always easy or quite complete. Nevertheless, an approximate idea of the number of red cells produced per blastoderm can be obtained from cell counts of the red cell suspensions made. Fig. 2 is a composite of four separate experiments and represents averages from 2-7 blastoderms for each point. The total red cell number increases for 6 days of culture. The leveling-off point in culture on day 7.5 occurs when the culture contains only 1-2% of the cells which the embryo will have at that age. It must be remembered that the erythropoietic area enlarges enormously during that

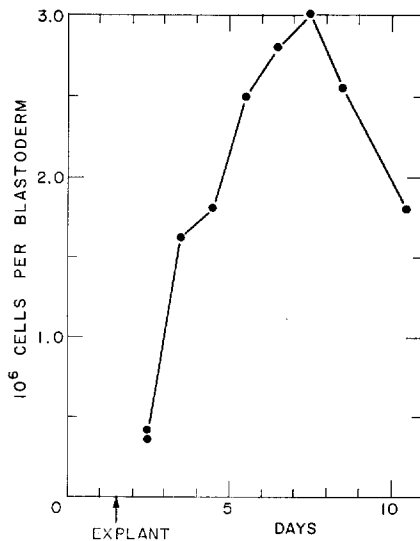


FIGURE 2 Red cell production in organ culture. The cell suspensions obtained from the cultures were counted in a hemocytometer and were judged to be 80% erythroid cells in slides made in a cytocentrifuge and stained with benzidine-May-Grünwald-Giemsa's.

TABLE I  
*In Ovo and In Culture Blastoderm Measurements*

Day	<i>In ovo</i> *		<i>In culture</i> ‡	
	RBC/embryo (P + D cells) × 10 <sup>6</sup>	cm <sup>2</sup> of area vasculosa	RBC/cm <sup>2</sup> of area vasculosa × 10 <sup>6</sup>	RBC/cm <sup>2</sup> of area vasculosa × 10 <sup>6</sup>
1.5	(0.05)§	1	(0.05)	(0.05)
2.5		13		0.4
3.5	9	22	0.4	1.6
4.5	33	30	1.1	1.8
5.5	67	34	2.0	2.5
6.5	111	39	2.9	2.8
7.5	226	42	5.4	3.0

\* RBC/embryo from Gail P. Bruns (1971). The extent of the area vasculosa from A. Romanoff, (1960, p. 1046), after Grodzinski (1934).

‡ Calculated from the data of Fig. 2, using the unchanging value of 1 cm<sup>2</sup> for the area vasculosa. § Calculated value, from the data in this present paper.

|| Measured in our experiments.

time *in ovo* (Table I); in culture it does not. The doubling time of approximately 12 hr for day 2.5-3.5 in Fig. 2 compares with the cell cycle time of 9 hr measured for the previous 24 hr period by thymidine pulse-labeling technique described below. After day 3.5, cell numbers increase more slowly, and after day 7.5 cell numbers diminish rapidly.

### Cell Cycle Time of Erythroid Cells in Early Culture

De-embryonated blastoderms from embryos with six to nine pairs of somites were cultured as usual, but in medium containing 1  $\mu$ Ci/ml of thymidine-<sup>3</sup>H. After 1 hr, the cultures were transferred three times within the next hour to cold medium containing 10<sup>-4</sup> M thymidine as a chase. Samples were taken at frequent intervals, harvesting one-third of a blastoderm each time (see Methods, Cell Morphology and Mitotic Index Measurements). Slides were prepared from the washed erythrocyte population obtained by teasing the tissue in buffered saline-1% bovine serum albumin. Radioautography was performed (see Methods) and labeled mitoses were counted (Fig. 3). Label appears in mitotic cells some 3 hr after the end of the pulse. At 5 hr, all the erythroid cells which are in mitosis are labeled, the mitotic index at that time being 6.0%. If all the cells are in cycle,

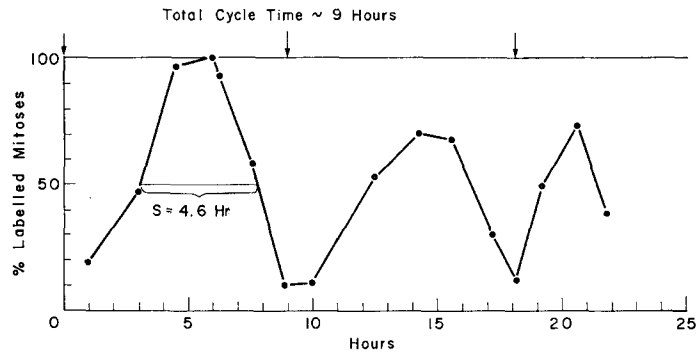


FIGURE 3 Cell cycle time by thymidine-<sup>3</sup>H pulse using de-embryonated chick blastoderms, six to nine somites.

TABLE II  
Thymidine-<sup>3</sup>H Uptake into Labeled Primitive and Immature Definitive Cells during Culture

Labeled on day	Immature definitive cells*	Labeled cells ‡		Grain counts of labeled cells ‡	
		Primitive	definitive	Primitive	definitive
	%	%	%	grains/nucleus per day of exposures	
2.5-3.5	—	98		8.5	
3.5-4.5	22.0	82	81	8.1	9.0
4.5-5.5	19.5	52	77	4.1	5.7
5.5-6.5	9.1	15	97	3.1	4.4
9.5-10.5	2	(6)	86	—	2.5

\* Between 1,100 and 3,600 total cells counted for each point. Immature definitive cells are benzidine-negative erythroid cells at these ages.

‡ Primitive cells include after day 3.5 some maturing definitive cells, both cell types being benzidine positive. Emulsions were exposed for 1 day (for harvest on days 3.5 and 4.5), 2 days (for harvest on days 5.5 and 6.5), and 5 days (for harvest on day 10.5). On day 10.5 the primitive cells are almost entirely without label. Grains in 200-300 cells were counted for each harvest point. The frequencies of grain counts were approximately normally distributed for all samples of immature definitive cells and mostly so for those primitive cells which were labeled.

the time for mitosis would be calculated at about 30 min. Since the doubling time for the next 24 hr (Fig. 2) and the cell cycle time here measured are fairly close (12 hr and 9 hr, respectively), it is likely that most of the erythroid cells are in cycle during day 1.5-3.5. A lengthening of cell cycle times in the primitive cell series as the cells become more mature has been described *in ovo* (Weintraub, et al., 1971).

### DNA Synthesis during Culture

The purpose was to test by a series of quantitative radioautographic experiments the changes in DNA synthesis during maturation of primitive and definitive red cells in culture (Table II). The de-embryonated blastoderms from six to nine somite embryos were cultured in the usual medium and exposed for 24 hr periods to deoxyadenosine-<sup>3</sup>H, 1  $\mu$ Ci/ml, before harvesting and radioautography. As usual, the blastoderms and the Millipore filters (Millipore Corp., Bedford, Mass.) on which they rested were transferred to new organ culture dishes with fresh medium every 2 days. For radioactive labeling, this fresh medium would contain the radioactive nucleoside. Tissues were labeled in this way, after explanting on day 1.5, during days 2.5-3.5, 3.5-4.5, 4.5-5.5, 5.5-6.5, and 9.5-10.5. On each occasion, two blastoderms were labeled, cells were harvested from both together, cells were counted in the hemocytometer, and slides were prepared in the Shandon cyto-centrifuge from 0.2 ml of a suspension containing approximately  $5 \times 10^6$  cells/ml. Radioautography was performed as described in Methods.

The proportion of immature definitive cells (erythroblasts) declines after day 5.5 and almost vanishes. However, those that do remain are nearly all capable of taking up the label and therefore are capable of DNA synthesis right up to day 9.5-10.5. On the other hand, the amount of precursor label per cell which is taken up remains constant during days 2.5-4.5 and then falls to one-half and even further. We assume, but have not tested, that our 24 hr uptake is not affected by factors such as changes in pool size of precursors.

The primitive cell class, which later includes some maturing definitive cells, ceases, with some

exceptions, to take up label after day 5, approximately. This is expected from the declining rate of red cell production at that time (Fig. 2). The rate of uptake of label during day 2.5–4.5 is at the same level as in the immature definitive cells after day 4. However, those primitive cells on day 4.5–5.5 which do take up label do so in diminished amount; the drop in uptake to one-half parallels the behavior of the immature definitive cells.

### Early Pulse Labeling of Erythroid Cell Precursors with Deoxyadenosine-<sup>3</sup>H

It is clear that the precursors of the primitive cell line are in the cell cycle during the first 24 hr of of culture. The purpose of the next experiment was to find out whether the precursors to the definitive cell line are also in cycle during that time, that is, during day 1.5–2.5 on the over-all scale, or

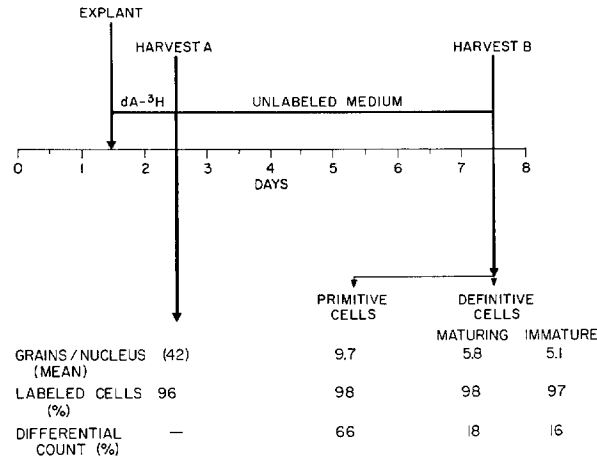


FIGURE 4 a

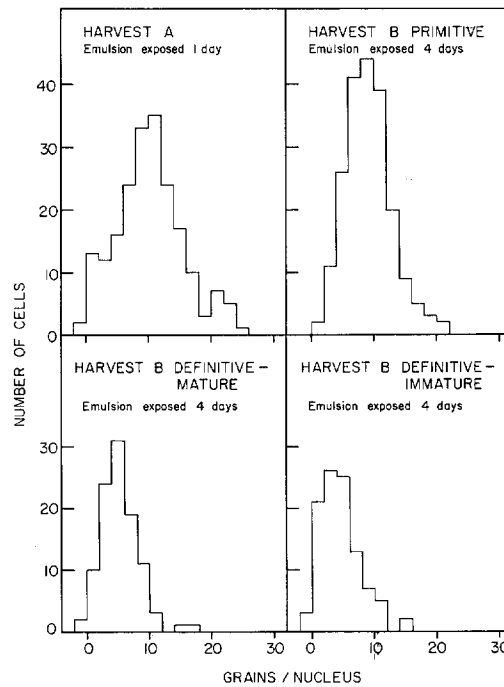


FIGURE 4 b

whether they are resting in  $G_0$ . Since there is no way of recognizing the definitive precursors at that stage, an indirect experiment was carried out as shown in Fig. 4.

De-embryonated blastoderms from six to nine somite embryos were cultured on Millipore filters on medium 199:egg white (3:1), and after 30 min at 37°C were moved to another dish of the same medium containing 1  $\mu\text{Ci/ml}$  of deoxyadenosine- $^3\text{H}$ . Two blastoderms were harvested after 24 hr of incubation at 37°C (harvest A, Fig. 4) and another two were transferred, with three changes at 20-min intervals, to medium without radioactive label. Culture was continued for a further 5 days with changes of medium after 1 and after 3 days. The final harvesting (B, Fig. 4) was done on day 7.5. Slides were made as usual in the cytocentrifuge, and radioautography was performed. The cells in harvest A were evaluated as one population; for harvest B, a distinction was made between primitive cells (all mature) and definitive cells. A further distinction was made (Fig. 5) between benzidine-negative "immature" definitive cells (proerythroblasts and basophilic erythroblasts) and "mature" definitive cells (early and late polychromatic erythroblasts). The radioautographs were scored for the occurrence of labeled cells and for the number of grains per cell.

The experiment shows that all (96%) of recog-

nizable erythroid cells are in cycle, at least to the extent of making DNA, during day 1.5–2.5 and that all the precursors (97–98%) of the definitive cell line are also in cycle at that time. Secondly, the results indicate that the primitive cells undergo on the order of two more cell divisions during days 2.5 and 7.5 before becoming erythrocytes, although Fig. 2 suggests three divisions during that time. If it is assumed that the definitive precursors take up as much label during day 1.5–2.5 as do the primitive precursors, then it would appear that the definitive cells have undergone one additional cell division on the average. In that case the calculated proportion of definitive precursors on day 2.5 would be 17%. The shape of the frequency distribution of grains per nucleus in harvest A (Fig. 4 *b*) is reasonably symmetrical, which would argue against the possibility that the precursors to the definitive cells form a subset of 17% of cells, with different labeling characteristics. It is assumed in these considerations that there has been no reutilization of label in these cells.

It should also be noted that harvest B contains 18.3% of erythroid cells of undetermined class (not shown in Fig. 4) that have so many grains as to be uncountable. Perhaps these are cells which picked up so much radioactivity during day 1.5–2.5, the high end of the distribution curve, as to suffer sufficient radiation damage to prevent them from dividing. The 4 day exposure of the emulsion

FIGURE 4 (a) Early pulse labeling of erythroid precursors with deoxyadenosine- $^3\text{H}$  (dA- $^3\text{H}$ ). A cell was scored as labeled if it contained more than 1 grain, the background value. For A, 1951 cells were scored; for B, 1088 cells. Grains per cell were counted after exposing the emulsion for 4 days in the case of harvest B. The grain counts for cells in harvest A were determined after 1 and 2 days of exposure, but the value given is extrapolated to 4 days of exposure. Since, on the second day, the numbers were precisely twice those obtained after 1 day, the extrapolation to an assumed "4 day" value was felt to be justified. The mean grain counts are derived from the frequency distributions shown in (b). "Immature" definitive cells are benzidine-negative cells showing the morphological criteria of proerythroblasts and basophilic erythroblasts. (b) Frequency distribution of grain counts for harvests A and B from (a).

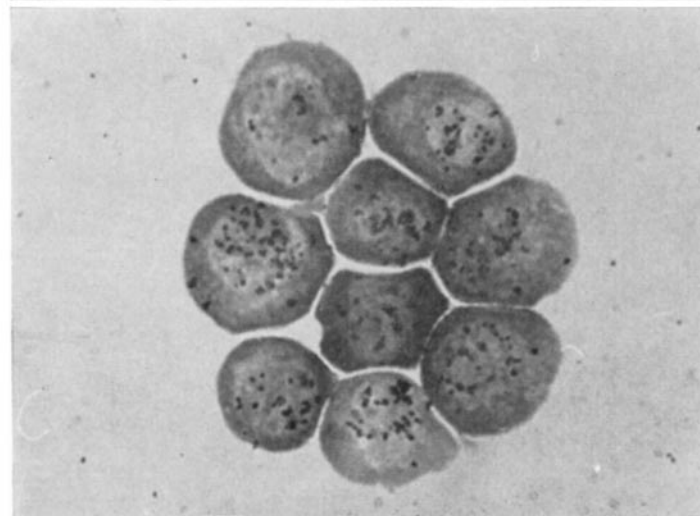
	No. cells counted	Exposure of emulsion day(s)
Harvest A	202	1
Harvest B		
Primitive	202	4
Definitive- mature	102	4
Definitive- immature	102	4



HARVEST A



HARVEST A



HARVEST B

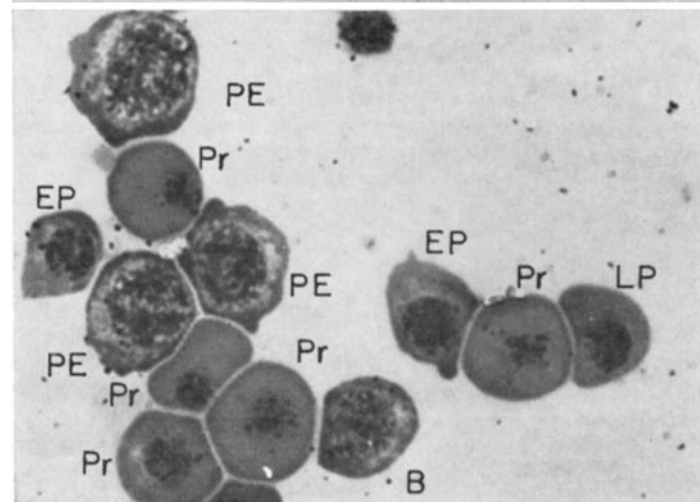


FIGURE 5

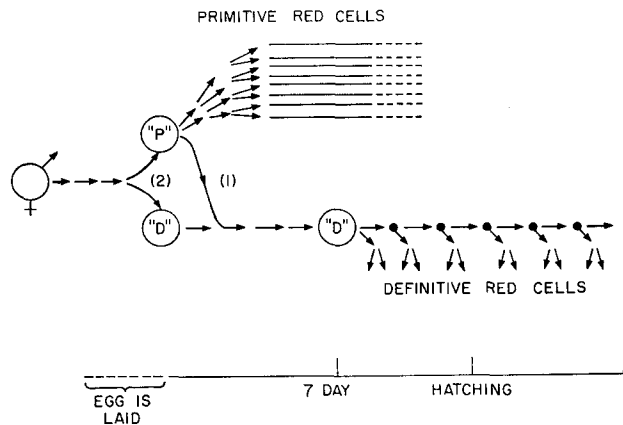


FIGURE 6 Scheme for the origin of primitive and definitive red blood cells. "P" and "D" denote precursor cells committed to forming primitive and definitive red blood cells, respectively (1) and (2) and alternate models. The diagram shows that a single cohort of primitive cells are produced, but that the definitive line has a self-perpetuating stem cell (●) compartment.

for harvest B cells would lead to an uncountably large number of grains per nucleus.

#### DISCUSSION

A possible scheme for erythroid cell formation in the chick embryo is presented in Fig. 6. The diagram shows the primitive red cell lines as a single cohort of cells arising in the area vasculosa which proliferate and mature as a group, then stop dividing and persist for a few days. Finally they disappear. The definitive cell line, on the other hand, becomes morphologically distinct later but has a self-perpetuating stem cell compartment from which a certain fraction mature as erythroid cells. Definitive cell production also arises in the yolk sac, the area vasculosa, but later has its stem cell compartment in the bone marrow with only a very brief involvement of liver, spleen, and the bursa of Fabricius. The distinction in red cell development is made here between a *determination* event which leads to cells committed to one or other erythroid cell line (definitive "D" or primitive "P" cells, in Fig. 6) and *differentiation* or *maturation* events of such committed cells which

pass through proerythroblast, basophilic, polychromatic, and orthochromatic erythroblasts to mature erythrocytes. We also distinguish two alternate routes of such determination, marked (1) and (2) in Fig. 6. Route 1 supposes that a multipotential precursor cell will become determined to a committed proerythroid cell of the primitive type and will later change its commitment to become a definitive cell type. Route 2 has the two pathways of determination diverge early, so that a cell line once committed will not change its commitment later. Both routes are possible and we do not yet have evidence for or against either scheme. The problem is a general one in vertebrate erythroid cell development, and schemes as in Fig. 6 can be usefully applied to the tadpole-frog metamorphosis, and to mouse or human fetal development.

An approach to evaluate the scheme is made by our studies in culture. A chick embryo with six to nine pairs of somites—after some 36 hr of incubation under our conditions—has virtually no detectable hemoglobin. On the other hand, a suspension made by disaggregating mechanically such an

FIGURE 5 Photographs of erythroid cells on day 2.5 and day 7.5 obtained from harvests A and B, respectively, in Fig. 4 a. In harvest A, different maturation stages are discernible, but are difficult to discern in photographs. In harvest B, the almost fully mature cells of the primitive series (Pr) are easily differentiated from the various maturation stages of the definitive cell series (PE—proerythroblast, B—basophilic erythroblast, EP—early, and LP—late polychromatic erythroblast). Stained with benzidine-May-Grünwald-Giemsa's; magnification  $\times 1180$ .

embryo will contain some cells which fit the expected morphological criteria for a very early proerythroblast. Such cells are also recognized in sections made of embryos at this stage (reviewed in Romanoff, 1960, p. 587).

After a few hours of incubation of the area vasculosa only, i.e. of a de-embryonated blastoderm from a six to nine somite embryo, blood islands and hemoglobin are formed, in confirmation of the earlier observations of the investigators cited in the Introduction. The cells first seen belong to the primitive cell series, and the first hemoglobins to appear are the early hemoglobins P and E. This clearly shows that the embryo itself is not needed for the production of the early red cells (confirming Settle's work, [1954]) and the early hemoglobins P and E. If the precursor cells are already committed at the time of explantation, a possible influence by the central portion of the embryo earlier in development, i.e. before de-embryonation, cannot be ruled out.

Since we were able to maintain the de-embryonated blastoderm in culture for up to 9 days—total age now 10.5 days—we could show that the area vasculosa, *without the embryo*, was able to form not only the early but *also the late embryonic* erythrocyte types and hemoglobin types. The information for this much later change is already contained in the explanted tissue and does not require stimulation by the embryo itself. The tissue contains some kind of "clock" which times the appearance of the later change in cell type. The question whether this mechanism is intrinsic to the erythroid precursor cells or whether it depends on interaction between the various cell types present as suggested by Miura and Wilt (1969) is the subject of our current experiments with dispersed cell cultures.

Red cell production proceeds in our blastoderm cultures from the time of explantation, day 1.5, to reach a maximum value of  $3 \times 10^6$  cells per embryo on day 7.5 (Fig. 2). Both primitive and definitive cells are included in these counts. After this maximum, there is rapid decay and disappearance of both cell types.

From these data we might infer that in the erythropoietic tissues explanted at six to nine somites the cell number doubles five times in the first 48 hr, which is the expected number of terminal cell divisions in the maturation of red cells in general and of the primitive red cell series in particular. Perhaps the last doubling between days 3.5 and 7.5 is due to stragglers in the primitive

cell series and to cell division among the definitive red cell series. It would appear to be the failure of the definitive red cell series to thrive which accounts for the decline of red cell production in our cultures.

Extrapolation of the cell numbers obtained in Fig. 2 to the time of explanting, using the cell cycle time of 9 hr obtained below as a doubling time, leads to an estimate that the erythropoietic area of the six to nine somite embryo has some 50,000 erythroid precursor cells.

At first sight, the value of  $3 \times 10^6$  cells per embryo on day 7.5 compares unfavorably with the  $120 \times 10^6$  cells per embryo found *in ovo* by Bruns (1971). Several possibilities could account for this low production *in vitro*; for example, the cells might fail to divide. However, the mitotic index in our cultures is high, 6% for day 1.5–2.5, 4% for day 2.5–3.5. Furthermore, the cell cycle time of 9 hr during day 1.5–2.5 and the doubling time of 12 hr for day 2.5–3.5 compare closely with the values obtained *in ovo* by Weintraub et al. (1971) by an entirely different method.

In our cultures, the explanted area vasculosa remains constant, whereas *in vivo* there is a dramatic increase over the same period (Table I). Calculated on an area basis, our production of red cells over the first 6 days of culture compares well with the *in ovo* situation, which, incidentally, suggests that cell death is not a very important factor in our cultures. The failure of our blastoderms to spread is probably due to the failure of the peripheral epitheloid cells to attach and to move centrifugally. Attempts to correct this situation by coating the filter with collagen were unsuccessful.

The important conclusion which emerges from these considerations is that production of red cells in the embryo is not merely due to the proliferation of the precursor cell population recognisable early, say on day 1.5, but must also be the result of massive *recruitment* (induction) from other mesodermal cells to the red cell precursor pool. We reach this conclusion because the six to nine somite embryo *in vivo* and the corresponding de-embryonated blastoderm, the common starting point, contain identical numbers of erythroid precursors. One might speculate that either the recruiting process itself, or the proliferation of the candidates for recruitment, are dependent upon the spreading of the blastoderm, perhaps dependent upon the tension which the centrifugal movement of the peripheral cells is known to set

up in the blastoderm. Is this a release from contact inhibition of mitosis through mechanical tension? It should be noted that in our organ cultures there is no noticeable increase of nonerythroid cells of the magnitude required to correspond to the 40-fold increase expected from the growth of the area vasculosa. It is hoped that the future study of suspension cell cultures will shed light on these possibilities.

Finally, one possible clock whereby the late definitive cell population delays its appearance would be the arrest of their early precursors in a G<sub>0</sub> or noncycling state. Such a scheme, though conceivable, is made less likely by our experiments which show the precursors to be fully active at least in DNA synthesis on day 1.5–2.5, and therefore probably in cell cycle. The same conclusion is reached by other work in this laboratory (Bruns, 1971) which shows in vivo that the precursors to the definitive cell line incorporate thymidine-<sup>3</sup>H label as early as at 48 hr of incubation. This hypothesis is therefore abandoned.

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