

THE RELATIONSHIP BETWEEN RNA SYNTHESIS AND HEMOGLOBIN SYNTHESIS IN AMPHIBIAN ERYTHROPOIESIS

Cytochemical Evidence

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ABSTRACT

A cytochemical study of the relationship between RNA synthesis and hemoglobin synthesis has been performed on splenectomized newts, *Triturus viridescens*. Employing radioautography, labeled cytidine was incorporated into the RNA of the early developmental stages but was not incorporated in the later stages. Labeled leucine was incorporated into the cellular protein of all stages except mature erythrocytes but was incorporated at a higher level in the later stages. Microphotometric measurements of azure B binding to cytoplasmic RNA revealed a sharp initial increase between the stem cell and proerythroblast followed by a rapid decrease between the basophilic and polychromatophilic stages. The loss of cytoplasmic RNA became more gradual in the late stages and, in the mature erythrocyte, little or no cytoplasmic RNA could be detected. Measurements of cytoplasmic total protein, using fast green staining at pH 2.0, and of heme showed that both curves increased similarly with development, indicating net hemoglobin synthesis. The results are compatible with the hypothesis that, as the stem cell differentiates along erythrocytic lines, a stable "messenger" RNA specifying the production of a given type or types of hemoglobin is formed. This complex probably becomes associated with ribosomal RNA and is retained throughout the process of RBC differentiation.

INTRODUCTION

The development of mammalian red blood cells is characterized by the early establishment and retention of a stable messenger or informational RNA complex which directs the synthesis of hemoglobin. This hypothesis is based on the observations that (a) RNA synthesis can be detected only in the earliest stages of development and appears to be absent in the later stages although the nucleus is still present (11); (b) hemoglobin synthesis continues to occur and, indeed, is most in-

tensive *after* cessation of RNA synthesis (9, 11, 14, 15, 21); and (c) actinomycin D does not inhibit Fe^{59} incorporation into heme or net hemoglobin synthesis in erythropoietin-treated cultures of rat bone marrow (8) although the concentration of actinomycin is sufficient to block RNA synthesis (18). The significance of these results is that, with the establishment of a stable informational complex in the early stages, erythropoietic cells are probably irreversibly committed to a specific de-

developmental pathway from which they cannot deviate. The result is a highly specialized cell which has lost not only its ability to synthesize proteins and to reproduce itself but also the structural means by which these activities are performed.

In lower vertebrates the mature erythrocytes retain their nuclei yet appear to be unable to synthesize protein (3) or to carry out DNA synthesis and mitosis (4, 12). In these respects this cell type is similar to the mature mammalian erythrocyte. Furthermore, certain morphological and tinctorial features characteristic of red blood cell differentiation in mammals seem to characterize the process of erythrocyte development in lower vertebrates. In view of the morphological similarities seen in vertebrate erythropoiesis, it is interesting to speculate that the chemical mechanisms underlying these similarities may be identical in widely different animals and, moreover, that perhaps a common pattern of erythrocytic differentiation exists among the various vertebrate classes.

This report presents cytochemical data on the relationship between RNA synthesis and hemoglobin synthesis in erythropoietic cells of the newt, *Triturus viridescens*. The results exhibit a high degree of similarity to those presented earlier by us in a cytochemical study of rabbit fetal erythro-

sis (11). Our data, combined with studies on avian and reptilian erythropoiesis, suggest that the pattern of erythrocytic differentiation may remain basically unaltered throughout the vertebrate phylum.

MATERIALS AND METHODS

The animals used for these experiments were adult newts, *Triturus viridescens* (obtained from Lemberger Company, Oshkosh, Wisconsin). Erythropoiesis in these amphibians normally occurs in the spleen. However, if the spleen is removed surgically, erythropoietic activity shifts to the peripheral blood where it is most intensive during the later weeks of January and during the normal breeding season of April to June (17). In our studies the production of red blood cells in the peripheral blood of splenectomized newts was minimal or absent at other times of the year.

In the fall newts were splenectomized after being anesthetized in a 0.5% solution of tricaine methanesulphonate¹ (MS-222) and maintained in spring water at 20–24°C. They were fed 1 to 2 times weekly on a diet of beef liver. Only animals exhibiting marked erythropoietic activity in the blood were used in these experiments.

¹ Obtained from Sandoz Pharmaceutical, Hanover, New Jersey.

FIGURES 1 to 8 Erythropoietic stages from peripheral blood of splenectomized newt. Stained in 0.025% azure B. All exposures were taken and printed at an equivalent time. $\times 1215$.

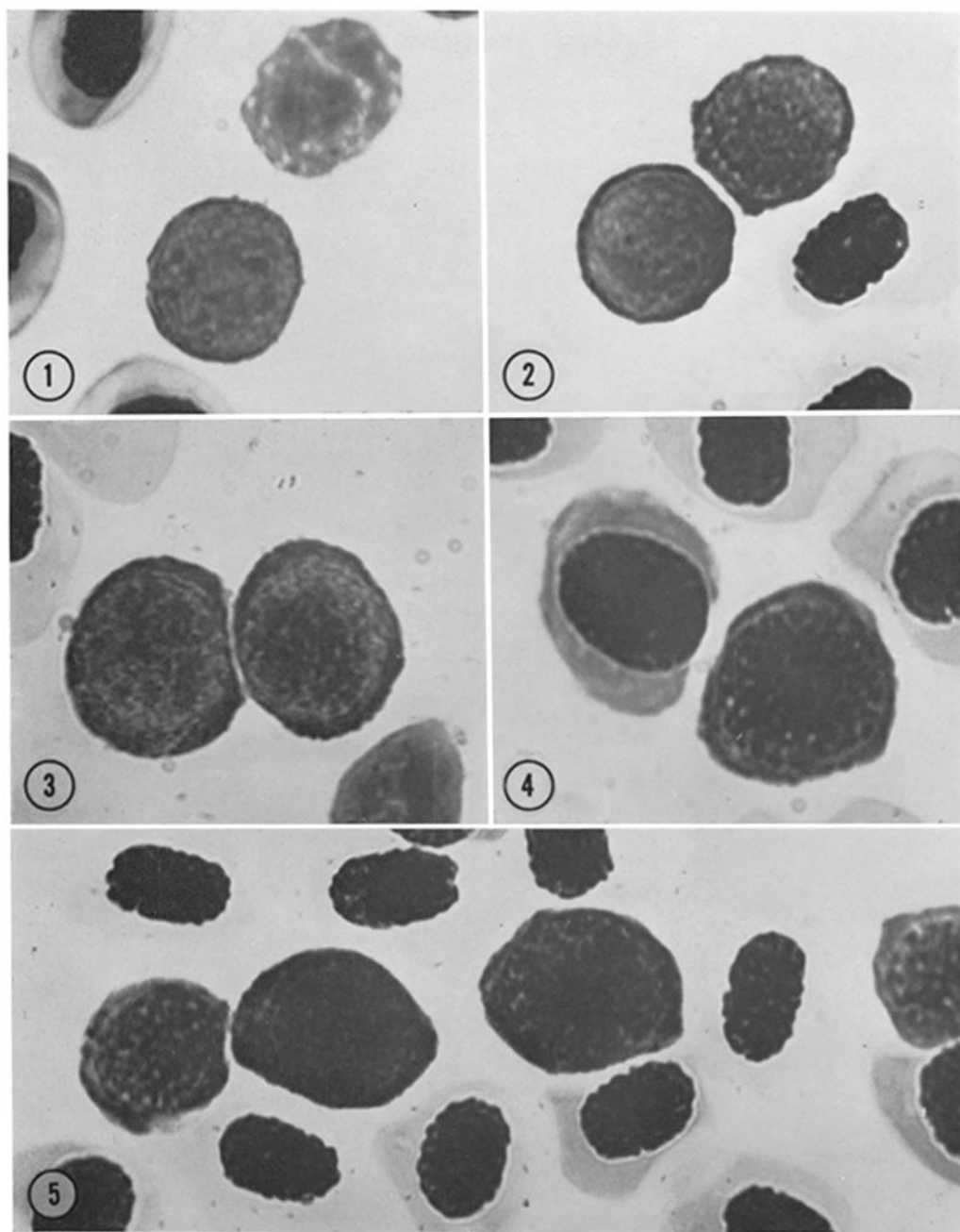
FIGURE 1 Lymphoid hemoblast.

FIGURE 2 The cell on the left represents a proerythroblast (PrE). Note intensity of cytoplasmic basophilia. The cell at right is a basophilic erythroblast (BE) and exhibits a more clumped chromatin than the proerythroblast. Otherwise both cell types are quite similar.

FIGURE 3 Two proerythroblasts (PrE). Note the close similarity of these cells to the basophilic erythroblasts. The chromatin is less condensed than that of basophilic erythroblasts.

FIGURE 4 The cell to the right is an early to midpolychromatophilic erythroblast in which the cytoplasm exhibits a moderate degree of basophilia. Note degree of chromatin clumping compared to cells in Fig. 3. The cell to the left is a late polychromatophilic erythroblast (LPE) and shows less cytoplasmic basophilia and a denser nucleus than the adjacent cell.

FIGURE 5 The two cells in which the intense cytoplasmic basophilia almost obscures their nuclei are basophilic erythroblasts (BE). Note the chromatin clumping compared to that in Fig. 3. To the extreme right and left are two midpolychromatophilic erythroblasts (MPE). The remainder of the cells are chiefly mature erythrocytes.



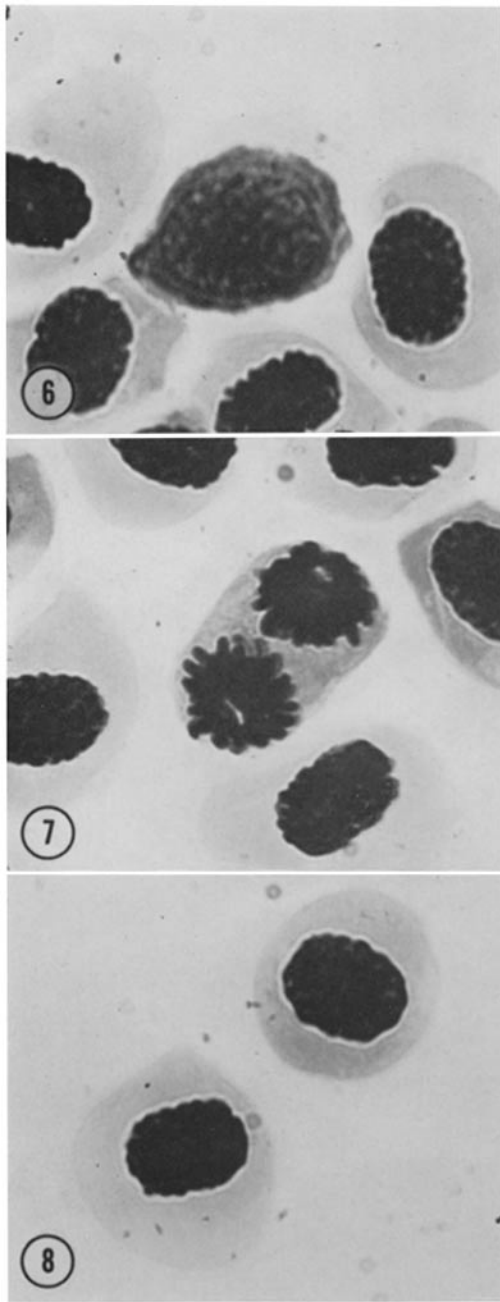


FIGURE 6 Midpolychromatophilic erythroblast (MPE).

FIGURE 7 A late polychromatophilic erythroblast in telophase. Note its darker cytoplasmic staining compared to that of the cell immediately to left. The latter is a mature erythrocyte.

Radioautography

For the study of the synthesis of RNA and protein, selected animals were injected intraperitoneally with 75 to 80 μ c of tritiated cytidine (specific activity, 1.75 c/mmole)² or tritiated leucine (specific activity = 0.5 c/mmole).² After 2, 4, and 8.5 hr exposure to isotope, blood was obtained by cutting the tip of the tail. Smears were prepared and fixed either by (a) freezing them immediately in liquid isopentane cooled to -170°C with liquid nitrogen followed by substitution in absolute methanol at -70°C for 1 to 2 days or by (b) air drying followed by immersion in absolute methanol at room temperature for 5 to 15 min.

To insure specific incorporation of cytidine- H^3 into RNA, representative slides were extracted in one of the following ways: (a) 5% trichloroacetic acid at $1-3^{\circ}\text{C}$ for 5 to 8 min; (b) DNase³ (0.2 mg/ml in 0.003 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 6.5) for $1\frac{1}{2}$ hr at 25°C followed by cold 5% trichloroacetic acid as described above; (c) RNase³ (0.2 mg/ml in distilled water at pH 6.0) for $1\frac{1}{2}$ hr followed by cold trichloroacetic acid; or (d) 5% trichloroacetic acid at 90°C for 30 min. Following extraction procedures, slides were coated with Kodak NTB-2 liquid emulsion, developed after 3 to 7 days' exposure, and lightly stained in 0.025% azure B at pH 4.0 to facilitate recognition of the various cell types. Slides treated by the various extraction procedures were then compared. Since cytoplasmic basophilia was used to recognize a given cell type, grain counts were made on smears extracted with DNase. The number of grains over each individual cell was counted and the area of the cell determined from camera lucida drawings with a polar planimeter. Incorporation was expressed as mean number of grains per μ^2 .

Leucine- H^3 -treated smears were extracted in cold 5% trichloroacetic acid and processed subsequently in a manner similar to that described above.

Microphotometry

For microphotometric determination of RNA, heme, and total protein, two groups of smears were obtained from different animals at a 1 year interval. Each group was fixed, stained, and measured by a

² Obtained from Schwarz BioResearch Co., Orangeburg, New York.

³ Obtained from Worthington Biochemical, Freehold, New Jersey.

FIGURE 8 The cell above and to the right is a reticuloocyte which exhibits a slightly denser cytoplasm than the mature RBC to the left. Note dense nucleus characteristic of these two stages.

TABLE I
Uptake of Labeled Precursors by Erythropoietic Cells

Stage	No. of cells	Cytidine-H ³		No. of cells	Leucine-H ³	
		Av. No. of grains after 2 hr			Av. No. of grains after 2 hr	
		Nucleus	Cytoplasm		Nucleus	Cytoplasm
PrE	77	0.276 ± 0.033	0.012 ± 0.002	—	—	—
BE	22	0.246 ± 0.036	0.011 ± 0.004	1	0.052	0.044
EPE	32	0.119 ± 0.043	0.006 ± 0.002	1	0.042	0.075
MPE	149	0.064 ± 0.005	0.002 ± 0.0007	25	0.067 ± 0.010	0.089 ± 0.011
LPE	—	0	0	17	0.074 ± 0.012	0.084 ± 0.007
RET	—	0	0	25	0.070 ± 0.009	0.115 ± 0.008
RBC	—	0	0	—	0	0

different investigator using different instruments. One group (animal 2) of smears was fixed wet in absolute methanol at room temperature for 5 to 15 minutes; the other group (animal 1) was air dried and immersed in methanol. After staining, microphotometric measurements were performed using the "plug" method (26).

For the determinations of cytoplasmic RNA, slides were stained in 0.025% azure B in McIlvaine's buffer at pH 4.0 (7) and measured at 530 or 540 m μ . Cytoplasmic heme was measured in the same cells at 412 m μ . At this wavelength the azure B stain did not interfere. Following these measurements, smears were immersed for 1 to 2 hr in distilled water at pH 2.0 to remove azure B and restained in 0.001% fast green FCF at pH 2.0 (24). Total cytoplasmic protein was then measured at 585 m μ in the same cells in which azure B binding and heme had been previously determined. For each set of measurements the preparations were mounted in a matching refractive index oil to minimize light scatter. The relative amount of bound dye or heme was calculated by the formula $M = EA$, where M = relative amount of absorbing substance, E = extinction, and A = area of the cytoplasm determined by planimeter tracings of camera lucida drawings. In practice the value of E was obtained from the average of 2 to 8 plug measurements.

The sequence in nomenclature of the various stages of red blood cell development which we employed represents a modification of that presented for avian (20) and amphibian (17) erythropoiesis:

Hemoblast (stem cell, or hemocytoblast) → proerythroblast (PrE) → basophilic erythroblast (BE) → early polychromatophilic erythroblast (EPE) → midpolychromatophilic erythroblast (MPE) → late polychromatophilic erythroblast (LPE) → reticulocyte (RET) → erythrocyte (RBC).

The relative duration of each developmental stage was determined by assuming that the number of cells at a given time is proportional to the amount of time

spent by the cells at that stage. This technique is similar to the method used to determine the relative duration time of mitotic stages (19, 28). Thus undamaged cells were counted in several smears and the percentage of cells at each stage determined. This result was then translated into relative per cent development time. In highly active animals the earliest stages (Hemoblast → EPE) exhibited a low frequency of occurrence whereas the later stages (MPE to RET) were frequently encountered. Support for the validity of this assumption was found when erythropoietic activity was decreased. At this time, few of the earliest stages could be found whereas the later stages were still abundant.

RESULTS

Summary of Morphologic Features

Generally, two features were used to identify the erythropoietic cells; the intensity of cytoplasmic basophilia and nuclear morphology. The lymphoid hemoblast, or stem cell, was characterized by a nucleus in which the chromatin appeared more or less homogeneously stained (Fig. 1). Several nucleoli were also visible. The cytoplasm consisted of a very thin, moderately basophilic rim (Fig. 1). Proerythroblasts were not easily distinguished from basophilic erythroblasts. The basophilic erythroblast (Figs. 2, 5) exhibited an intense cytoplasmic basophilia which almost obscured the nucleus. The nucleus showed evidence of chromatin clumping which was the criterion by which proerythroblasts and basophilic erythroblasts were separated. The proerythroblast (Fig. 3) did not exhibit the same degree of chromatin clumping seen in the BE but its cytoplasm was also considerably basophilic. Subsequent stages (Figs. 4 to 8) were characterized by (a) a decrease in cyto-

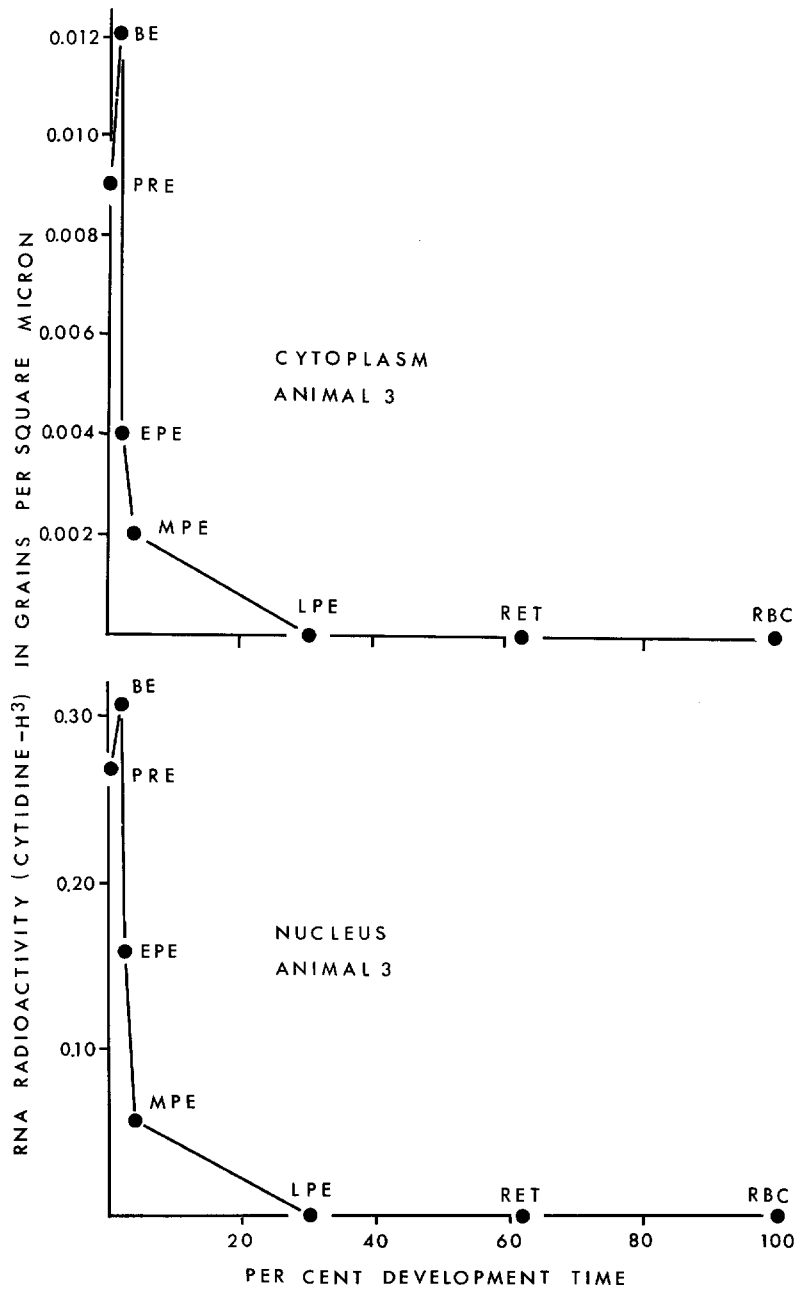


FIGURE 9 Radioactivity of nuclear RNA and cytoplasmic RNA expressed as grains per square micron following administration of cytidine-H³.

plasmic basophilia, (b) a decrease in nuclear size accompanied by increasing chromatin condensation, (c) a general increase in cell size so that the ratio of cytoplasm to nucleus tended to increase

with differentiation and, (d) a decrease in the size and number of nucleoli, although 1 to 2 small nucleoli were still evident in early reticulocytes. Mitotic figures were often seen (Fig. 7).

Radioautographic Studies of H³ Cytidine-Incorporation

Two hours after injection the average number of grains was highest in the nuclear RNA of the proerythroblast and basophilic erythroblast (Table I; Figs. 9 to 12). Successive stages exhibited a sharp decrease in incorporation of labeled precursor (Table I; Figs. 9, 13, 14) until in the later stages (LPE to RBC) no label was detected (Figs. 9, 15). Accompanying the decrease in level of incorporation was a decrease in the percentage of labeled cells at a given stage. Thus, whereas 70% or more of the cells were labeled in the earliest stages (PrE to BE), approximately 30 to 40% of the intermediate stage (MPE) were labeled. In all the labeled cells, the cytoplasmic label was considerably lower than the nuclear label. After 8.5 hr, the label over the cytoplasm was increased but the later stages still revealed no labeling.

In newts in which erythropoietic activity was absent, no label was observed in mature erythrocytes even 48 hr after injection of cytidine-H³. However, in these preparations some cells, presumably lymphoid cells, were so heavily labeled that they were almost completely obscured.

Radioautographic Studies of H³ Leucine-Incorporation

These preparations were made in late June when erythropoietic activity had greatly decreased. Thus, it was difficult to find the early stages although late stages were still abundant. The average number of grains over the cells showed a gradual increase as differentiation proceeded reaching its highest level in the reticulocyte (Table I; Figs. 16 to 19). All stages were labeled except the mature red blood cell. The label occurred over both the nucleus and cytoplasm although the ratio of cytoplasmic to nuclear grains increased with the degree of differentiation. However, some of the nuclear grains might be attributed to a small rim of cytoplasm which overlies the nucleus in these preparations.

Microphotometric Measurements

AZURE B BINDING: Measurements of relative amounts of azure B binding to cytoplasmic RNA (animal 2) showed a rapid rise from the hemoblast to the basophilic erythroblast (BE) and a sharp drop between the latter stage and the midpolychromatophilic erythroblast (MPE)

(Table II; Fig. 20). Beyond this point, a more gradual decrease in cytoplasmic RNA was observed. In this preparation, some dye uptake was seen in the cytoplasm of the mature erythrocyte and probably represented dye binding to hemoglobin acid groups or to trace amounts of RNA. In a separate group of measurements (animal 1), the decline in cytoplasmic RNA occurred between the PrE and MPE and, as in the previous group, was followed by a more gradual decrease (Table II; Fig. 20). Measurements at 540 m μ of unstained erythrocytes obtained from the same animal indicated that most of the absorption by these cells on the stained slides was due to absorption by heme. It was concluded that little or no cytoplasmic RNA occurred in mature erythrocytes and that most of the absorption was probably due to the presence of hemoglobin. Electron micrographs of mature erythrocytes support this conclusion since few ribosomes, if any, can be seen (6, 12, 27).

The differences between the two sets of measurements might be attributed to type of fixation (animal 1 was air dried prior to methanol immersion whereas animal 2 was fixed wet) and to the problem of staging of the earliest cell types which were difficult to recognize. However, the differences observed are probably not significant since the general trend in both sets of measurements not only tended toward a sharp decrease in the earliest stages, but included a gradual decrease in the later stages. In both groups these decreases were relatively comparable.

TOTAL PROTEIN AND HEME: Cytoplasmic total protein determined by fast green staining increased in both concentration and relative amounts per cell as differentiation proceeded. This increase appeared to occur in three steps (Table III; Fig. 21): (a) a short rapid rise from the hemoblast to midpolychromatophilic erythroblast, (b) a more gradual and sustained increase from the MPE to reticulocyte, and (c) another relatively rapid rise terminating in the mature erythrocyte. The amount of heme measured in the same cells followed a curve (Fig. 22) whose shape was similar to that obtained for total protein, indicating that most of the fast green staining represented hemoglobin.

DISCUSSION

The results of the present study show that, in the absence of cytidine-H³ incorporation which is considered to indicate the lack of RNA synthesis, leucine-H³ continues to be incorporated into cellu-

lar protein (Fig. 16). This incorporation represents mainly net synthesis of hemoglobin rather than turnover, since the relative amounts of cytoplasmic heme and total protein reveal a similar increase with development (Figs. 21, 22). Accompanying the loss of cytidine incorporation is a gradual decrease in cytoplasmic RNA which probably represents the loss of ribosomal RNA (Fig. 20). Essentially similar results have been reported in the chick (3) and rabbit (11).

Although the techniques employed in this study do not permit a distinction to be made between messenger RNA and ribosomal RNA (soluble RNA is ruled out since it is probably lost during preparative procedures), the results may be explained by postulating that the messenger or informational RNA which directs hemoglobin synthesis is formed at the time of stem cell differentiation and that it is stable and retained throughout the course of red blood cell development (11). Alternatively, very minute amounts of messenger RNA undetected by our methods may be synthesized in the later stages to complement the massive synthesis of messenger in the earlier stages.

The latter possibility is weakened by the results of several experiments which have been directed towards a study of RNA and hemoglobin synthesis in control and actinomycin D-treated erythropoietic systems. If erythropoietin, which is be-

lieved to stimulate RNA synthesis in primitive precursor cells (8, 18), and actinomycin D were added, at the same time or at varied intervals of time, to cultures of rat bone marrow, the effect of actinomycin D on hemoglobin synthesis was progressively reduced as the interval between addition of erythropoietin and addition of actinomycin was increased. When the interval was of 24 hr duration, no effect on Fe^{59} incorporation was obtained, suggesting that the messenger had been established during the preceding 24 hr period and that it was stable (8). Since, in a cytochemical study of *in vivo* mammalian erythropoiesis (11), only the earliest stages exhibited cytidine- H^3 incorporation, actinomycin D is probably repressing RNA synthesis in these cells but has no effect on protein synthesis until the supply of cells, in which the messenger complex is already established, is exhausted.

Bruns et al. (2), studying nuclear RNA in duck reticulocytes, were unable to identify a DNA-like RNA fraction in these relatively late stages. In addition, in the reticulocytes of duck (13), chick (25), and turtle (25), actinomycin D did not affect incorporation of labeled amino acids into protein. Thus, both cytochemical and biochemical studies of erythropoiesis in amphibians, reptiles (25), avians (3, 25), and mammals (8, 9, 11, 14, 15, 18, 21) suggest that a stable messenger RNA is prob-

FIGURES 10 to 15 Erythropoietic cells from an animal exposed for 2 hr to 75–80 μc of tritiated cytidine. Smear was fixed by freeze substitution, extracted with DNase, and lightly stained in 0.025% azure B. These photographs show the characteristic labeling pattern of the various stages with cytidine. $\times 1050$.

FIGURE 10 A probable hemoblast exhibiting grains primarily over the nucleus. A small nucleolus is visible at lower left of nucleus.

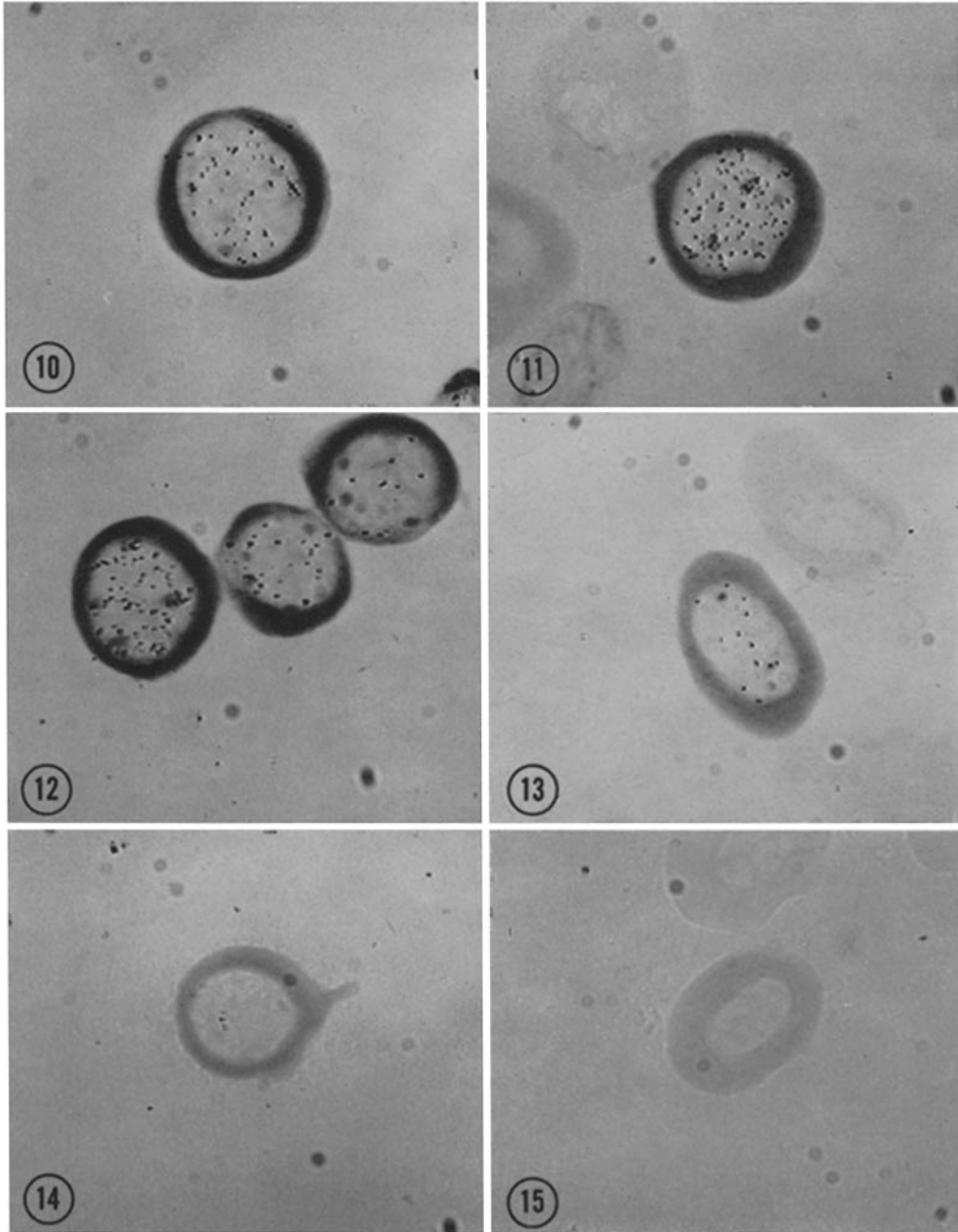
FIGURE 11 An early stage, PrE or BE, showing incorporation primarily over nucleus. The cell to the left and above is a mature erythrocyte in which grains are absent.

FIGURE 12 Three cells representative of early stages exhibiting nuclear grains. Note the large nucleoli in these cells.

FIGURE 13 A midpolychromatophilic erythroblast showing incorporation of cytidine. Grain counts are markedly less than those found in preceding stages. About 30 to 40% of MPE exhibit this amount of labeling. To the right and above is a mature erythrocyte in which no grains can be seen.

FIGURE 14 A midpolychromatophilic erythroblast exhibiting only 1 to 3 grains. This appearance is characteristic of the majority of cells at this stage.

FIGURE 15 A late polychromatophilic erythroblast showing no incorporation of cytidine.



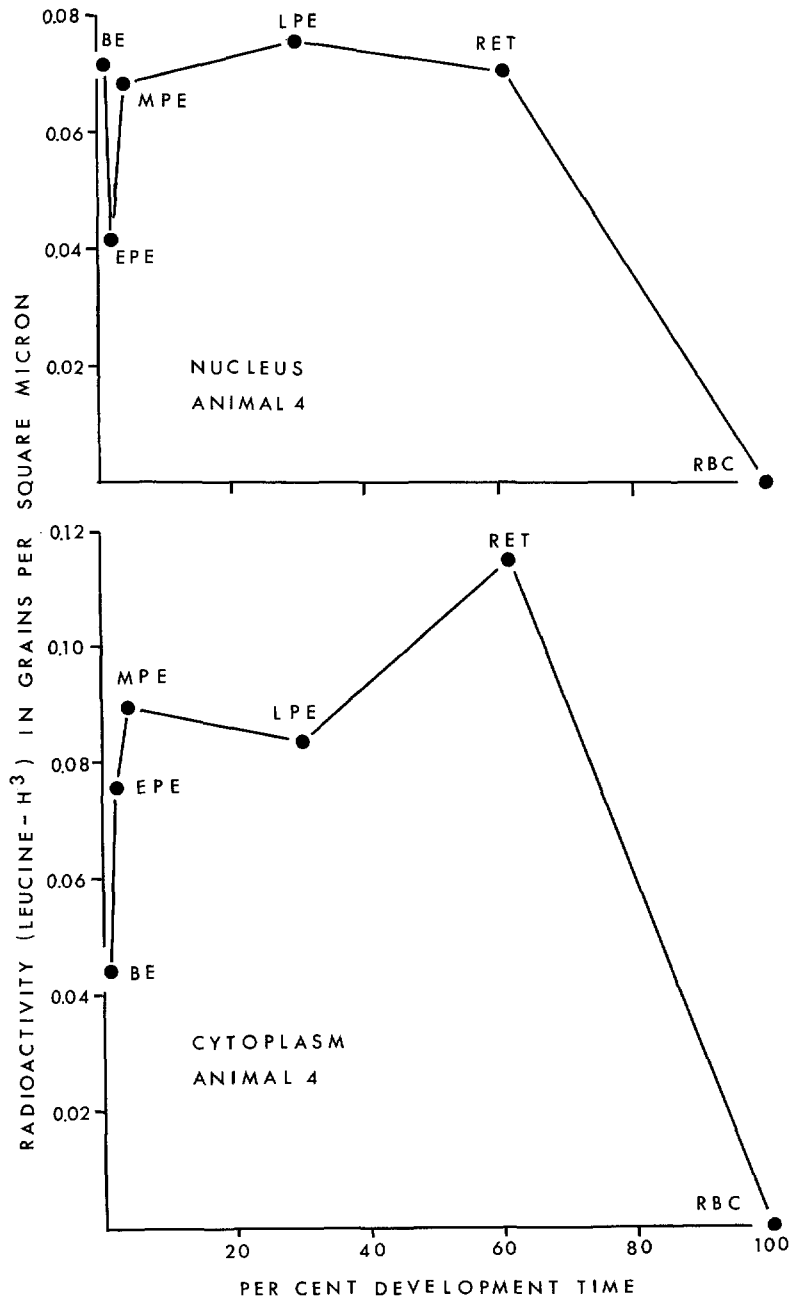
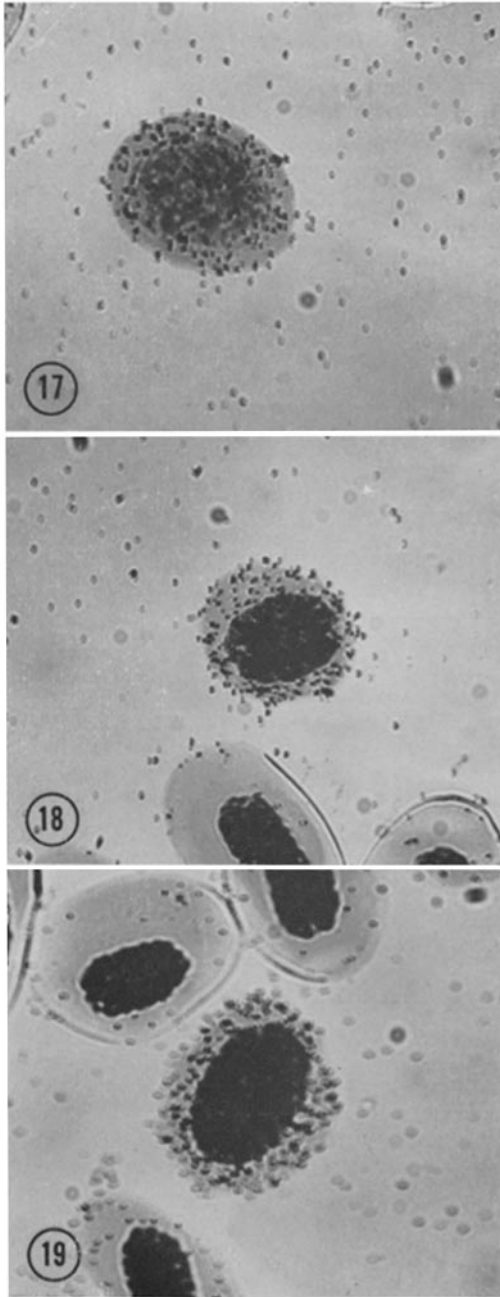


FIGURE 16 Incorporation of leucine- H^3 by cytoplasmic and nuclear proteins during blood cell development.

ably a recurring feature of the erythropoietic process throughout the vertebrate phylum.

Since the later stages appear to play an insignificant role in synthesis of messenger, the earliest

stages become the focus of attention because it is here that the synthetic mechanisms necessary for hemoglobin formation are probably established. Our data show that, accompanying differentiation



FIGURES 17 TO 19 Erythropoietic cells from animal given 75 to 80 μc of leucine- H^3 for 2 hr. Smears were fixed dry in methanol, extracted with cold 5% trichloroacetic acid, and deliberately overexposed to emphasize grain counts. $\times 1050$.

FIGURE 17 A midpolychromatophilic erythroblast.

of the stem cell, there is a burst of RNA synthesis in the earliest stages. Although we cannot distinguish messenger RNA and ribosomal RNA, part of this newly synthesized RNA probably contributes to the ribosomes since a measurable increase in cytoplasmic basophilia is detected between the stem cell and proerythroblast stage (Fig. 20). The remainder of this new synthesis is probably messenger.

It might be postulated that, following this initial burst of synthesis which results in newly formed messenger RNA and ribosomal RNA, certain transformations begin to occur as the cell proceeds to synthesize hemoglobin. From the PrE to MPE (Fig. 20) or the BE to MPE (Fig. 20) stages, a sharp decrease in cytoplasmic basophilia is observed which reflects loss of ribosomal RNA. Electron microscope examination of these stages in mammals (10) reveals a large decrease in cytoplasmic ribosomes from the PrE to MPE. Furthermore, while most of the ribosomes are single in the earliest stages, association into tetrad polysomes seems to predominate in the intermediate and later stages (10, 23). Thus, one might envision that, during the early stages, the cell readies itself for the bulk of hemoglobin synthesis by ridding itself of "inactive" ribosomes and by forming numerous templates for ensuing hemoglobin formation.

Once an erythropoietic line characterized by a given type or types of hemoglobin is established, the cells of this line are probably irreversibly committed to the production of red blood cells containing the specified hemoglobin. Any change to a red cell population characterized by a "new" type of hemoglobin, such as that which occurs during amphibian metamorphosis, would require establishment of a new cell line. This could be accomplished by repressing the synthesis of the "old," previously formed messenger and stimulating formation of a "new" messenger specifying the new hemoglobin type. The question then arises: at what level of differentiation does the stimulation of new messenger occur, or to what extent does the old pre-existent erythropoietic line contribute to the change? The results, presented here and previously

FIGURE 18 Late polychromatophilic erythroblast. The adjacent mature erythrocytes show grain counts which are equivalent to background.

FIGURE 19 Reticulocyte. Grains over adjacent mature erythrocytes are not significantly above background.

TABLE II
Average Relative Amounts of Bound Azure B Determined Microphotometrically

Cell type	Animal 1		Animal 2	
	No. of cells	Relative amount	No. of cells	Relative amount
Hemoblast	—	—	2	93 (95.8; 90.8)
PrE	21	72.9 ± 3.38	5	122 ± 22.8
BE	17	61.5 ± 3.59	13	140 ± 8.81
EPE	10	61.2 ± 2.59	12	114 ± 14.46
MPE	82	42.6 ± 1.00	54	93.7 ± 5.71
LPE	46	28.1 ± 1.16	25	69.8 ± 3.65
RET	17	18.8 ± 0.73	31	41.1 ± 2.43
RBC	48	0	20	29.2 ± 2.37

TABLE III
Average Relative Amounts of Bound Fast Green and Heme Determined Microphotometrically

Cell type	Animal 1			Animal 2		
	No. of cells	Relative amount of bound fast green	Relative amount of heme	No. of cells	Relative amount of bound fast green	Relative amount of heme
Hemoblast	—	—	—	3	54.0 ± 7.00*	11.7 ± 2.78
PrE	19	21.3 ± 2.05	18.5 ± 1.62	5	95.6 ± 15.42	16.1 ± 2.97
BE	16	18.7 ± 2.97	20.6 ± 1.49	10	73.5 ± 11.1	21.5 ± 2.01
EPE	12	25.4 ± 3.35	29.8 ± 1.42	6	74.0 ± 8.52	21.2 ± 2.26
MPE	76	29.2 ± 1.37	35.5 ± 1.07	27	118 ± 10.99	20.9 ± 1.68
LPE	37	41.0 ± 2.05	44.0 ± 2.33	21	161.3 ± 11.90	26.1 ± 1.79
RET	16	56.2 ± 4.12	75.3 ± 3.47	31	195 ± 9.99	33.2 ± 1.69
RBC	44	109.5 ± 4.88	145.1 ± 4.44	18	271.2 ± 19.99	62.7 ± 3.26

* Standard error of mean.

in rabbits, indicate that the intermediate and later erythropoietic stages show little or no RNA synthesis. Moreover, these cells are already well differentiated elements, both morphologically and biochemically. Since the earlier stages show intense RNA synthesis, establishment of a new cell line would, of necessity, occur in these precursor cells. The precursor cell most likely to be involved is the stem cell which is considered to represent an "undifferentiated" cell type.

These conclusions are compatible with morphological and biochemical studies on erythropoiesis. In mammals, morphological evidence indicates that during fetal development the liver and eventually the bone marrow produce a secondary or definitive erythrocyte which differs from the primary erythrocyte formed in the yolk sac. The change in erythropoietic site from the yolk sac to the liver can also be correlated with a change in hemoglobin characteristics (1, 5). We have observed that the sinusoids of rabbit fetal liver con-

tain many primary erythrocytes for some time after the liver has assumed erythropoietic activity. However, these cells never incorporate labeled RNA precursors and are being phagocytized by the reticuloendothelial (RE) cells of the liver. Therefore it seems that, when the new hemoglobin appears in the mammalian fetus, it is associated with a new cell line originating from stem cells of the liver.

In amphibians a similar situation occurs at metamorphosis. Moss and Ingram (22) have shown that tadpole erythrocytes contain a different type of hemoglobin than adult frog erythrocytes. Moreover, thyroxine repressed the synthesis of tadpole hemoglobin but induced, after 10 to 15 days, the formation of adult frog hemoglobin. These authors concluded that thyroxine acts not by stimulating the relatively mature red cells, but acts on the precursor cells which then release a new line of red cells into the circulation after 13 to 15 days. Early morphological work showed that during

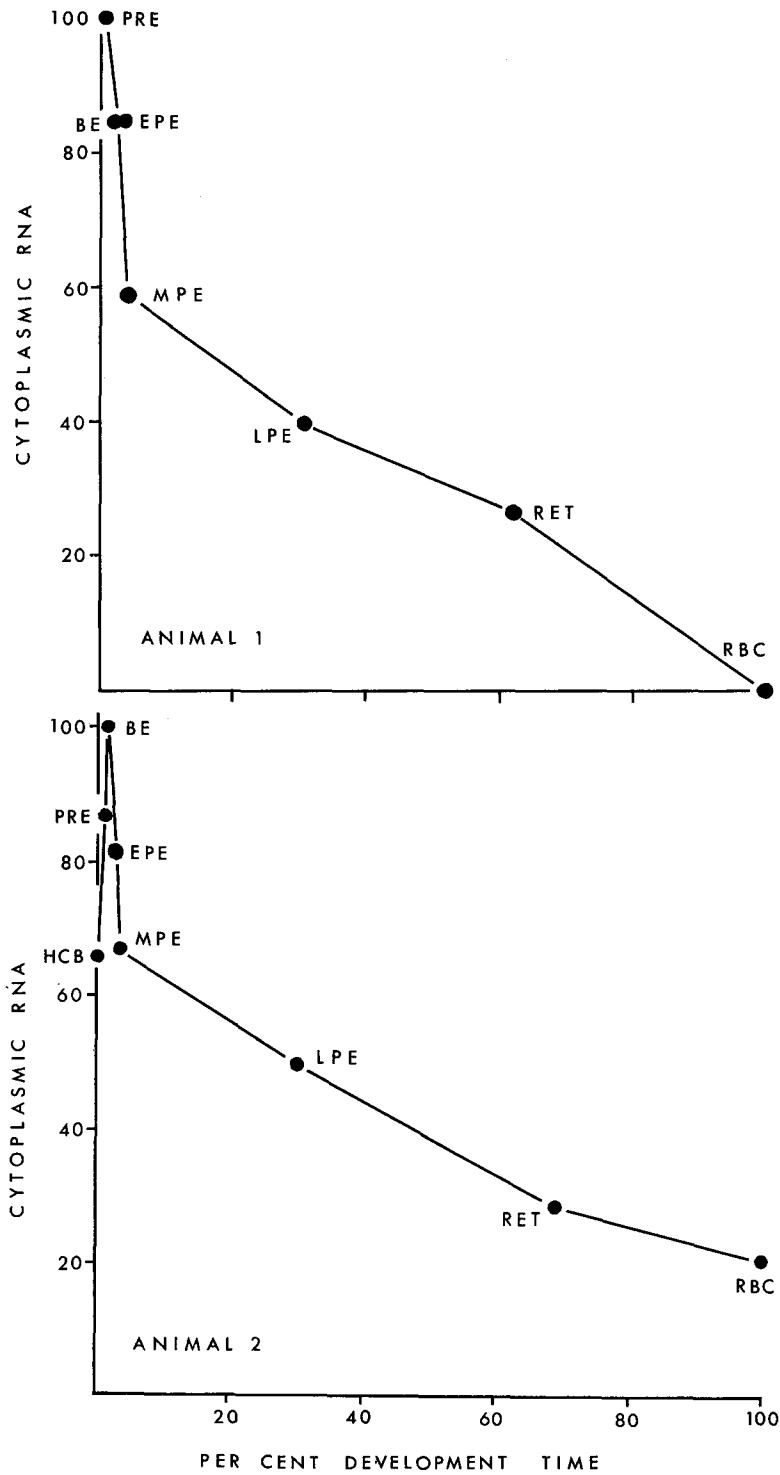


FIGURE 20 Relative amounts of cytoplasmic RNA (azure B) during blood cell production. The highest value was arbitrarily set at 100 units, and values for other stages were changed accordingly.

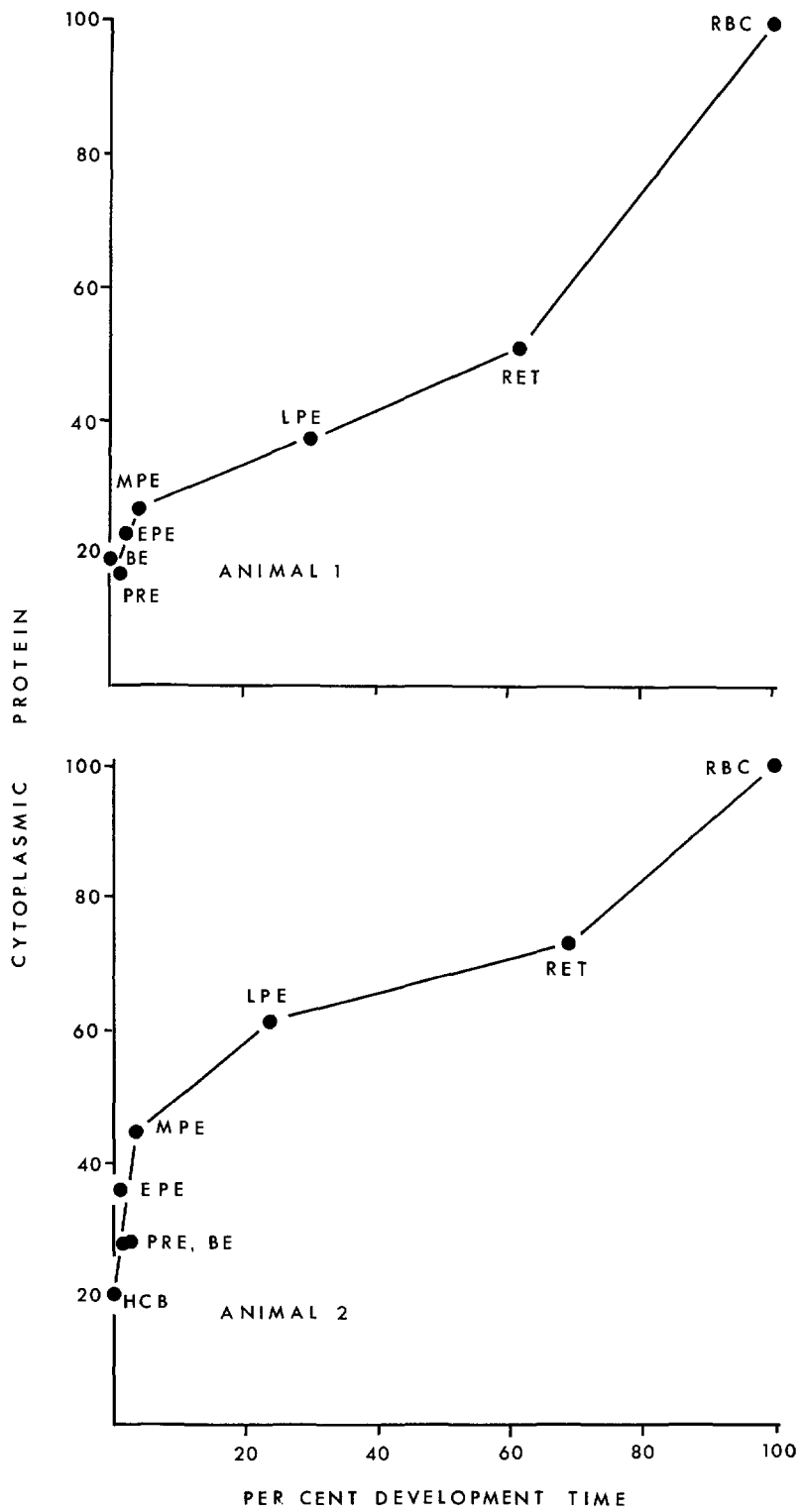


FIGURE 21 Changes in the amount of cytoplasmic protein (fast green at pH 2) during blood cell development. The highest value was arbitrarily set at 100 units, and values for other stages were changed accordingly.

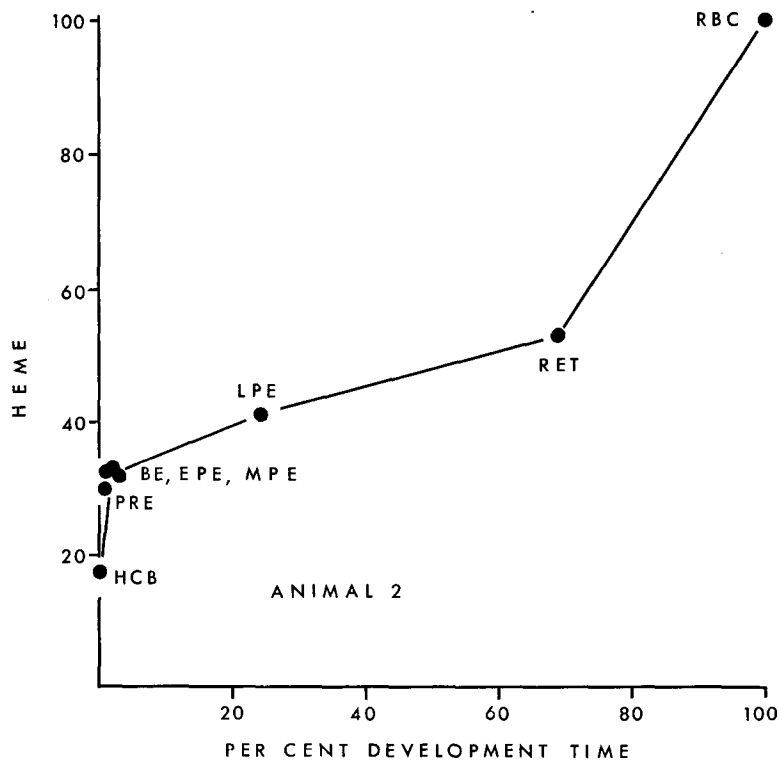
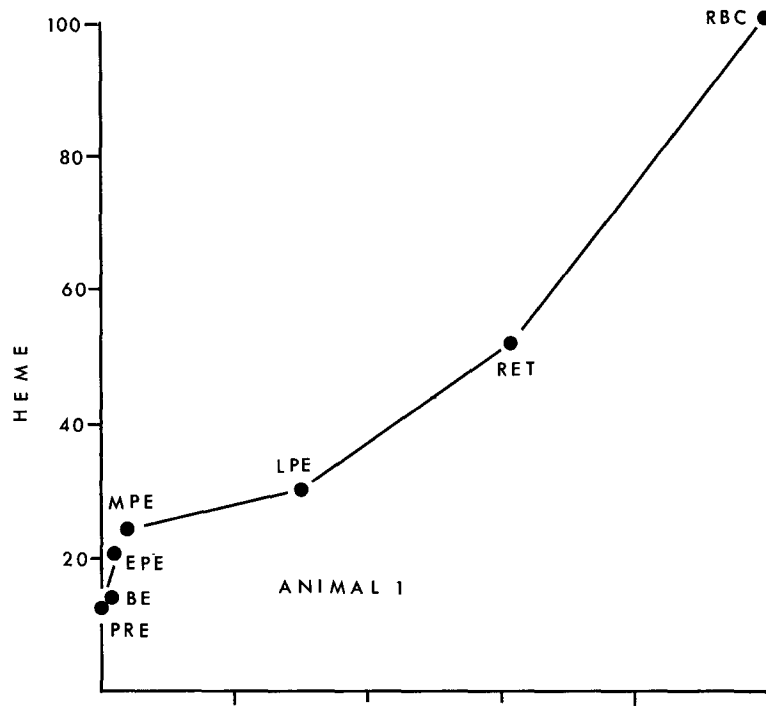


FIGURE 22 Heme amounts during the course of blood cell production. The highest value was arbitrarily set at 100 units, and values for other stages were changed accordingly.

metamorphosis the site of erythropoietic activity shifted from the kidney to the spleen (16), indicating that the "new" cell line characteristic of adult frogs is established from the stem cells of the spleen.

We propose the following model for the differentiation of erythrocytes: differentiation of an uncommitted stem cell results in, or is accompanied by, the synthesis of new messenger RNA and ribosomal RNA, the former possessing relatively stable characteristics. In the cytoplasm, shortly before the massive synthesis of hemoglobin begins, the messenger becomes associated with ribosomes forming polysomes while "inactive" ribosomes may be deleted from the cell. The nuclei are initially in-

involved in the formation of cellular RNA, whereas in later stages they lose this ability and serve, through mitosis, as the means by which the system undergoes proliferation (11, 12). Establishment of a new line of cells containing different hemoglobin probably occurs by the synthesis of a new messenger in the stem cells which directs the synthesis of "new" hemoglobin.

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