DNA SYNTHESIS AND MITOSIS IN ERYTHROPOIETIC CELLS

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ABSTRACT

Studies of newt (*Triturus* or *Diemictylus viridescens*) erythropoietic cells showed that DNA synthesis and mitosis normally occur throughout most of the developmental process. Mitotic divisions were found in all immature precursor stages from the proerythroblast to the highly hemoglobinized reticulocyte. Mitoses were absent in mature erythrocytes. Radioautographic examination of thymidine-^{3}H incorporation into DNA revealed that all erythroid cells except the mature erythrocyte were labeled. Microphotometric measurements of Feulgen-stained smears showed that all immature stages were undergoing DNA synthesis whereas the mature erythrocyte was inactive. The results obtained from three independent methods clearly demonstrate that (*a*) no loss of DNA or of chromosomes occurs during erythrocytic development and (*b*) highly hemoglobinized and, therefore, well-differentiated cells normally do undergo DNA synthesis and mitosis.

INTRODUCTION

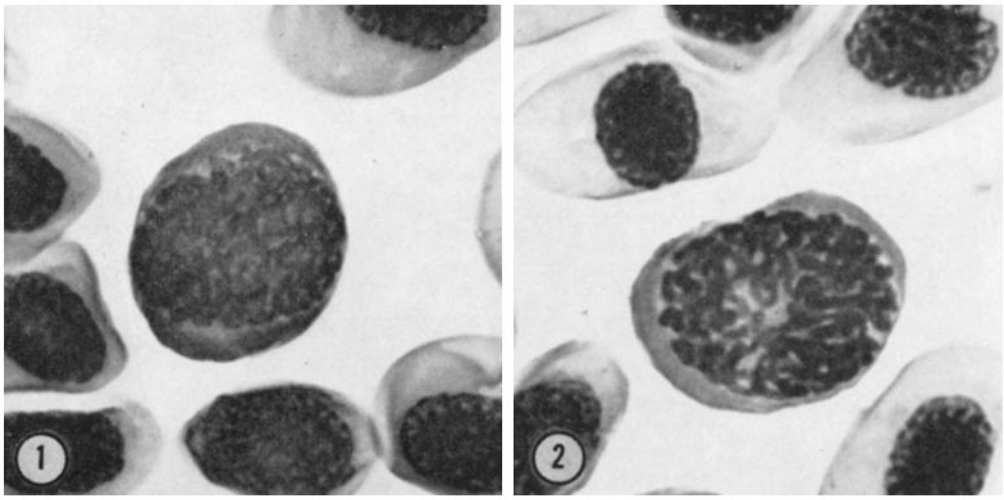
The relationship between cell division and the differentiated state has been examined in several different tissue types. In myogenesis, DNA synthesis and mitosis appeared to cease in the mononucleate myoblast prior to the appearance of myofilaments and the fusion of the mononucleate cells to form myotubes (21). In explants of pancreatic acinar tissue, cell divisions were not found in those cells containing specific granulation but most proliferative activity was limited to less differentiated cells at the periphery of the explant (28). These observations are consistent with the thesis that cells synthesizing a specific cell product seldom divide. However, differentiated cell types cultivated in vitro have often been induced to undergo division although eventual loss of the specific phenotypic characteristics has been reported (12, 13).

In an earlier report (10), rabbit erythropoietic cells in situ were shown to undergo DNA synthesis and mitosis throughout most of their development. Proliferation of these cells persisted even in well-advanced developmental stages and ceased prior to the expulsion of the nucleus. The present study, which was undertaken to examine the extent of DNA synthesis and mitosis in newt erythropoiesis, shows that, as in the rabbit, mitotic division is normally characteristic of most erythropoietic stages. The significance of these results is that (*a*) contrary to some earlier reports, no loss of DNA or of chromosomes accompanies erythrocytic development, and (*b*) highly hemoglobinized and, therefore, well-differentiated cells do frequently divide without loss of the differentiated state.

MATERIALS AND METHODS

Proliferative activity in newt[1] (*Triturus* or *Diemictylus viridescens*) erythropoietic cells was examined by three different techniques: (*a*) direct observation of

[1] Obtained from Lemberger Company, Oshkosh, Wis.



FIGURES 1–5 Mitotic figures in erythropoietic cells at various stages of development. The smear of peripheral blood was obtained from an unsplenectomized newt and stained with Wright's stain. × 1353.

FIGURE 1 An early polychromatic erythroblast in early prophase.

FIGURE 2 A mid-polychromatic erythroblast in late prophase.

mitotic figures in smears stained with Wright's stain, (*b*) radioautography after exposure to tritiated thymidine (^{3}HTdR), and (*c*) microphotometric measurement of Feulgen-stained preparations. Most of the animals had been previously splenectomized, a procedure reported to result in erythropoietic activity in the peripheral blood (15). However, peripheral erythropoietic activity was observed in some unoperated animals during the middle weeks of January and during the breeding season (April–June). All experiments reported in this study were performed on peripheral blood obtained by cutting the tail. All animals were maintained in spring water at 20–23°C.

The mitotic index of the total erythropoietic population was obtained by counting randomly the number of mitotic figures (late prophase to telophase) per 1000 cells. In two smears which exhibited a high total mitotic index, the mitotic index for several individual stages was determined in the same way. However, too few cells existed in the very early developmental stages so that a meaningful mitotic index could not be obtained for these stages. For radioautographic studies of DNA synthesis, 75–80 μc of tritiated thymidine (specific activity = 1.5 c/mmole)[2] was injected intraperitoneally into splenectomized newts, with 30 or 33 gauge hypodermic needles. Blood smears were prepared after 2–24 hours' exposure to thymidine-^{3}H, air dried, and fixed in absolute methanol for 5–15 min. Individual slides were extracted with DNase,[3] RNase,[4] or cold (5°C) 5% trichloracetic acid (TCA) and dipped in Kodak NTB-2 liquid emulsion. After exposure for 1–2 wk, radioautographs were photographically processed and stained in cold (5°C) 0.025% Azure B (9) for 2–3 min. Comparison of the variously extracted slides indicated that the labeled precursor was incorporated into DNA.

Microphotometric measurements of the amount of Feulgen-DNA per nucleus were performed on cells in which measurements of cytoplasmic RNA and hemoglobin had been previously carried out (animals 1 and 2 in reference 11). Thus, precise staging of the cells was possible. Smears were hydrolyzed for 12 min in 1 N hydrochloric acid at 60°C and then immersed for 1 hr in Schiff's reagent. After dehydration and clearing, the smears were mounted in an oil, the refractive index (1.568) of which closely matched that of the stained nuclei. Measurements of the amount of dye bound to DNA in individual nuclei were made with the "plug" method (25) at a wavelength of 560 mμ or with the two-

[2] Obtained from Schwarz BioResearch Co., Orangeburg, N. Y.

[3] Obtained from Worthington Biochemical Corporation, Freehold, N. J.

[4] Obtained from Worthington Biochemical Corporation, Freehold, N. J.

wavelength method (25) at wavelengths of 500 and 540 mμ. Measurements were performed by each author working independently on smears obtained from different animals. The amount of Feulgen-DNA per nucleus was obtained from the average of two–three measurements of the same nucleus and plotted as a histogram.

The sequence of erythropoietic stages employed was: hemoblast → proerythroblast (PrE) → basophilic erythroblast (BE) → early polychromatic erythroblast (EPE) → mid-polychromatic erythroblast (MPE) → late polychromatic erythroblast (LPE) → reticulocyte (RET) → mature erythrocyte (RBC). The criteria used for identification of these various stages have been presented in a previous report (11). It should be pointed out, however, that reticulocytes in the present report were identified on the basis of two features: (*a*) a cytoplasmic acidophilia closely approximating that of the mature erythrocyte, and (*b*) the presence of a slight amount of cytoplasmic basophilia. In Azure B-stained preparations, the latter criterion alone was used. In other respects, the reticulocyte was essentially similar to the mature red blood cell, regardless of staining procedure.

RESULTS

Morphological Observations

Mitotic divisions were seen in cells representative of each erythropoietic stage, from the proerythroblast through the reticulocyte (Figs. 1–5) but were absent in mature erythrocytes. The distribution by erythropoietic stage of 500 randomly encountered mitotic figures is shown in Table I. A high percentage of these mitoses was found in mid- and late polychromatic erythroblasts. Mitotic figures in reticulocytes, an advanced stage in erythrocytic development, accounted for approximately one-fifth of all observed mitoses. Although fewer divisions (~4%) were distributed among the more immature cell types (PrE → EPE), these data should not be interpreted to indicate that mitotic frequency increased in the later stages. The larger number of mitoses found in the latter was due to the fact that the later stages (MPE → RET) comprised approximately 98% of the total erythropoietic population (see column 4 in Table I), thereby increasing the probability of chance encounter of mitotic figures in these stages.

The mitotic index of the total erythropoietic population (excluding the nonmitotic mature erythrocytes) varied considerably from animal to animal. In some animals little or no mitotic activity was observed, whereas in others some 2–2.5% of the immature erythroid cells was found in mitosis. Since the earlier stages comprised a small fraction of the total population, the values obtained represented mainly the mitotic index of the later developmental stages (MPE–RET). The mitotic index of the mid- and late polychromatic erythroblasts ranged from 2.3 to 2.7% whereas that of the reticulocytes fell to between 1.0 and 1.4% (Table I). In general, mitotic activity was most conspicuous in blood samples drawn during the late morning hours. However, a careful study of the relationship between sampling time and mitotic activity was not undertaken.

In peripheral blood smears prepared from erythropoietically active animals, binucleate mature erythrocytes were often found (Figs. 6, 7). This condition was apparently derived from nuclear budding or "division." In most cases, the "daughter" nuclei were unequal in size. Occasionally, mature cells were seen in which concomitant fission of the nucleus and cytoplasm was suggested (Fig. 8). Another frequent finding in mature erythrocytes was the presence of nuclear fragments. These fragments were observed either as fine, dust-like granules, 0.3–0.6 μ in size (Fig. 10), or as large clumps of varying size (Figs. 9, 10). Both types of fragments were stained positively by the Feulgen reaction (Figs. 9, 10). These processes of nuclear budding and fragmentation,

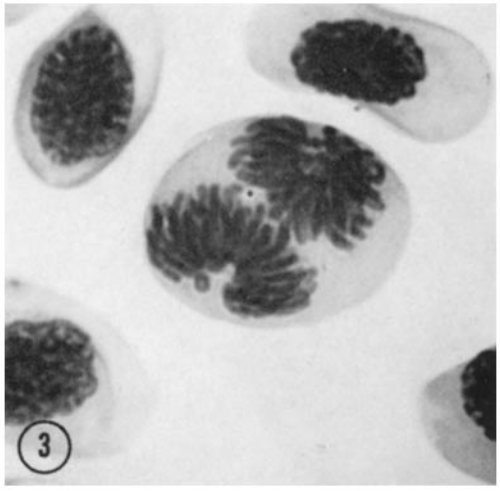
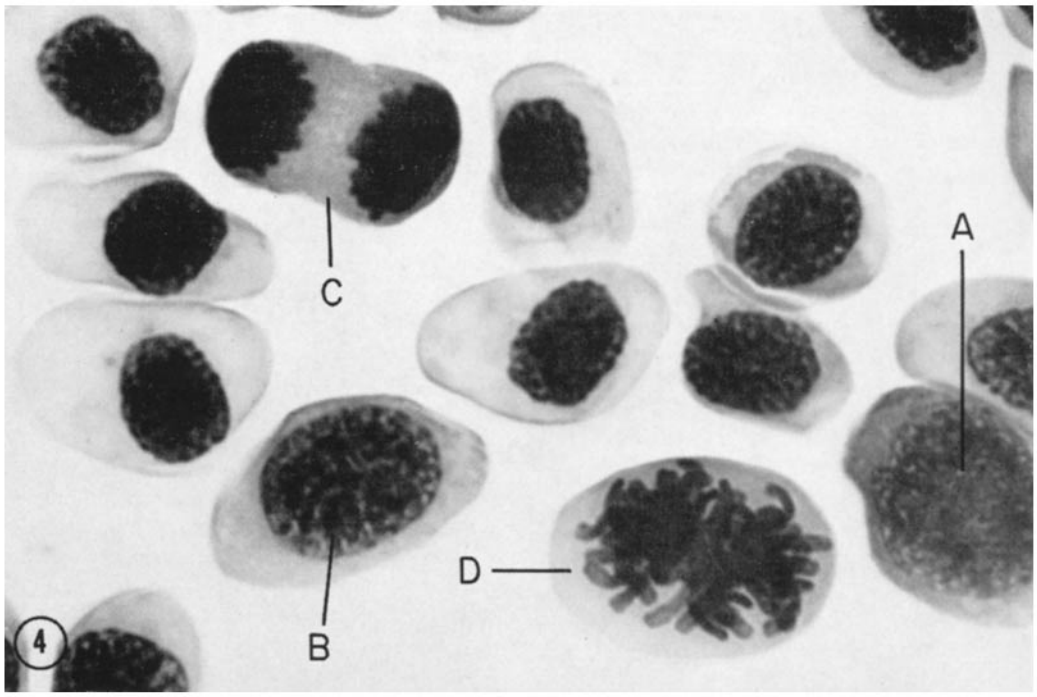


FIGURE 3 A reticulocyte in anaphase. Its cytoplasmic features are essentially similar to those of the surrounding mature erythrocytes, indicative of a high content of hemoglobin.



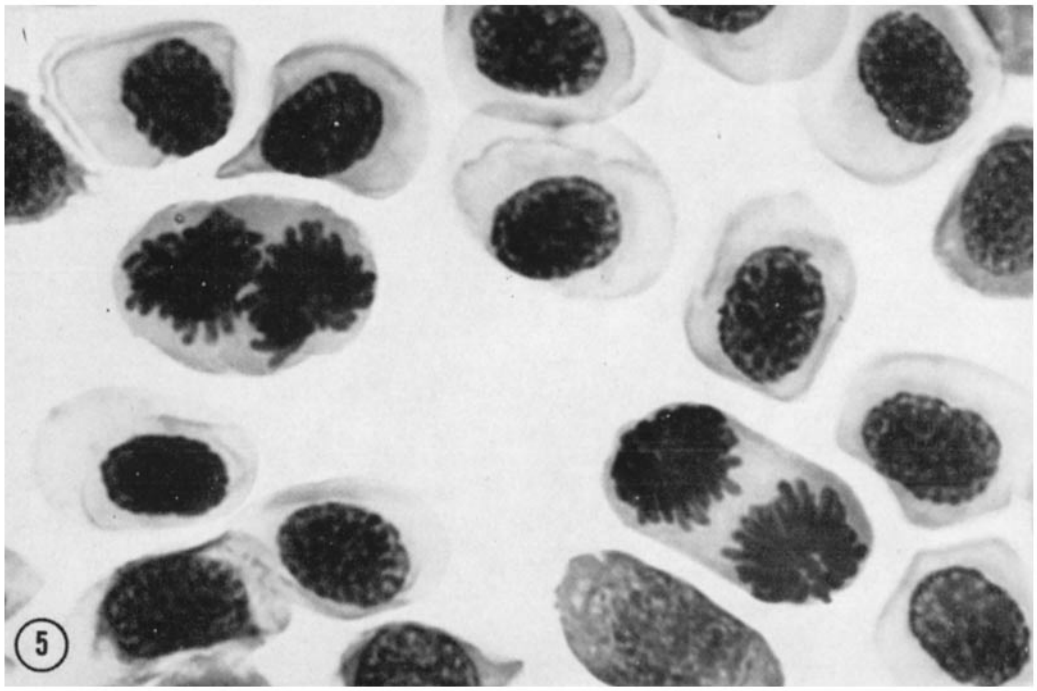


FIGURE 4 Cell *A* is a mid-polychromatic erythroblast in interphase whereas *B* is probably an early reticulocyte. At *C*, a late polychromatic erythroblast can be seen in early telophase. At *D*, a reticulocyte, probably in metaphase, is indicated. Note that the cytoplasmic acidophilia of cell *D* closely approximates that of the surrounding mature erythrocytes, indicating high hemoglobin content.

FIGURE 5 Two late polychromatic erythroblasts in mitosis. Both cells contain a moderate amount of cytoplasmic acidophilia.

TABLE I

Distribution of Mitotic Figures by Developmental Stage

Stage	No. of mitoses	Total Mitoses	Rel. frequency of stage*	M. I.‡
		%	%	
PrE	2	0.4	0.71	§
BE	6	1.2	0.11	§
EPE	10	2.0	1.30	§
MPE	174	34.8	24.0	2.3–2.7%
LPE	197	38.4	38.0	
RET	111	22.2	36.0	1.0–1.4%
RBC	0	0	—	
Total	500	100	100	

* This value represents the percentage of cells in each given stage as obtained from random counts of 1000 cells.

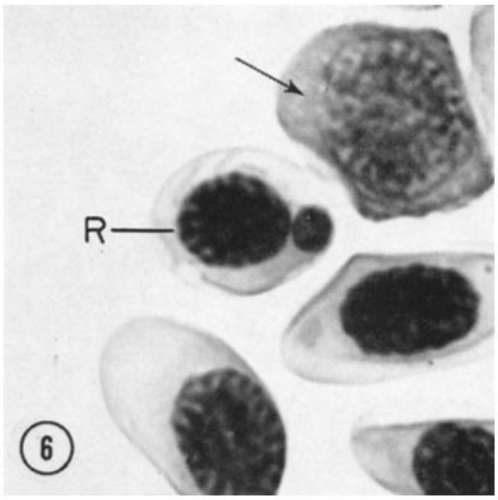
‡ Mitotic Index. Obtained by counting randomly the number of mitotic figures per 1000 cells.

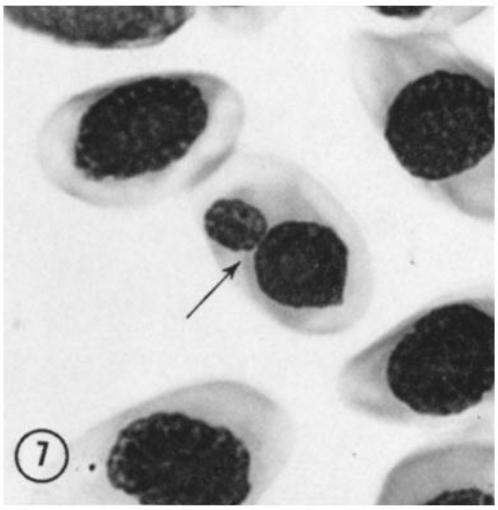
§ Too few cells to be significant (total of 42 cells for the three stages).

which have been called amitosis (5, 8), probably indicate incipient degeneration of mature erythrocytes and were not considered to contribute to an increase in the size of the erythroid population. Nuclear budding and fragmentation were rarely observed in immature erythroid cells. In numerous blood smears prepared from erythropoietically inactive newts, these nuclear alterations have not been seen, thereby excluding the possibility that they represent artifacts of smear preparation.

Incorporation of Tritiated Thymidine (^3HTdR)

Cells in all stages of erythrocytic development (except the mature erythrocyte) incorporated labeled thymidine (Figs. 11–14) which was shown





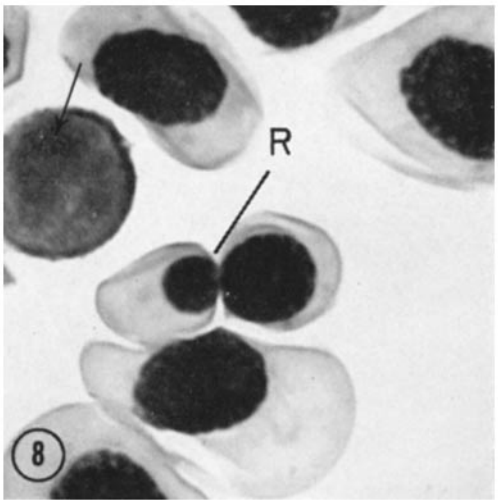


FIGURE 6 A nature erythrocyte (*R*) showing process of nuclear budding or "division." A mid-polychromatic erythroblast is indicated at the arrow. Wright's stain. × 615.

FIGURE 7 A binucleate mature erythrocyte (arrow). Note that the two nuclei are unequal in size. Wright's stain. × 615.

FIGURE 8 A mature erythrocyte (*R*) in which both the cytoplasm and nucleus appear to be undergoing fission. Cell at arrow is a lymphocyte. Wright's stain. × 615.

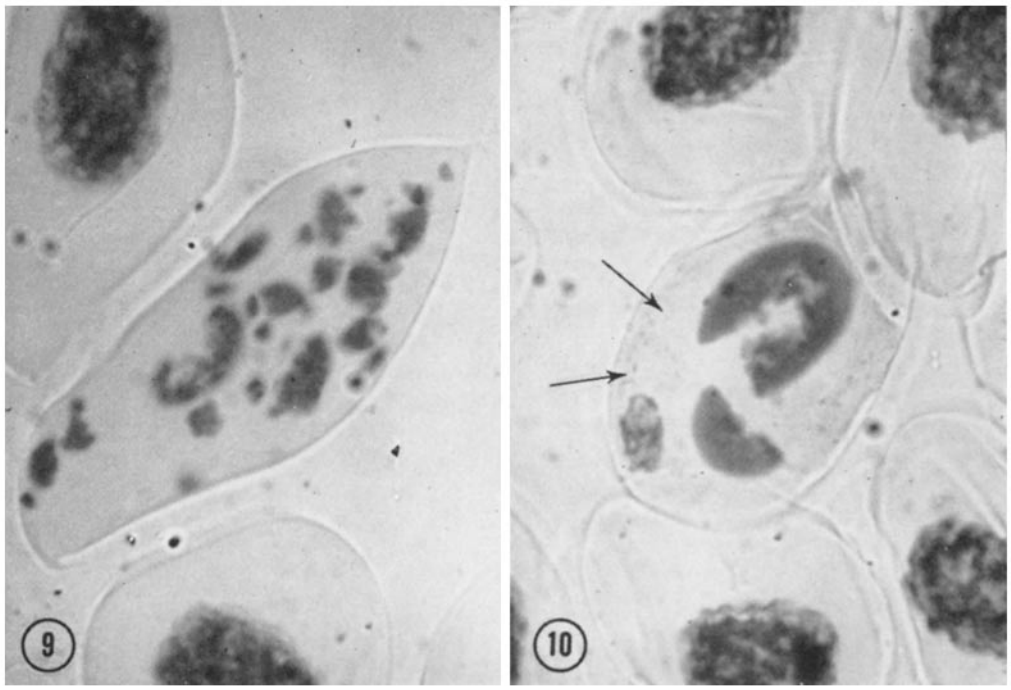


FIGURE 9 A mature erythrocyte exhibiting nuclear fragmentation. Note the varying size of the clumps and their positive reaction to Feulgen staining. Feulgen preparation. × 2200.

FIGURE 10 A mature erythrocyte showing different aspects of nuclear fragmentations. Around the cell periphery, small Feulgen-positive granules can barely be seen (around arrows). In the center and at lower left of cell are large, Feulgen-positive clumps. Feulgen preparations. × 2200.

to be present in DNA. After 2 hr of exposure to precursor, approximately 17–28% of all erythropoietic cells was labeled (Table II). By 24 hr, 22–32% of these cells was labeled (Table II). Since most of the cells in these preparations represented the later developmental stages (MPE–RET), these data reflect predominantly the percentage o lafbeled cells in these stages. Labeled late polychromatic erythroblasts (Fig. 13) and reticulocytes (Fig. 14) were very frequent. Mature erythrocytes showed no incorporation of labeled thymidine (Figs. 13, 14).

Microphotometric Measurements

Measurement of the amount of Feulgen dye that binds to DNA in individual mature erythrocytes revealed a unimodal distribution of values which was interpreted to represent the diploid class (Figs. 15, 16). This distribution is characteristic of a nondividing cell population and substantiates both the lack of mitotic figures and the nonlabeling of erythrocytes after exposure to labeled thymidine. Measurements of all immature erythroid stages showed a bimodal distribution of values into distinct 2C and 4C classes and a number of intermediate values (Figs. 15, 16). Such a distributional pattern was found for each developmental stage, from proerythroblasts through reticulocytes, and is indicative of a cell population carrying out DNA synthesis and mitosis.

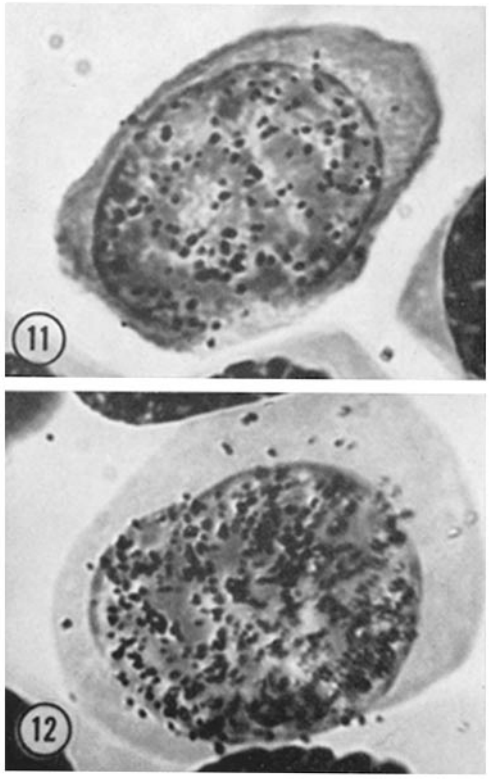
DISCUSSION

The results of this study show that, in newts, DNA synthesis and mitosis occur in essentially all stages of erythropoiesis. Proliferative activity is not limited to the earlier, more immature cell types but is frequently observed in late developmental stages which, by virtue of their high hemoglobin content (11), can be clearly regarded

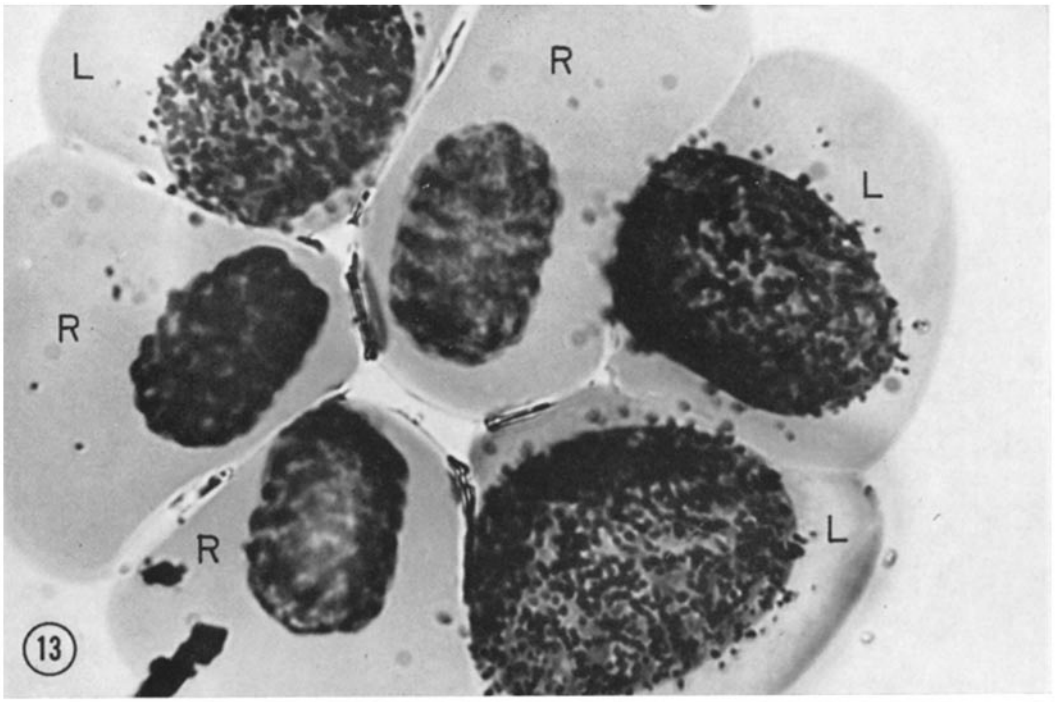
as "well-differentiated" elements. In newt erythropoiesis, mitotic activity presumably ceases during the transition of the reticulocyte into a mature erythrocyte. This transitional stage, which is difficult to distinguish morphologically from its immediate predecessor, might be called a "late reticulocyte." In rabbit fetal (10) and adult human (18) erythropoiesis, all mitotic activity ceases in a distinct stage called the "late normoblast," a cell type analogous to the newt "late reticulocyte"; both cell types represent highly "differentiated" and nearly terminal stages in erythrocytic development. These results combined with our previous studies on erythrocytic differentiation (10, 11) indicate that the erythropoietic process within two widely different vertebrates occurs in an essentially similar manner.

Several authors have reported that in mammalian erythropoiesis DNA synthesis and mitosis cease in the polychromatic erythroblast (1–3, 18), i.e. that all erythroblasts except the late orthochromatic erythroblast or late normoblast are capable of cell division. Our previous study of DNA synthesis in rabbit erythroblasts is essentially in agreement with this conclusion. However, since the polychromatic erythroblast also marks the point at which the main bulk of hemoglobin synthesis begins, the impression sometimes conveyed is that the proliferative activity of erythroblasts ceases before or at the time the most intensive period of hemoglobin production begins (cf. reference 12 for general discussion of this relationship; 26). Our data, both in rabbits and in newts, do not agree with this viewpoint. In both instances, it is clear that highly hemoglobinized cells, irrespective of the nomenclature applied to them, normally undergo DNA synthesis and mitosis. These data are in agreement with the results of early morphological studies both in amphibia (7, 8, 14) and in mammals (20).

Moreover, our results show that, contrary to some reports (17; see also reference 3 for review), no loss of DNA accompanies erythrocytic development. The viewpoint for DNA loss is largely based upon a microphotometric study of human bone marrow smears (17) in which the *average* value of DNA in a number of cells at a given developmental stage was considered to represent the relative amount of DNA per cell. The results obtained from this approach suggested that DNA was gradually lost in successive developmental stages, the largest decrease occurring between the polychromatic erythroblast and the late normoblast. However, this interpretation is not valid, for several reasons: (*a*) The study in question was performed at a time when DNA synthesis was believed to occur during prophase rather than in interphase. Thus, it was assumed that all interphase nuclei were in a "resting" stage equivalent to G_1 and not synthesizing DNA, an invalid assumption (24). (*b*) Consequently, the *average* amount of DNA per nucleus in those erythropoietic stages undergoing DNA synthesis and mitosis will be higher than the true diploid level since all nuclei, ranging from diploid to twice the diploid level, will be included in its determination. Moreover, the average will vary depending upon the proportion of diploid, tetraploid, and intermediate values encountered. Conversely, the *average* amount of DNA per nucleus in a non-



FIGURES 11–14 Radioautographs of erythropoietic cells from newts exposed for 2 hr to tritiated thymidine (^{3}H-TdR). Preparations were briefly treated with cold TCA prior to application of emulsion and stained in cold Azure B after photographic processing. × 2200.
FIGURE 11 An early polychromatic erythroblast.
FIGURE 12 A mid to late polychromatic erythroblast.



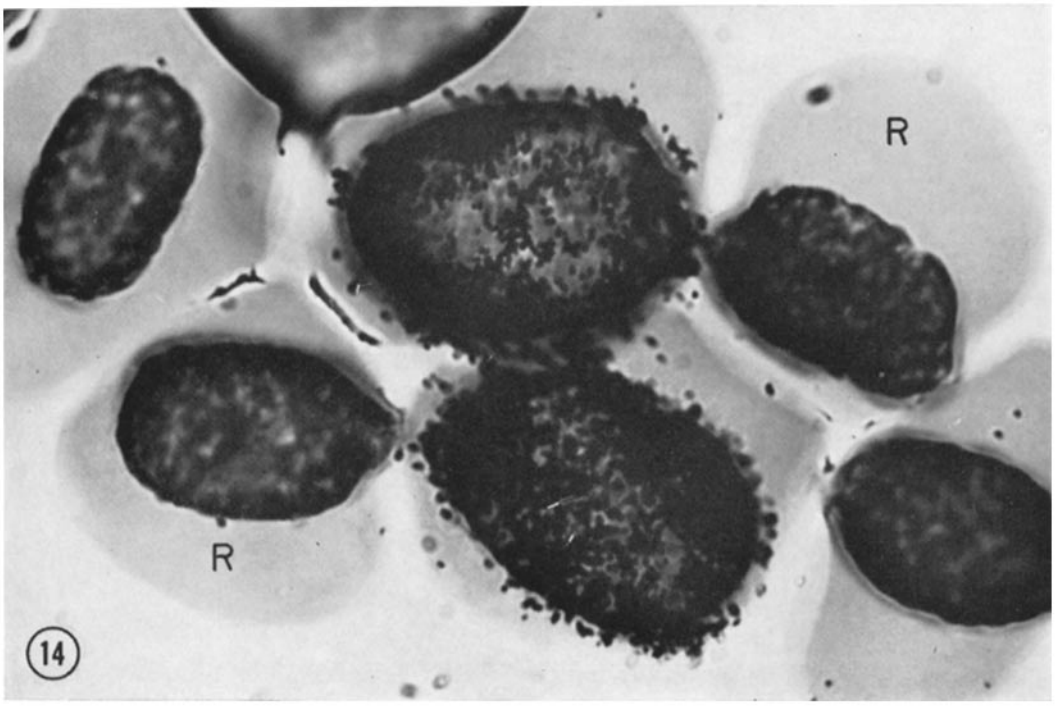


FIGURE 13 Three labeled late polychromatic erythroblasts (*L*). Note lack of grains over adjacent mature erythrocytes (*R*). The degree of cytoplasmic staining is indicative of a cell containing a moderate amount of hemoglobin.

FIGURE 14 Two labeled reticulocytes occupy the center of the field. Grains are absent over adjacent mature erythrocytes (*R*). The cytoplasmic density of the reticulocytes closely approximates that of the mature red blood cells.

TABLE II

Percentage of Labeled Erythropoietic Cells after Thymidine-^3H

Hours after TdR-^3H	Animal IX			Animal IaX			Animal 2X		
	No. of cells	No. labeled	%	No. of cells	No. labeled	%	No. of cells	No. labeled	%
2	1300	226	17.3	662	135	20.4	1181	336	28.5
9	1201	228	19.0						
24	565	124	22.0				530	172	32.5

dividing cell population (late normoblast) will approximate the true diploid level. Comparison of the two populations, based on average values, will erroneously suggest that a DNA loss has occurred. Yet the lower average of a nondividing population simply reflects the absence of DNA-synthesizing cells. Previous microphotometric measurements of Feulgen-DNA amounts in late normoblasts of mouse have clearly shown that these cells represent a diploid population (24).

The postulate for DNA loss has received support from reports that a gradual reduction in chromosome number accompanies erythrocytic maturation (16, 27). This reduction is assumed to result from the loss of chromosomal segments or of whole chromosomes. A chromosomal loss is difficult to reconcile with our observations that the relative diploid and tetraploid DNA levels are maintained throughout erythropoiesis. One would have to postulate that the chromosomes remaining after each loss become more polytene so that the amount of DNA in the lost chromosomes is restored. Alternatively, a gradual and unusual fusion or a pairing of chromosomes might be used to explain the maintenance of DNA levels. Both phenomena, however, would be quite unique.

Although mitosis occurs frequently in the later erythropoietic stages, erythroid cells in mitosis do not lose their morphologic and tinctorial characteristics. Indeed, the only parameter affected by mitosis appears to be the size of the daughter cells, which are greatly reduced in size with respect to the parent cell. However, even this parameter is eventually restored with further maturation. These observations indicate that the differentiated state of the parent cell is transmitted to each daughter cell. Similar conclusions concerning the heritability of the differentiated state, i.e. of the stability of specific phenotypic characteristics in the presence of mitotic activity, have been reported for embryonic chondrocytes (6) and retinal pigment cells (4) grown in vitro.

The persistence of erythroid phenotypic traits in the presence of mitotic activity can perhaps be explained on the basis of the intrinsic stability characteristic of erythropoietic cells. Previous studies have shown that the bulk of RNA syn-

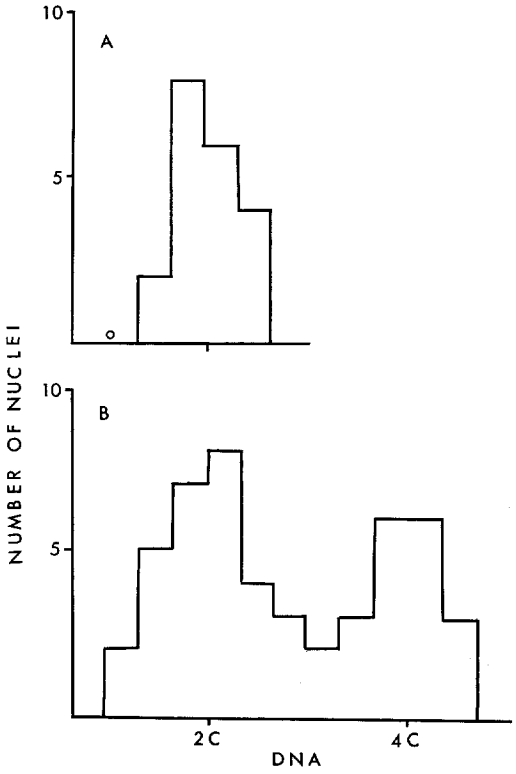


FIGURE 15 Histograms obtained from microphotometric measurements of Feulgen-stained newt blood cells (cf. animal 2 in reference 11). Graph A depicts DNA amounts in mature erythrocytes. Note that the values fall around the 2C mode indicative of a nondividing population. Graph B represents DNA amounts in immature erythroid cells (proerythroblast to reticulocyte) and shows a distribution of values characteristic of a dividing cell population. Each individual stage, prior to the mature erythrocyte, exhibits the distribution seen in Graph B.

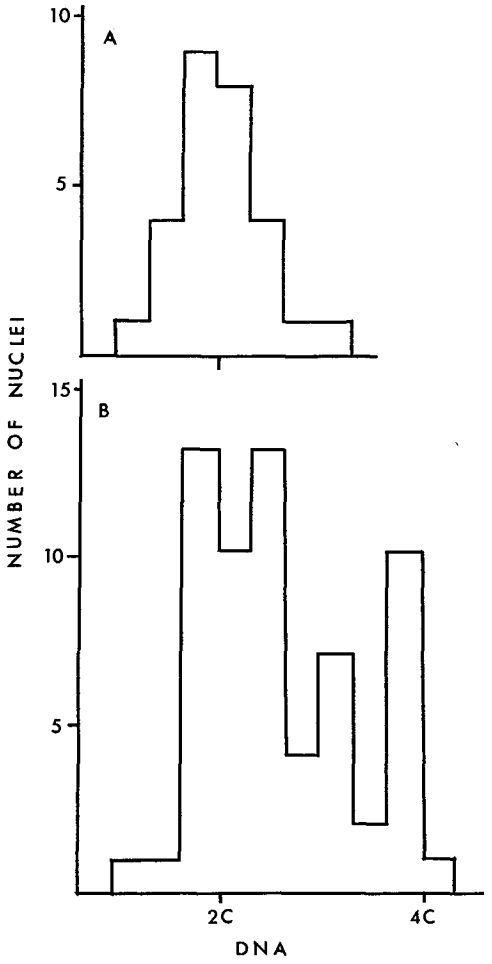


FIGURE 16 Histograms obtained from microphotometric measurements of Feulgen-stained newt blood cells. Similar to Fig. 15 except that measurements were done on a different animal (animal 1 in reference 11) and by a different person.

thesis takes place in the earliest stages (PrE, BE), whereas in subsequent stages the production of RNA is greatly diminished or absent (2, 3, 10, 11). The restriction of RNA synthesis to the earlier cell types suggests that the messenger RNA responsible for subsequent hemoglobin formation is largely produced in the most immature cell types. Furthermore, since the most intensive period of hemoglobin production occurs in the relative absence of RNA synthesis, hemoglobin messenger RNA has been considered to possess high stability (10, 11, 19, 23). The termination of specific gene transcription for hemoglobin-producing cells prior to the onset of intensive hemoglobin synthesis (MPE–RET) suggests that erythroid cells become irreversibly committed to a specific developmental line. It is conceivable that, because of these conditions, mitosis has no effect upon the differentiated state. Since RNA synthesis is greatly reduced or absent in the later stages, transmission of the differentiated state from the parent to its progeny apparently occurs at an epigenetic level, presumably at the level of the ribosomes and previously synthesized, stable messenger RNA. Moreover, the lack of detectable RNA synthesis in the late developmental stages still capable of mitosis would indicate that transmission of the differentiated state from parent to daughter cells involves maintained repression of this nuclear function.

The role of mitosis in erythropoietic cells appears to serve as a means by which the size of the population is increased. In view of this function, it is interesting to note the recent suggestion (22) that failure of erythropoietic cells to undergo mitosis may be a contributing factor in the etiology of megaloblastic anemias, blood dyscrasias which, in part, are characterized by a deficiency in the numbers of red blood cells. In addition, due to the dilution of ribosomes brought about by division of a non-RNA synthesizing cell, mitosis may partially be responsible for the gradual decrease in cytoplasmic basophilia so characteristic of the erythropoietic process.

Thus, in two widely divergent species, the erythropoietic process can be temporally subdivided into four phases: (*a*) "induction" of an "undifferentiated" stem cell, resulting in massive synthesis probably of both messenger and ribosomal RNA, (*b*) cessation of RNA synthesis followed by intensive hemoglobin production (both phases 1 and 2 include the occurrence of mitotic activity although proliferation and synthesis of hemoglobin may not occur concomitantly), (*c*) cessation of proliferative activity, and (*d*) terminal maturation characterized by the gradual loss of hemoglobin synthetic ability. In mammalian erythropoiesis, terminal maturation is more specialized in that the nucleus and all organelles are absent in the end product.

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