ERYTHROPOIESIS DURING AMPHIBIAN METAMORPHOSIS

I. Site of Maturation of Erythrocytes in *Rana catesbeiana*

GEORGE M. MANIATIS and VERNON M. INGRAM

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

ABSTRACT

The site of maturation of the erythroid cells in premetamorphic, metamorphic, and adult stages of *R. catesbeiana* has been investigated by comparing the percentages of immature, hemoglobin-containing erythroid cells in circulating blood, liver, spleen, kidney, and bone marrow. In both premetamorphic and thyroxine-treated tadpoles, the liver was found to be the main site of maturation of the red cells. The corresponding site in the frog is the bone marrow.

INTRODUCTION

During ontogeny most vertebrates exhibit one or more changes in the pattern of their hemoglobins (5). In amphibia the transition from the larval to the adult type of hemoglobin constitutes part of the developmental process characterized as metamorphosis (reviewed in reference 3). In many instances the developmental changes in the hemoglobin pattern coincide in time with a shift of the site of erythropoiesis (e.g., see reference 11). In mammals, for example, erythroid cell differentiation takes place first in the yolk-sac blood islands and subsequently in the fetal liver and spleen. Eventually, in the normal adult, it is restricted to the bone marrow. During the same time there is a transition from the embryonic hemoglobins to the fetal, and finally to the adult, type (reviewed in reference 12). The question of a possible correlation between the site of erythropoiesis and the type of the hemoglobin produced is of obvious interest.

In the bullfrog (*R. catesbeiana*), Jordan and Speidel (9) found that the chief erythropoietic organ is the spleen, with the bone marrow being active only in the spring. In the tadpole, on the other hand, the main site of erythropoiesis was considered to be the intertubular regions of the kidney. The same authors (10) found that, during thyroxine-induced metamorphosis, there was a shift of erythropoiesis from the kidneys to the spleen. Hollyfield (7) studied the origin of erythropoietic tissue in mid-larval *Rana pipiens* by reciprocal transplantation of the prospective hemopoietic anlagen between diploid and triploid embryos at the stage when the neural folds have just closed to form the neural tube. He concluded that the stem cell originates in the pronephric or mesonephric anlage.

Because of the criteria used for the identification of the erythroid cells, the validity of the conclusions drawn by Jordan and Speidel (9, 10) seems questionable (see Discussion). For this reason we decided to investigate the question of the site where the erythroid cell matures, particularly during the metamorphosis of *R. catesbeiana*. The findings reported here point to the liver as the organ where the erythroid cell matures both before and during metamorphosis.
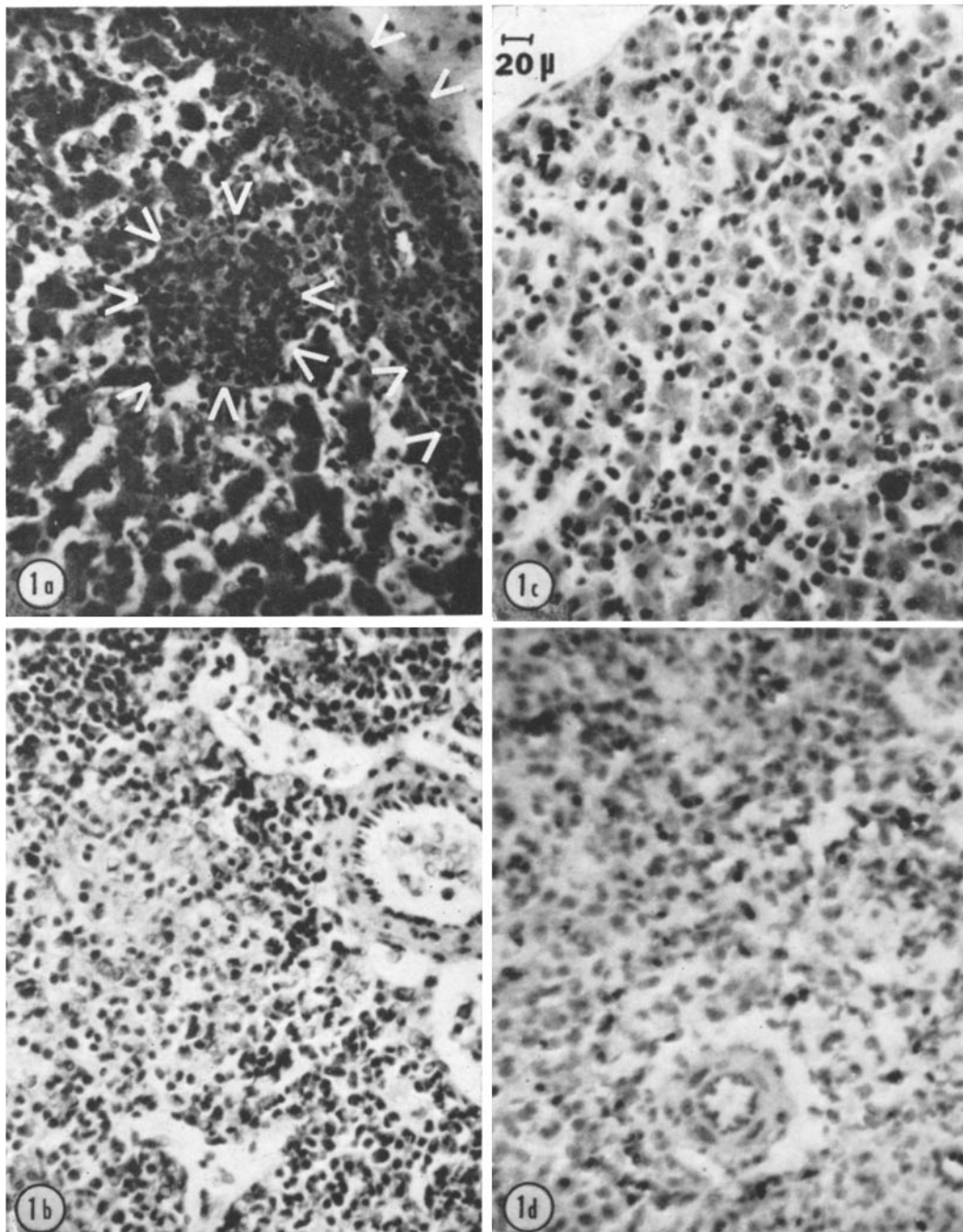
FIGURE 1 Sections of paraffin-embedded tissues. Fig. 1 *a* shows a section of the liver from a premetamorphic tadpole, 8 μm, stained with hematoxylin-eosin. The arrows indicate areas suggestive of hematopoietic activity. × 128. Fig. 1 *b* shows a section of the spleen from a premetamorphic tadpole, 8 μm, stained with hematoxylin-eosin. × 200. In Fig. 1 *c* a section of the liver from an adult frog, 10 μm, stained with hematoxylin-eosin, is shown. × 200. Fig. 1 *d* depicts a section of frog spleen, 6 μm, stained with hematoxylin-eosin. × 200.

MATERIALS AND METHODS

Source of Animals

Larval and adult *R. catesbeiana* were purchased from the Connecticut Valley Biological Supply Co., Southampton, Mass. All tadpoles used for thyroxine-induced metamorphosis had hind legs less than 5 mm long. Tadpoles were kept in groups of 5–10 in plastic containers (32 × 28 cm) filled with aged tap water to a height of 3 cm. They were fed either canned spinach or boiled romaine lettuce. To induce metamorphosis, the animals were placed in a 2.5 ×10⁻⁸ M solution of L-thyroxine. The frogs were kept in a terrarium, the floor of which was covered with continuously running tap water. They were fed *Tenebrio* larvae. The room temperature was kept constant at 20°C (19°–22°C) with a light-dark cycle of 12 hr (8 a.m.–8 p.m.).

Collection of Blood

Tadpoles were immobilized first by immersion in a 1:3000 solution of tricaine methanesulfonate (MS-222, Sandoz, Inc., Hanover, N. J.). They were decapitated and the heart was allowed to pump blood into a heparinized amphibian Ringer's solution (19) (0.01% heparin sodium, USP). Frogs were bled by heart puncture with a heparinized syringe.

Tissue Sections

Pieces of tissue were fixed and embedded in paraffin according to the method of Sainte-Marie (20). They were stained with Delafield's hematoxylin-eosin (17).

Smears of Red Blood Cells

The cells were washed in Ringer's and then diluted with 5% bovine serum albumin in Ringer's and smeared. Alternatively, the tail of a tadpole was cut off at the tip and a drop of blood was placed on a slide and smeared. (Fig. 4 *a*).

Tissue Touch-Imprints

The liver, kidney, spleen, and bone marrow from exsanguinated animals were cut into small pieces (1–2 mm thick) with fine dissecting scissors, blotted on filter paper to remove excess fluid, and then used to make touch-imprints on microscope slides. Smears or tissue touch-imprints were first dried in a stream of air, then fixed in methanol for 20 min and stained. The smears were stained with benzidine (18) and counterstained with Wright-Giemsa (13).

OBSERVATIONS

Histology

Sections of liver, kidney, spleen, and frog bone marrow were examined. The spleen in both tad-

pole and frog contains hemopoietic tissue (Figs. 1 *b* and 1 *d*); the bone marrow of the adult frog also has islands of hemopoietic tissue (Fig. 2 *c*). The sections of the frog liver (Fig. 1 *c*), as well as
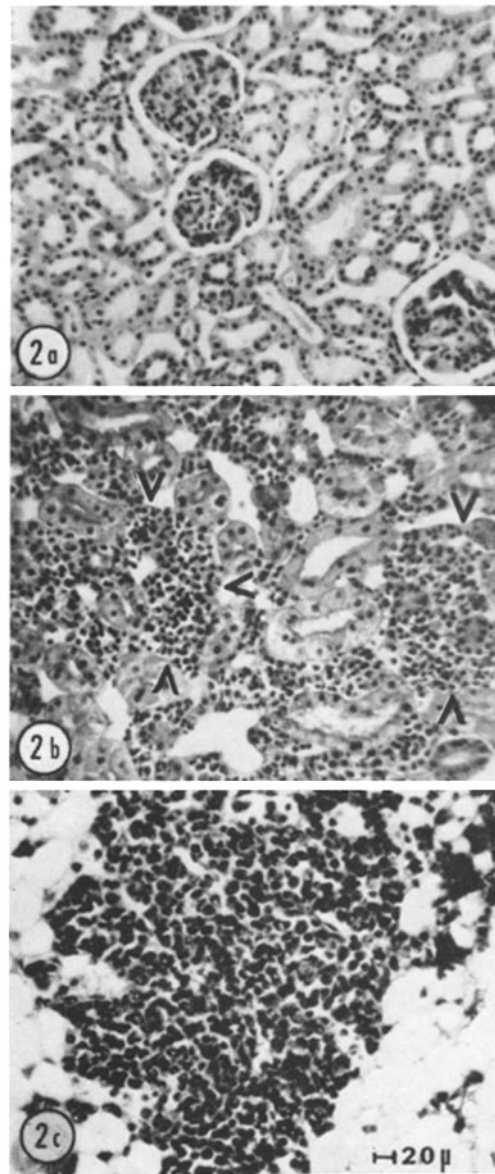

FIGURE 2 Sections of paraffin-embedded tissues. Fig. 2 *a* is a section of frog kidney, 6 μm thick, stained with hematoxylin-eosin. × 128. Fig. 2 *b* is a kidney section from a premetamorphic tadpole, 6 μm, stained with hematoxylin-eosin. The arrows indicate areas suggestive of hematopoietic activity. × 200. In Fig. 2 *c* bone marrow from the femur of an adult frog is shown, 6 μm, stained with hematoxylin-eosin. × 200.

the sections of the kidney (Fig. 2 a), show only parenchymal tissue and do not suggest hemopoietic activity. By contrast, the tadpole liver is infiltrated, particularly in the subcapsular region, with clusters of mononuclear cells suggesting hemopoietic activity (Fig. 1 a). The same holds true for the intertubular regions of the tadpole kidney (Fig. 2 b). The picture remains essentially unchanged during metamorphosis when frog hemoglobin first appears. Therefore, the liver, spleen, and kidney of the tadpole are the most likely sites of maturation of the red cells. Because it is difficult to differentiate hemopoietic cells in tissue sections, the following approach was also used.

Morphology of Imprints

Tissue touch-imprints were used for the detection of maturing cells of the red cell series. The ratio of immature to mature erythrocytes in each organ was determined and compared with the corresponding ratio in the peripheral blood. An organ with a ratio of immature to mature erythrocytes significantly higher than that of the circulating blood is most likely to be the site where

maturation of red cells takes place, although the possibility that this organ is the site of sequestration of immature cells can not be ruled out. The following criteria were used for identification of a cell as an erythroid one: (a) nuclear morphology (chromatin clumping), (b) cytoplasmic basophilia, (c) shape of the cell, (d) ratio of nuclear to cytoplasmic areas, (e) positive staining with benzidine. To avoid subjective judgement early erythroid cells, which may be confused with other immature cells (e.g., of the lymphoid series), were not counted. Only cells stained positively with the benzidine reagent for heme protein were included in the counts. Since the preparations were fixed in methanol, which inhibits both the cytochrome system and the peroxidases (6), the staining was specific for hematin compounds (pseudoperoxidases).

For the preparation of the tissue imprints the animals were first bled exhaustively by decapitation. A certain amount of blood, however, remained in each organ and varied from animal to animal. Therefore, the only meaningful comparison is the one between the ratios of immature/mature erythroid cells from the peripheral blood

TABLE I
Cell Counts from Blood Smears and Organ Imprints

Hind leg/tail	Days in thyroxine*	Immature erythrocytes in the blood	Immature erythrocytes in the liver	Immature erythrocytes in the spleen	Immature erythrocytes in the kidney
		%	%	%	%
0–0.14	0	0–1.0	2.5–4.5	0–0.9	0–1.4
0.14	8	0.3	0.9	0.5	0.4
0.19	9	0.3	1.3	0.3	0.5
0.24	10	0.2	0.2	0.3	0.2
0.29	11	0.0	1.0	0.1	0.0
0.25	11	0.0	0.7	0.1	0.1
0.22	13	0.0	1.4	0.2	0.3
0.27	13	0.1	1.8	0.3	0.0
0.32	15	0.0	3.0	0.2	0.0
0.27	17	0.1	2.5	0.3	0.0
0.38	18	0.2	2.8	0.2	0.0
0.31	19	0.0	1.4	0.0	0.0
0.36	20	0.5	2.9	0.8	0.5
0.36	21	0.3	2.0	0.3	0.6
0.65	22	0.4	5.0	0.9	0.5
0.62	24	4.1	21.0	5.0	3.5
0.61	25	2.5	33.0	3.5	2.5
0.85	27	21.0	29.0	23.0	20.0
0.93	28	19.0	40.0	23.0	22.0

* 2.5×10^{-8} M L-thyroxine.

From each preparation 1000–2000 cells were counted.

% = percentage of benzidine-stained cells that are immature.

and the organ imprints of the *same* animal. In the cell counts shown in Table I the characterization of a cell as immature was based on two criteria (e.g., Figs. 3 *a*, 3 *b*, and 3 *c*): (*a*) nucleus showing chromatin clumping but not completely condensed as in mature erythrocytes, and (*b*) diameter of the nucleus larger than two-thirds of the smaller axis of the cell. Table I shows that in premetamorphic tadpoles the liver contains a higher proportion of immature, benzidine-positive erythroid cells than the blood, the kidneys, or the spleen. In the thyroxine-treated tadpoles there is an initial decrease followed by an increase in the number of immature, benzidine-positive cells in the liver. When the animal enters the metamorphic climax (day 24 under the conditions of the

experiment) the liver is flooded with maturing erythroid cells. This precedes by 3 days the appearance of a large number of immature cells in the peripheral blood (Fig. 4 *c*), whereas the increase of immature cells in the kidneys and spleen coincides with their increase in the blood.

The following additional morphological observation was made: mitotic figures were noticed only in the bone marrow of the adult frog and in the liver of the tadpole. They were particularly abundant during metamorphosis. Although immature erythrocytes were seen in the other organs, *clusters* of immature cells were observed only in the liver (Fig. 4 *d*).

During metamorphosis relatively mature erythropoietic cells were noted that had a large numbe
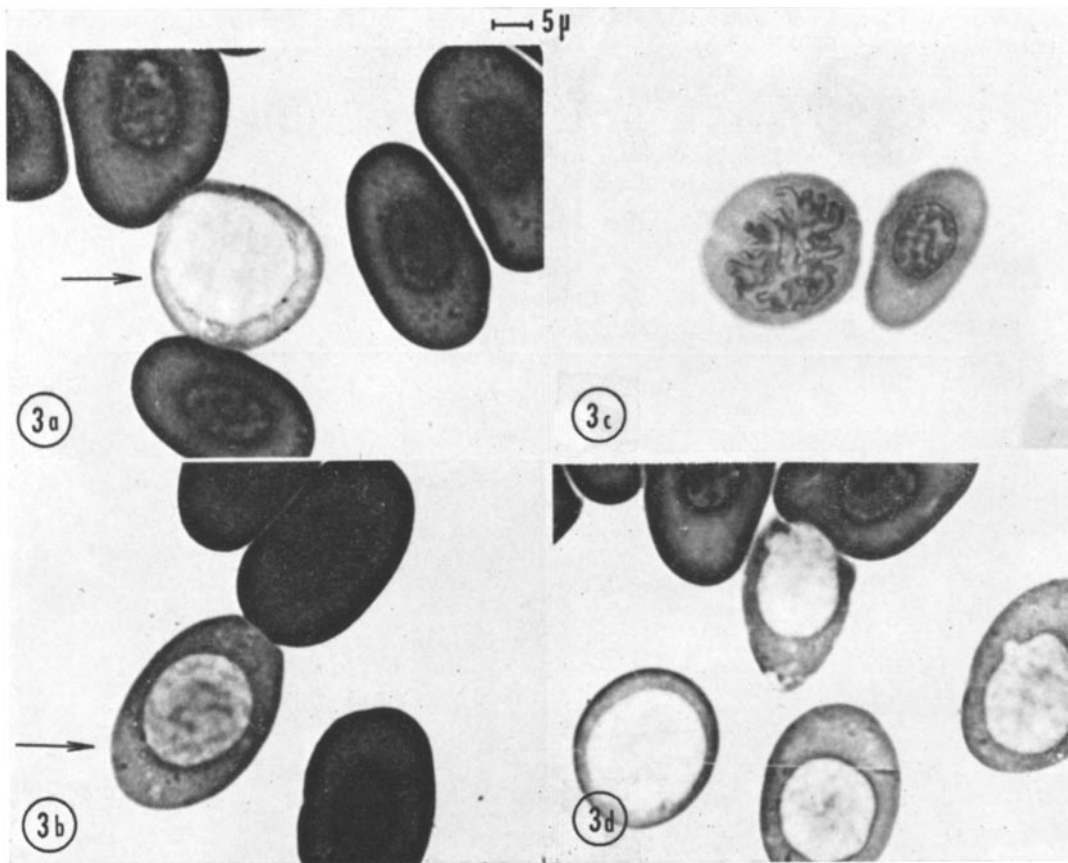

FIGURE 3 Frog erythroid cells, benzidine and Wright's stain. × 1250. Fig. 3 *a* is the imprint of bone marrow from the foreleg of an adult frog (prepared in June), showing an early polychromatic erythroblast (arrow) together with mature erythrocytes. Fig. 3 *b* is the same as Fig. 3 *a*, showing a late polychromatic erythroblast (arrow). Fig. 3 *c* is a frog bone marrow imprint, showing a hemoglobin-containing cell in metaphase. In Fig. 3 *d* a frog bone marrow imprint is shown; polychromatic erythroblasts of various degrees of maturation are represented.

of vacuoles in their cytoplasm (Fig. 4 d). In premetamorphic tadpoles, maturing erythrocytes with a few vacuoles were occasionally observed but not in the numbers appearing in the metamorphosing animals.

When the first immature erythroid cells appear in the liver there is no detectable hemopoietic activity in the bone cavity. In the adult frog the bone marrow is the only place where erythropoietic activity is visible.

DISCUSSION

The morphological observations described indicate that in both premetamorphic and metamorphosing *R. catesbeiana* tadpoles the erythroid cells reside mainly in the liver during the maturation process. By contrast, in the adult frog maturing erythroid cells were found, almost exclusively, in the bone marrow. It should be emphasized again that only cells containing hemoglobin were counted, i.e., cells beyond the basophilic erythroblast stage. Therefore, these results do not elucidate the origin of the hemoblast or stem cell; this may well originate somewhere else and be transferred to the liver where it resides during the later stages of maturation. From Table I it is apparent that during thyroxine-induced metamorphosis the percentage of immature erythroid cells in the liver falls to a minimum and then becomes more or less constant until around day
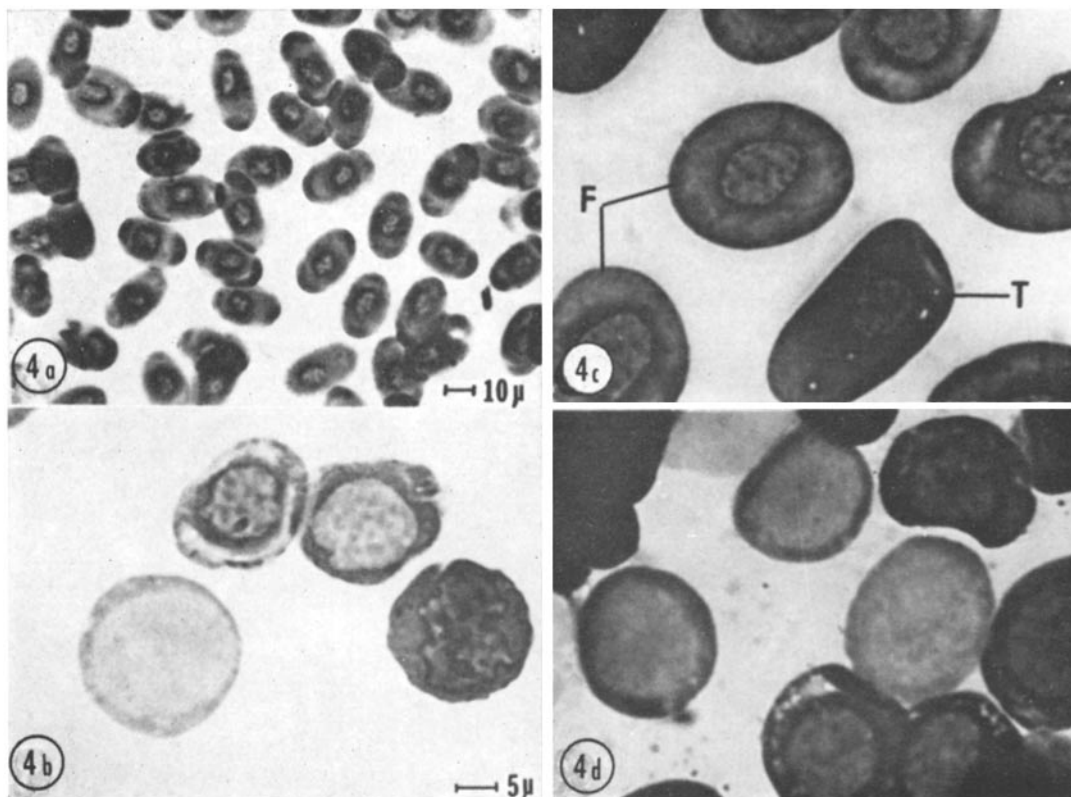

FIGURE 4 Erythroid cells from tadpoles before and during metamorphosis. In Fig. 4 a circulating red blood cells from a premetamorphic tadpole are shown. × 504. Fig. 4 b shows a liver imprint from a premetamorphic tadpole. Immature red cells at various stages of maturation are evident; one cell is in mitosis. × 1250. Fig. 4 c shows circulating red cells from a tadpole at metamorphic climax (hind leg/tail = 0.93) (thyroxine treated). There are two populations of red cells morphologically distinct; the oval, homogeneously stained cells (T) resemble those of the premetamorphic tadpole, while the round cells (F) appear during metamorphosis and presumably contain frog hemoglobin. × 1250. Fig. 4 d is a liver imprint from metamorphosing tadpole at metamorphic climax (hind leg/tail = 0.9) (thyroxine induced). Maturing erythrocytes at various stages of development appear; benzidine and Wright's stain were used. × 1250.

20 (after thyroxine treatment) when there is a sudden increase. The fact that one does not see a gradual increase in the number of immature erythroid cells, as one would expect for a proliferating population of cells, is probably due to the criteria used for the identification of cells. Since only cells with hemoglobin were counted, the sudden increase indicates that the cells are relatively synchronized in their development. In contrast to the liver, both the spleen and the kidneys do not contain maturing erythrocytes in percentages significantly higher than that in the circulating blood. In agreement with the morphological observations are the results of the immunological detection of frog hemoglobin in the metamorphosing tadpoles (14, 15). In all of the animals undergoing either natural or thyroxine-induced metamorphosis in which frog hemoglobin was detectable, the liver homogenate contained more frog hemoglobin than the circulating blood.

The above results appear, in part, to contradict the earlier observations (8–10). Jordan and Speidel (10), studying the effect of feeding tadpoles with thyroid extract, observed a "stimulation of the differentiation of erythrocytes from lymphocytes" 2 days after the first feeding. The spleen was characterized by the "presence of large numbers of young erythrocytes together with many intermediate stages between these and their lymphocyte progenitors." In later stages, however, the percentage of lymphocytes to erythrocytes in the spleen increased. The authors attributed this to the exhaustion of the spleen. The discrepancy between our results and those of Jordan and Speidel is, probably, due to the different criteria used for cell identification. They assumed that the lymphocyte is the progenitor of the red cell, and considered erythroid cells with "a large, spheroidal, lilac-colored nucleus" as senile erythrocytes (Jordan, 1919). The fact that the above authors observed stimulated erythropoiesis in the spleen 2 days after thyroxine treatment makes it doubtful that they were really looking at erythroid cells. The results of our study seem to be more in agreement with the observation made in *R. temporaria* by Maximow (16), who concluded that in later larval life the lymphocytes accumulate in the blood sinuses of the liver where they develop into erythrocytes; in postlarval stages erythropoiesis is limited to the bone marrow. The finding of Jordan that the bone

marrow of the frog is active only after hibernation may indicate that erythropoiesis is carried out only during the spring and is not a continuous process. The finding that in adult *R. pipiens* kept at 24–26°C the life span of the red cell was approximately 200 days (2) is compatible with this hypothesis. Our results do not contradict those of Hollyfield (7) since he studied only the embryological origin of the erythropoietic tissue and not the site of maturation of red cells.

Another conclusion drawn from our observations is that highly hemoglobinized cells undergo mitosis (Figs. 3 c and 4 b), contrary to some reports for mammalian erythroid cells (reviewed in reference 1) and in agreement with the observations for the newt *Triturus* (4).

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