ERYTHROPOIESIS DURING AMPHIBIAN METAMORPHOSIS

III. Immunochemical Detection of Tadpole and Frog Hemoglobins (*Rana catesbeiana*) in Single Erythrocytes

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ABSTRACT

Rabbit antibodies specific for the major tadpole and frog hemoglobin components of *R. catesbeiana* were used for the detection of the two hemoglobins inside single cells. The antisera, after fractionation by ammonium sulfate precipitation and diethylaminoethyl (DEAE)-cellulose chromatography, were conjugated with fluorescein isothiocyanate for the antifrog hemoglobin serum and tetramethylrhodamine isothiocyanate for the antitadpole hemoglobin serum. The conjugated fractions, refractionated by stepwise elution from a DEAE-cellulose column, were used for the fluorescent staining of blood smears, liver tissue imprints, and smears of liver cell suspensions. Both simultaneous and sequential staining with the two fluorescent preparations indicated that larval and adult hemoglobins were not present within the same erythrocyte during metamorphosis. In other experiments, erythroid cells from animals in metamorphosis were spread on agar containing specific antiserum. Precipitates were formed around the cells which contain the particular hemoglobin. The percentages of cells containing either tadpole or frog hemoglobin were estimated within the experimental error of the method. The data showed that the two hemoglobins are in different cells. It is concluded that the hemoglobin change observed during the metamorphosis of *R. catesbeiana* is due to the appearance of a new population of erythroid cells containing exclusively frog hemoglobin.

INTRODUCTION

An important question concerning the ontogeny and differentiation of the erythroid cell and their control is whether embryonic, fetal, and adult hemoglobins coexist within a single red cell. Betke and Kleihauer (3), using their acid-elution method, concluded that both human hemoglobins A (adult) and F (fetal) are present in a single red cell during the change-over period. More recently, Kleihauer et al. (22) suggested the coexistence within the same cell of human embryonic and fetal hemoglobins. Tomoda (38), Hosoi (19), and Dan and Hagiwara (8), using fluorescein-conjugated antibodies against human hemoglobins A and F, concluded that in normal human adults a few erythrocytes contain both hemoglobins A and F. The last authors found that 5% of human adult erythrocytes contain both hemoglobins A and F. Gitlin et al. (15), using an immunochemical technique, found that 0.5–0.9% of the normal adult erythrocytes contain small amounts of

hemoglobin F. For the human, therefore, all of the existing evidence indicates that both fetal and adult hemoglobins can be present within the same erythrocyte. Also, in metamorphosing *Xenopus laevis*, Jurd and Maclean (20) found by immunofluorescent labeling that up to 25% of the blood red cells contain both adult and tadpole hemoglobins.

This, however, may not be a general phenomenon. Fantoni et al. (12) presented indirect evidence that in the mouse the switch in the type of hemoglobins formed during embryonic development is associated with the substitution of one erythroid cell line for another. More recently Rosenberg (34) found, by electrophoresis of hemoglobin from individual cells, that circulating erythrocytes of metamorphosing *R. catesbeiana* tadpoles contain either tadpole or frog hemoglobin but not both.

The experiments reported here deal with the question of the simultaneous presence of tadpole and frog hemoglobins within the same erythrocyte during metamorphosis of the bullfrog (*R. catesbeiana*). Both tadpole and frog have multiple hemoglobins (1, 2, 13, 31, 32). The major frog and tadpole hemoglobin components, unlike the human hemoglobins, do not share a common polypeptide chain.

The basis for our study was the observation of Moss and Ingram (30) that a tadpole, when treated with thyroxine, shows an initial decrease in the rate of tadpole hemoglobin synthesis by circulating erythrocytes. This is followed by an increased rate of frog hemoglobin synthesis. Such a pattern of hemoglobin synthesis could reflect the changes in activity within individual erythrocytes or, alternatively, could indicate that the cells producing tadpole hemoglobin cease functioning and a new population of cells, producing exclusively frog hemoglobin, appears. If the first alternative is correct then there should be cells during metamorphosis that contain both tadpole and frog hemoglobins. If the second alternative is correct, the two hemoglobin types should reside in different cells.

For the detection of tadpole and frog hemoglobins, use was made of the specific antisera characterized previously (27). Two methods were employed with preparations from circulating blood and liver imprints: (*a*) immunofluorescent staining and (*b*) the microprecipitation method described in references 14 and 15.

MATERIALS AND METHODS

The preparation and properties of the rabbit antisera against the major frog and tadpole hemoglobins used in this study have been described previously (27).

Chromatography

DEAE-cellulose (Selectacel type 40, Schleicher & Schuell, Inc., Keene, N.H.) was used (0.93 meq/g) (Lot No. 1763). The cellulose was treated with 1 N NaOH and 1 N HCl alternately, and finally suspended in 0.01 M sodium phosphate buffer, pH 7.5.

Conjugation of Immunoglobulins with the Fluorescent Dyes

Fluorescein isothiocyanate (FITC), Lot No. 7091492, was purchased from Baltimore Biological Laboratories, Baltimore, Md. Tetramethylrhodamine isothiocyanate (TRITC), Isomer R, Lot No. T-2054, was obtained from Mann Research Labs. Inc., New York. The reagents were kept in an evacuated desiccator over Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) at 2°C in the dark (29). Conjugation was carried out essentially as described in references 4, 16, and 41 using 30 μg/mg of immunoglobulin for TRITC and 15–25 μg/mg for the FITC. The antisera were first fractionated in 40% ammonium sulfate saturation at 2°C. The ammonium sulfate–precipitated globulin was subsequently fractionated on DEAE-cellulose into two fractions; the first (referred to as pool A) was eluted at 0.01 M sodium phosphate, pH 7.5, and the second (pool B) at 0.05 M sodium chloride, 0.01 M sodium phosphate, pH 7.5.

In our experiments we used fluorescein isothiocyanate for labeling the fractionated antibodies against frog hemoglobin and tetramethylrhodamine isothiocyanate for the antibodies against tadpole hemoglobin. Two fractionated antisera were labeled for each category. In addition, samples of fractionated normal rabbit globulin were labeled with each dye separately and served as controls. The coupling reaction was carried out with immunoglobulin at 10 mg/ml in a 13 × 100 mm Pyrex tube (Corning Glass Works, Corning, N.Y.) at 2°C with continuous stirring with a a Teflon-coated magnetic rod. The pH was controlled with a Radiometer-Copenhagen autotitrator (London Co., Cleveland, Ohio) with a temperature compensator, and kept at pH 9.5 by the addition of 0.1 N NaOH through a thin piece of polyethylene tubing at the bottom of the tube. After 2 hr the tubes were sealed and left at 2°C for 16 additional hr. The reaction mixture was diluted 10-fold with 0.01 M sodium phosphate buffer, pH 7.5, and refractionated on DEAE-cellulose. Fig. 1 shows the elution patterns of fluorescein conjugated

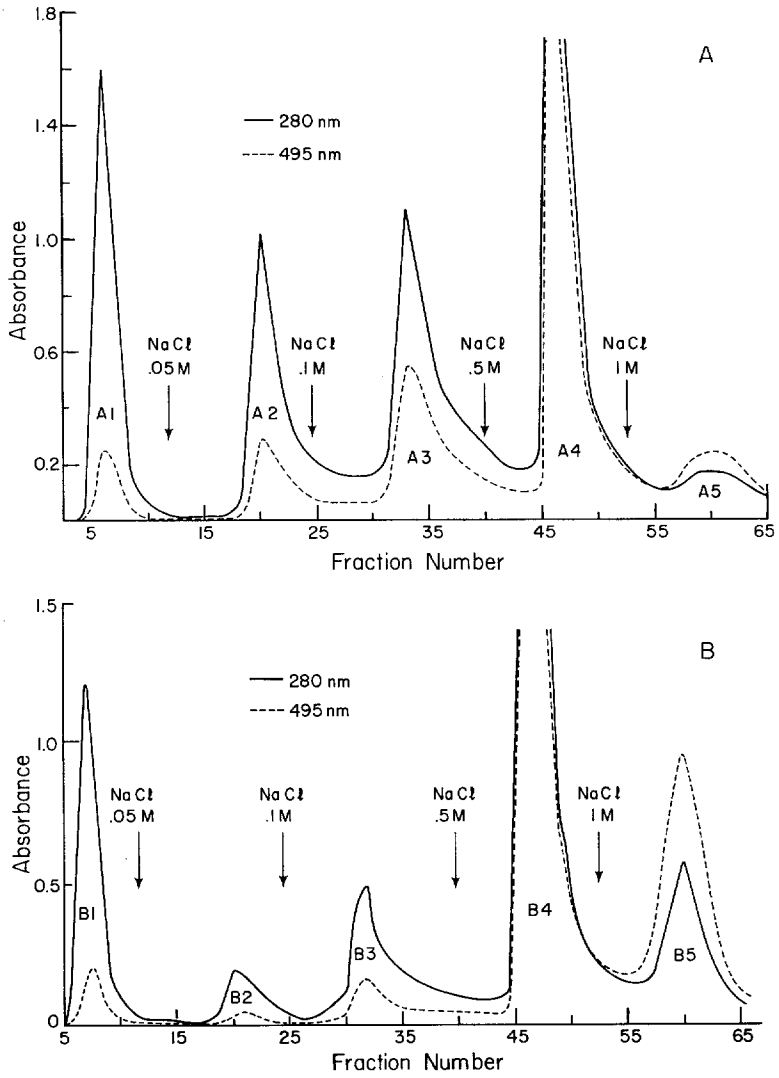

FIGURE 1 Fractionation of rabbit fluorescent immunoglobulins. Fig. 1 A shows rechromatography on DEAE-cellulose of pool A after conjugation with fluorescein isothiocyanate. Fluorescein isothiocyanate and its hydrolysis products are retained by the anion exchanger. The sample in a volume of 8 ml was applied on the column. Buffer was 0.01 M sodium phosphate, pH 7.5. Fractions of 2 ml were collected every 5 min. Fig. 1 B shows rechromatography of pool B after conjugation with FITC. Sample volume was 6 ml; flow rate was 2.6 ml/5 min.

pools A and B. The first conjugated globulin to be eluted from the column is the one with the lowest fluorochrome content. The pooled fractions from each of the first four peaks of both preparations (A1–A4 and B1–B4) had antibody activity as detected by immunodiffusion in agar. They reacted only with the homologous antigen.

The rhodamine-labeled globulin, after the completion of the reaction, was passed through a column of Sephadex G-50 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) to separate the conjugated protein from the unreacted dye and its low molecular weight derivatives, which are not retained by the DEAE-cellulose. The fractions corresponding to the conjugated protein were pooled and refractionated by DEAE-cellulose chromatography. Fig. 2 shows the patterns of stepwise elution of these preparations. The pooled fractions of the first two peaks of both preparations retained their antibody activity against tadpole hemoglobin (homologous antigen) and did not

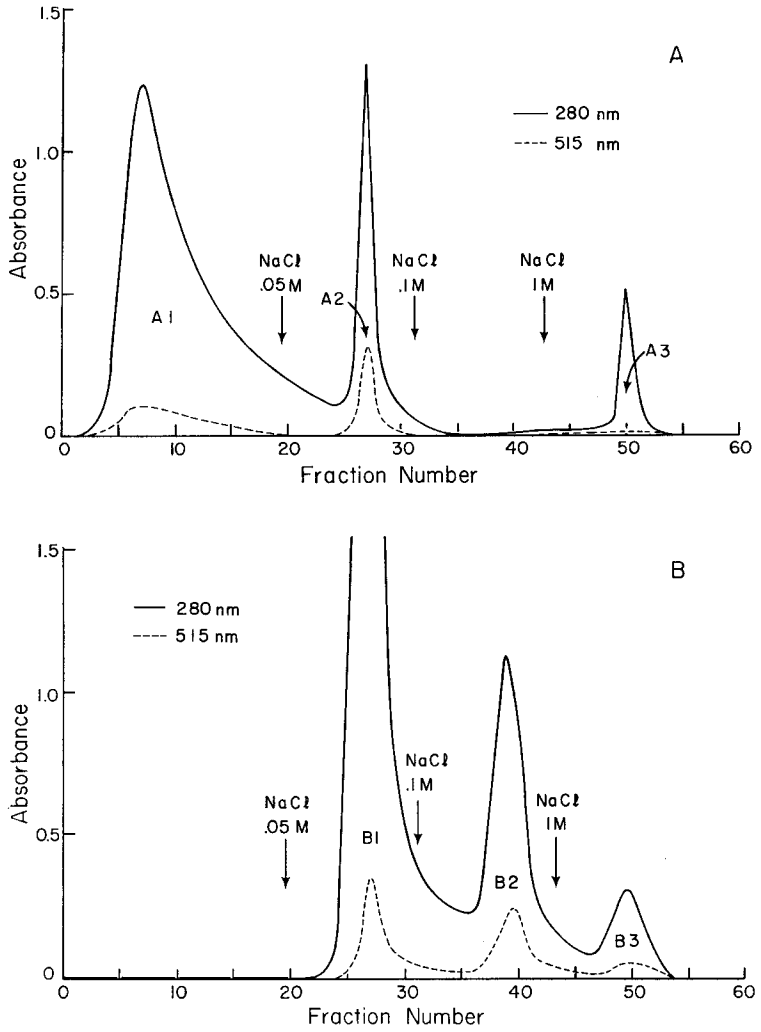

FIGURE 2 Fractionation of rabbit fluorescent immunoglobulins. Rechromatography of rhodamine-conjugated immunoglobulins after gel-filtration, on DEAE-cellulose equilibrated with 0.01 M sodium phosphate, pH 7.5, is shown. Column, 1 × 26 cm; flow rate, 2.3 ml/3.5 min Fig. 2 A, pool A; Fig. 2 B, pool B.

cross-react with frog hemoglobin. No antibody activity could be detected by immunodiffusion in the third peak (A3 and B3).

Fractions of conjugated immunoglobulin eluted at a given salt concentration were pooled, concentrated by vacuum dialysis, and filtered through a sterile 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.).

The following formulae were used to estimate the protein concentration of conjugates:

for FITC conjugates,

$$\frac{\text{OD } 280 - 0.35\ (\text{OD } 495)}{1.4} = \text{mg/ml (44)};$$

for TRITC conjucates,

$$\frac{\text{OD } 280 - 0.56\ (\text{OD } 515)}{1.4} = \text{mg/ml (4)}.$$

Tissue Sections

Pieces of tissue were fixed and embedded in paraffin according to the method of Sainte-Marie (36).

Smears of Red Blood Cells

Red blood cell smears were prepared as described in reference 26.

Tissue Cell Smears

Liver, spleen, and kidneys from exsanguinated animals were washed with phosphate-buffered sodium chloride $\mu = 0.02$ (3.212 g Na_2HPO_4, 1.24 g $NaH_2PO_4 \cdot H_2O$, and 2.432 g NaCl per liter). They were then teased apart with needles and shaken in a tube with a Vortex mixer (Scientific Industries, Inc., Queens Village, N.Y.). The large pieces of tissue were allowed to sediment and the supernatant was centrifuged in a clinical centrifuge. The sedimented cells were washed three more times with the above buffer, redispersed in 5% bovine serum albumin in this buffer, and smeared.

Tissue-Touch Imprints

Tissue-touch imprints were prepared as described in reference 26.

Staining of Sections and Smears with Fluorescent Antibodies

The slides to be stained with fluorescent antibodies were, after fixation in methanol, immersed in a solution of 3% Tween 80 (Fisher Scientific Company, Pittsburgh, Pa.) in the phosphate buffer described above, left in the mixture for 16 hr at room temperature, and then washed for 1 hr with frequent changes of the buffer. About 50 μl of the fluorescent globulin solution were placed in a small area of the smear. The slide was incubated in a humid chamber either at room temperature or at 37°C for 30 min, rinsed with buffer, and immersed in a large volume of buffer on a gyrating table for 1 hr. The buffer was changed twice during that period. The slides were drained and the imprints were mounted for examination in buffered glycerin (FA mounting fluid, Difco Laboratories, Detroit, Mich.) under coverslips.

Sequential Staining with Different Fluorescent Antibodies

The technique described in reference 17 was followed. The slide was first stained for frog hemoglobin–containing cells. The particular field was identified by vernier reading and photographed. The same slide was then treated with antitadpole-Hb antibodies. The same field was located and examined for the presence of yellow cells or a shift in the color of cells previously stained.

Microscopy

A Zeiss (Carl Zeiss, Inc., Oberkochen, W. Germany) standard GFL microscope equipped with an Osram HBO 200 high-pressure mercury lamp was used. Both a bright- and a dark-field ultracondenser were used. The exciting light was filtered through a heat-absorbing filter (KG 1/3.2 mm) and then through filters BG-38 and BG-12 (3.2 mm) or UG-1 (3.2 mm). The following were used as barrier filters to distinguish green, red, and mixed color cells: Kodak Wratten No. K2 (yellow) for general observation, Kodak Wratten No. 23A (red), Kodak Wratten No. 57A (green). The immersion oil used was type A (low viscosity) ($^nD^{25°} = 1.515$) (R. P. Cargille Laboratories, Inc., Cedar Grove, N. J.), which has a very low fluorescence.

Controls for the Specificity of the Immunofluorescent Staining

In order to identify the fluorescence observed when cells were treated with fluorescent antibodies as an immunologic reaction, the following controls were used: (*a*) Conjugated antibodies were absorbed with the homologous antigen; this treatment abolishes the fluorescence of treated cells. (*b*) Absorption of the heterologous antigen; this did not affect the staining of cells. (*c*) Treatment of the slides with unlabeled homologous antiserum followed by staining with the fluorescent antibodies greatly diminishes the intensity of the fluorescence of treated cells. (*d*) Identical treatment with unlabeled heterologous antiserum did not affect the observed fluorescence. (*e*) No staining was observed when labeled globulin from a nonimmunized rabbit was used (the fraction on DEAE-cellulose corresponding to the one used for the specific staining).

Sensitivity of Double Staining

One of the drawbacks of the fluorescent staining method is the lack of an absolute measure of the sensitivity of the method, i.e., what is the lowest percentage of the minor antigen that the method can detect? In order to get a rough and indirect estimate of the sensitivity, frog and tadpole hemoglobins were mixed in various proportions to a final total concentration of 2 mg/ml. A drop of the mixture was placed on a microscope slide, dried, fixed with methanol, and then stained sequentially with fluorescent antibodies to frog and tadpole hemoglobins. The slide was then observed under the microscope for fluorescence. It was thus determined that frog hemoglobin was detectable in the presence of a 400-fold excess of tadpole hemoglobin when the mixture was stained with only antifrog-Hb antibodies. When the slides were stained simultaneously with both conjugated antibodies, frog hemoglobin was detectable in the presence of 200-fold excess of tadpole hemoglobin.

Quantitation of Hemoglobin in Single Erythrocytes by Microprecipitation in Antibody-Agar Plates

The technique used was essentially the one described in references 14 and 15, with slight modifications. The antiserum-agar mixture contained (per 100 ml) 1.5 g agar (Purified, Difco Laboratories, Detroit, Mich.) in 0.1 M borate buffer, pH 8.6, 0.05 M NaCl, and amounts of antibodies sufficient to give distinct precipitates (previously determined). The final dilution of the antiserum in the agar was 1/8 for the antiserum against frog hemoglobin and 1/4 for the antiserum against tadpole hemoglobin. The size of each gel on a microscope slide was 25 mm × 15 mm. On each gel, 1–3 drops of a washed erythrocyte suspension in amphibian Ringer's (concn 1 × 10^6 cells per ml) were applied. To facilitate recognition of the microscopic field after the lysis of the cells and incubation, particles of styrene divinylbenzene copolymer latex (Dow Chemical Co., Bioproducts Department, Midland, Mich.) were added to the antibody-agar mixture before gelation. 3 μl of a 10% suspension (particle diameter 25–55 μm) were added per milliliter of antibody-agar. After the addition of the erythrocytes to the surface of the gels the cells were photographed at 80 × with a phase objective and the position of each field was registered by vernier readings. Because of the thickness of the wet preparations, it was, usually, necessary to take more than one picture per field, focusing at different depths in order to record all of the latex particles present in a particular field. The microscope slides were subsequently placed in a toluene atmosphere for 30 min to lyse the cells and then incubated for 7 days in a humidity chamber at 21°C.

The recognition of the microscopic fields after incubation was based on both vernier readings and the presence of latex particles in the field (the particles were not affected by the toluene treatment) (Fig. 5).

The diameter of the precipitates was measured on the projected photographic negatives. As a reference, we used a picture of a hemocytometer projected under conditions identical to those for the precipitates. The conversion of the diameter of the microprecipitates to absolute amounts of specific hemoglobins per cell was done according to the method described in reference 14, with some corrections. It was determined that the diameters of precipitates formed around 2-mm wells in the antibody-agar plates, prepared and treated the same way as those used with the erythrocyte suspension, gave a straight line when plotted *versus* the logarithm of the concentration of hemoglobin solution placed in the well (11). For tadpole hemoglobin the highest concentration in the tadpole red cell lysate tested was 34 mg/ml. For frog hemoglobin this concentration was 110 mg/ml.

Microphotography

For photography of fluorescent preparations, pictures were taken either with a Polaroid camera (black and white) or with a Zeiss camera (color). A polaroid type 47 film (ASA 3000) was used for black and white pictures; Anscochrome 500 (daylight type), GAF Corp., New York, was used for color slides.

The cells plated on agar and the microprecipitates formed in the agar were photographed with Tri-X Pan (400 ASA) Kodak film. For the microprecipitates a dark-field condenser and an electronic flashlight were used.

The microscope was mounted on a vibration-absorbing base, (Leitz, Wetzla-Germany).

OBSERVATIONS

Cellular Localization of Hemoglobins by Immunofluorescence

In order to minimize the main drawback of the immunofluorescent techniques, i.e. the nonspecific staining, the antibodies had been fractionated before and after conjugation with the dyes. The fluorescent antibodies were used mainly for the examination of the immature erythrocytes in liver imprints and liver cell suspensions, since it was shown (26, 27) that the immature cells and frog hemoglobin are detectable first in the liver and later, simultaneously, in the blood, spleen, and kidney. The rationale for concentrating on the liver preparations was as follows. We assumed that if the erythroid cell were able to switch on frog hemoglobin production, while tadpole hemoglobin synthesis was in progress, this would occur not in the mature erythrocyte but at an earlier maturation stage while the nucleus was still active. The large amounts of frog hemoglobin already present in the circulating mature red cell could interfere with the detection of tadpole hemoglobin.

TISSUE SECTIONS: No systematic studies were done with frozen sections because of the danger of diffusion of a soluble, unfixed antigen during the preparation which could result in ambiguous double staining. Kagen and Gurevich (21) experienced such difficulties in trying to localize myoglobin in muscle. Sections of ethanol-fixed, paraffin-embedded tissues were used instead. Sections of kidney, liver, spleen, and bone marrow from premetamorphic and metamorphosing tadpoles, as well as from adult frogs, were examined. Premetamorphic tadpoles' and adult frogs' sections stained only with the homologous

fluorescent antibodies in both sequential and simultaneous staining with the two fluorescent preparations. The staining of sections from animals undergoing metamorphosis (natural and thyroxine induced) gave the following results (animals at metamorphic climax): (*a*) Spleen: The sections were mainly stained with the antitadpole-Hb preparation (orange). There were, however, several cells which stained with the antifrog-Hb (green) preparation; only the cytoplasm was stained, the nuclei were not. These cells were individually spaced and not in clusters. No doubly stained cells could be seen. (*b*) Liver: The sections gave good staining with both antifrog and antitadpole preparations (green and orange). No yellow areas (doubly stained) were observed, however. Most of the orange cells (stained with antibodies against tadpole hemoglobin) were seen inside the blood vessels while the green cells were outside. There were clusters of cells stained with the antifrog-Hb antibodies. (*c*) Kidney: A few green cells were seen; otherwise the sections stained with the antitadpole preparation. (*d*) Bone marrow: The majority of the stained cells in these sections were stained green with the antifrog-Hb antibodies. No double-stained cells were found.

TISSUE IMPRINTS OR SMEARS OF CELL SUSPENSIONS: The search for doubly stained cells was mainly done in imprints and smears of blood and of various organs, particularly of the liver of metamorphosing animals, for the reasons mentioned before. It was considered necessary to stain the cells on slides after fixation and not in suspension, to avoid contamination of the cells in vitro with the second antigen which could result in double staining. Smears of peripheral blood were used first to test the method. Tadpole red cells were stained faintly with the rhodamine-conjugated antitadpole-Hb antibodies. Frog red cells, however, did not stain with any of the preparations. It was thought that the reason for the failure of fluorescent staining of frog red cells was probably that the hemoglobin was not exposed sufficiently to the antibodies; that is, the membrane of red cells was not disrupted enough by air drying and methanol treatment. Only after pretreatment of the fixed smears with Tween 80 was positive fluorescent staining obtained (25). Therefore, all preparations were treated with Tween 80 as described in Materials and Methods. This treatment does not increase autofluorescence.

Frog red cells stained mainly over the nuclear region (Fig. 3, top). One explanation for the peculiar pattern of staining is that light absorption by the relatively large amounts of heme present in the cell interferes with the fluorescence of fluorescein. This chromophore absorbs at a wavelength of 490 nm and fluoresces at 520 nm (33); this is near the region of maximum absorption for heme compounds. The nuclear region of the erythrocyte, because of the shape of the cell (protruding nucleus), has less hemoglobin underlying it than the cytoplasmic region. In favor of this explanation are the following observations: (*a*) In thin sections (4 μm) of frog tissues the cells stained with fluorescein-conjugated antifrog-Hb antibodies fluoresce in the cytoplasmic but not in the nuclear area. (*b*) Morphologically immature cells from imprints of adult frog bone marrow and of livers of metamorphosing tadpoles are stained mainly in the cytoplasm. Cells in more advanced maturation stages stain in the nuclear region as do the mature erythrocytes (Fig. 4). (*c*) Smears of frog erythrocytes fixed in cold acid-acetone did not stain over the nucleus but in the cytoplasm. Treatment with acid-acetone is known to dissociate the heme from the hemoglobin (35) (Fig. 3, bottom). This treatment damages the erythrocytes severely; therefore, it could not be used routinely for the purpose of this study. (*d*) Solutions of frog hemoglobin and of the globin derived from it were prepared in concentrations of 10, 2, 1, and 0.5 mg/ml. 1 drop from each was placed on a microscope slide, dried, fixed in methanol, and stained with the fluorescent antifrog-Hb antibodies. The intensity of the fluorescence of the hemoglobin solutions decreased as the concentrations increased, while the intensity of the fluorescence of the globin solutions paralleled the concentration.

STAINING OF IMPRINTS FROM PREMETAMORPHIC TADPOLES: In all preparations examined (blood, liver, kidney, spleen) the cells retained only the rhodamine-conjugated antitadpole-Hb antibodies. The immature erythroid cells present in the liver preparations stained in the cytoplasm with rhodamine very well. No cells which stained green (for frog hemoglobin) could be seen, nor were there cells whose orange fluorescence changed after the application of the green antifrog-Hb antibodies. The only exception was the bright green fluorescence of the granules of the cells similar to the eosinophils. Nonspecific staining of these granules by fluorescein-conjugated antibodies has been reported repeatedly (17, 28). From each preparation 200–300 in-

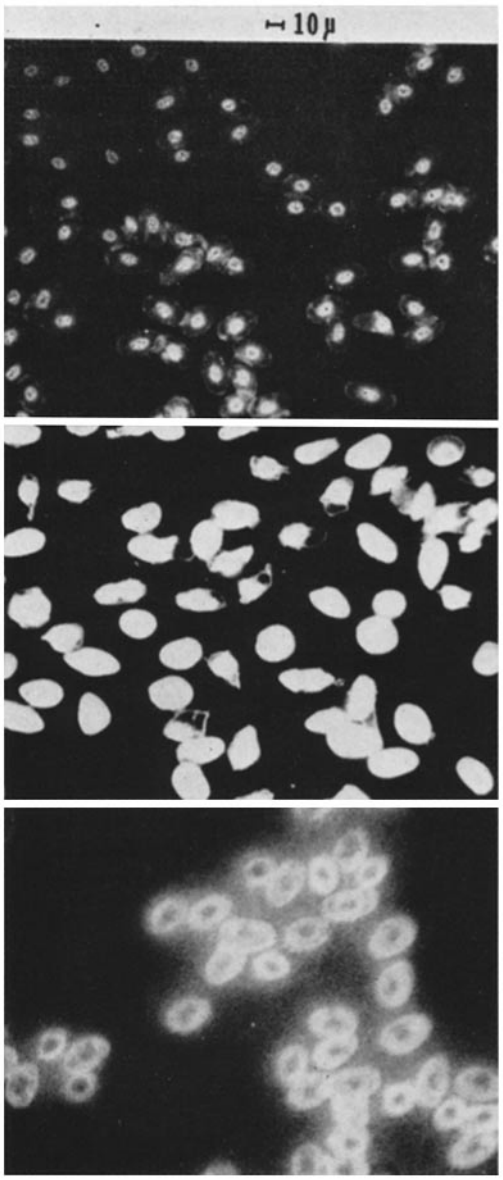

FIGURE 3 Staining of erythroid cells with fluorescent antibodies. Top, adult frog red blood cells, treated with Tween 80 and stained with antifrog-Hb fluorescent antibodies (fraction A2, see Fig. 1). × 200. Middle, premetamorphic tadpole red blood cells, treated with Tween 80 and stained with rhodamine-labeled antibodies (fraction B1, see Fig. 1). × 200. Bottom, frog red blood cells, fixed in cold acid-acetone and stained with fraction A2 of antifrog-Hb antibody. × 200.

dividual cells were examined with both sequential staining and the use of filters (see Table I).

IMPRINTS FROM ADULT FROGS: Imprints of liver, kidney, spleen, and bone marrow, as well as blood smears, were examined. As with the premetamorphic tadpoles, no doubly stained cells were found. Morphologically immature cells which stained with green fluorescent antibodies (antifrog-Hb) were found in abundance in the bone marrow. These cells stained primarily in the cytoplasmic region, as mentioned above.

PREPARATIONS FROM TADPOLES UNDERGOING METAMORPHOSIS: Animals undergoing both natural and thyroxine-induced metamorphosis were studied. Although imprints from spleen, liver, kidney, and bone marrow were examined, particular attention was paid to the imprints or smears of cell suspensions from the liver. More specifically the maturing cells were carefully searched for double staining. In the sequential staining the fluorescein-labeled antibody to frog Hb was applied first, because it is easier to detect the green fluorescence in the presence of orange fluorescence (5). Artificial mixtures of premetamorphic tadpole and adult red blood cells in known proportions were smeared and stained with both fluorescent preparations. The proportion of cells of each type determined by immunofluorescence was as expected. From each preparation two imprints or smears were examined and at least 300 individual cells were looked at. Table I shows cell counts from a series of naturally metamorphosing tadpoles. Only the percentage of the immature erythroid cells relative to all erythroid cells is given. In both series of experiments, during natural and thyroxine-induced metamorphosis, no doubly stained cells were found. It was striking that when the "green" (stained for frog hemoglobin) immature cells first appeared there were no "orange" cells (stained for tadpole hemoglobin) of comparable maturation (Fig. 4). All of the "orange" cells were of considerably more advanced maturation stages. In total, more than 10,000 individual cells from the liver of metamorphosing tadpoles were examined.

Microprecipitation in Antibody-Agar Plates

For each experiment three types of agar plates were usually employed. The first plate contained a mixture of both antisera at the appropriate dilution (one against frog hemoglobin and one against tadpole hemoglobin); the second plate contained antibodies to tadpole hemoglobin only; and the third contained antibodies specific for frog

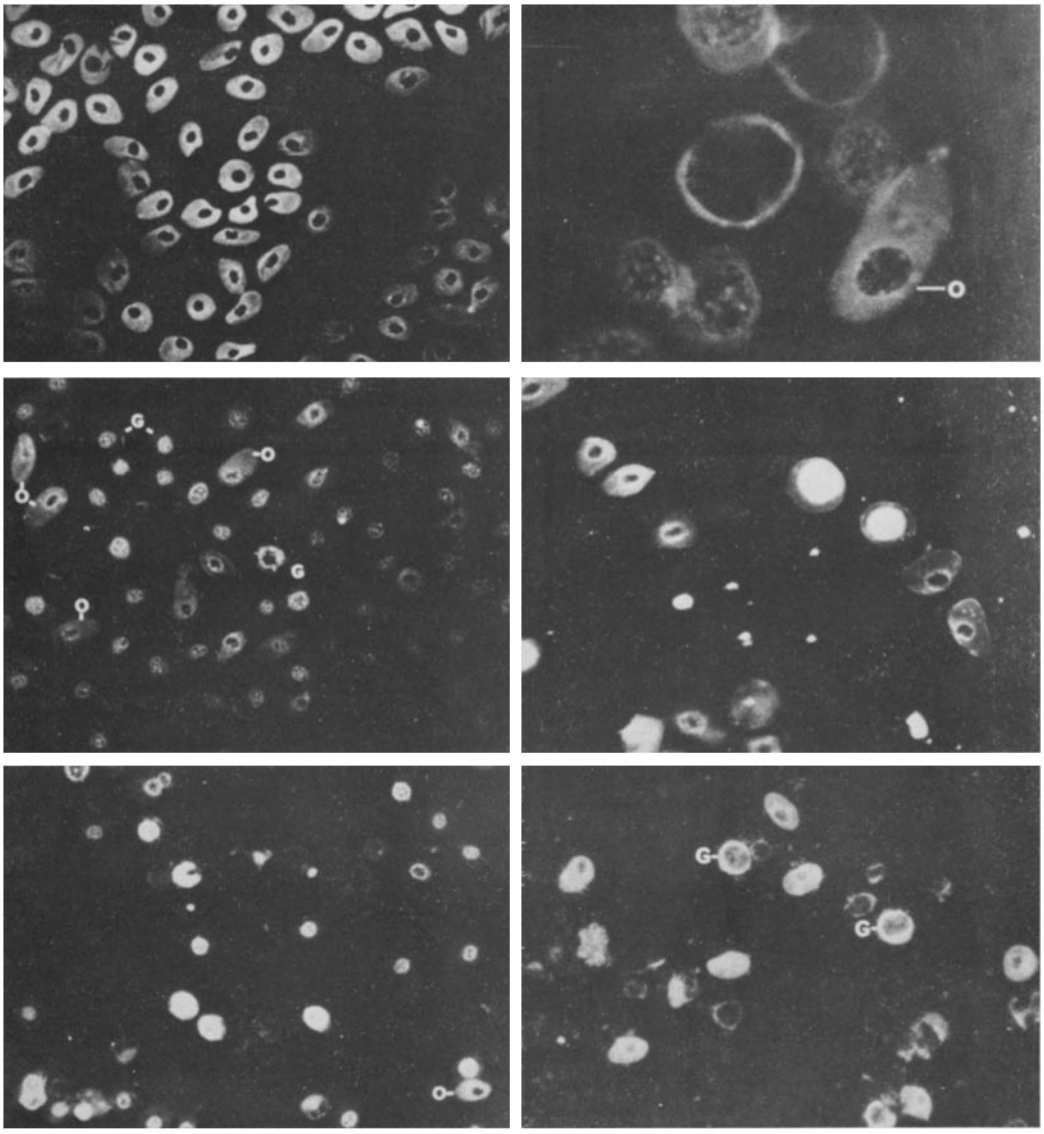

FIGURE 4 Top left, tadpole red blood cells, Tween treated, stained with rhodamine-conjugated anti-tadpole-Hb antibodies. × 200. Center left, red cells from the circulating blood of a naturally metamorphosing tadpole (hind leg/tail = 6.7), stained with both rhodamine- and fluorescein-conjugated antibodies. Two populations of cells are shown; those stained mainly in the cytoplasm were orange (*o*) while those stained in the nucleus were green (*G*) and morphologically less mature. × 200. Bottom left, liver cell suspension from the same animal as above, doubly stained. There is only one red blood cell stained orange (*o*); all the others stained green. × 200. Top right, liver cell suspension from a naturally metamorphosing tadpole (hind leg/tail = 0.80), Tween treated and doubly stained. All cells, except the one marked (*o*), were stained green. Note that the more immature cells are stained mainly in the cytoplasm. × 800. Center right, liver cell suspension from thyroxine-induced tadpole (5×10^{-8} M L-thyroxine for 22 days, hind leg/tail = 0.62). Doubly stained; the two bright cells are green. × 312. Bottom right, liver cell suspension from a naturally metamorphosed tadpole (hind leg/tail = 0.6); doubly stained. All cells are stained orange except for the two marked (*G*) which were green. × 200.

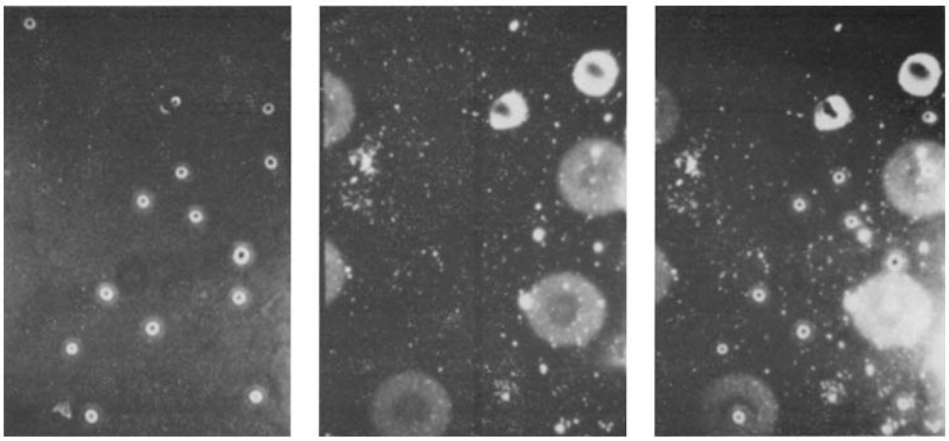

FIGURE 5 Left, red cells on agar gel before lysis. Center, the same field as left, but after lysis of the red cells and incubation for 7 days. The red cells come from a thyroxine-treated tadpole. The agar contains antiserum to frog hemoglobin. Some red cells have reacted with the antiserum, and microprecipitates have formed around them. The sharp circles are latex particles added in the agar to facilitate recognition of the microscopic field after lysis of the cells. × 80. Right, left and center superimposed.

TABLE I

Natural Metamorphosis

	% Immature "green cells" (antifrog-Hb antibodies)		% Immature "orange cells" (antitadpole-Hb antibodies)	
Hind leg/tail	Liver	Blood	Liver	Blood
0.04	0	0	6	1
0.01	0	0	3	0.4
0.32	0	0	1.5	0.5
0.60	2	0	0.5	0
0.63	5	0.5	0	0
0.74	40	6	0	0
0.76	++	++	0	0
0.80	50*	10*	0	0
6.7	80*	70*	0	0

* The number includes mature cells also.

hemoglobin. The first plate gives the percentages of cells containing tadpole or frog hemoglobin, respectively. If some erythrocytes contained both tadpole and frog hemoglobins they would be capable of forming precipitates in either plate. In that case the sum of the percentages of cells reacting with either antifrog or antitadpole-hemoglobin antibodies should be larger than the percentage of cells forming precipitates in the plate containing the mixture of the two antisera. If, on the other hand, tadpole and frog hemoglobins are in different cells, the sum of the two percentages should be equal to the percentage of cells reacting with the mixture of the antisera. Table II shows that blood cells from adult frogs reacted only with the agar containing the antifrog-Hb serum. Similarly, blood cells from tadpoles with hind legs shorter than 5 mm formed precipitates only in agar containing the antitadpole-hemoglobin antibodies.

In order to test the accuracy of the method, artificial mixtures of tadpole and frog blood cells were tested. Each blood preparation was tested separately, before mixing, to ascertain that it contained cells reacting only with the corresponding antiserum. Table II shows the results of this test. Although the starting population of cells reacted only with one antiserum, the percentages of cells reacting with antifrog- or antitadpole-Hb serum do not add up exactly to the percentage of cells reacting with the mixture of the two antisera. In one case (mixture 3) the difference is considerable. This can be attributed to the fact that some cells lyse quickly after plating (before being photographed), thus introducing an error. The magnitude of this error probably varies depending on such factors as the time interval between plating and photography, humidity of the air, etc. Another possible source of error is the fact that the optical background of the agar plates containing the mixture of the two antisera is high, making the detection of small precipitates difficult. Thirdly, occasionally two cells adhere to each other and photograph as single dots.

The smallest precipitate observed in this study had a diameter of 50 μm. This corresponds to an

TABLE II

Sample		A % of cells reacting with a mixture of antibodies to frog and tadpole Hbs	B % of cells reacting with antibodies to tadpole Hb	C % of cells reacting with antibodies to frog Hb	Overlap (B + C − A)
Frog blood	1		0 (451)*	99.1 (110)	
Frog blood	2		0 (542)	100.0 (370)	
Premetamorphic tadpole					
I Blood			99.3 (269)	0 (305)	
II Liver			73.3 (389)	0 (419)	
Artificial mixtures of adult frog and premetamorphic tadpole blood cells	1	98.5 (270)	36.9 (344)	63.1 (282)	+1.5 (SD 3.95)‡
	2	98.1 (312)	48.1 (524)	48.8 (482)	−1.2 (SD 3.25)
	3	98.3 (466)	86.0 (228)	20.7 (590)	+8.4 (SD 2.90)
	4	98.5 (329)	70.6 (574)	28.5 (583)	+0.6 (SD 2.75)

* The numbers in parentheses indicate the number of cells on which the percentage is based.
‡ SD = standard deviation.

estimated amount of 1.2×10^{-12} g/cell for frog hemoglobin and 10×10^{-12} g/cell for tadpole hemoglobin (see Materials and Methods). These amounts then represent the lower levels of sensitivity of the method.

Table III gives the results obtained with metamorphosing (thyroxine induced) tadpoles. The comparison of the percentages of cells containing frog hemoglobin in the circulating blood and in the liver cell suspension of the same animal (animals No. 1, 2, 3, 4, and 5) shows that the liver preparations contained always a higher proportion of erythroid cells with frog hemoglobin. This is in agreement with the conclusion drawn on the basis of morphological and independent immunological evidence that during metamorphosis the erythrocytes mature in the liver (26, 27). The mean frog hemoglobin content of erythrocytes of thyroxine-treated animals is 28.5 pg for the circulating cells and 9.4 pg for liver cells (Table III; Fig. 6). For adult frog erythrocytes this mean is 235 pg/cell. The percentages of cells, which the data indicate should contain both frog and tadpole hemoglobins, range from −3.2 to + 6.4. Except for the 6.4 value, the others fall, apparently, within the experimental error of the method and are probably not significant. The 6.4 value is difficult to assess in view of the results obtained with one of the artificial control mixtures (Table II, 3). It should be noted that in the animals for which the data are not complete (Table III, No. 1–5), the added percentages of cells reacting with either antifrog-Hb or antitadpole-Hb serum do not exceed 100. Animals No. 11 and 12 were two tadpoles in a survey of 30 untreated tadpoles, which contained immunologically detectable frog hemoglobin. Their blood cells were tested on agar-antibody plates, and they were subsequenly treated with thyroxine and tested again. Tadpole No. 11 was estimated to have, before thyroxine treatment, 3.7% frog hemoglobin-containing cells and tadpole No. 12, 15.5%. The percentage of frog hemoglobin in their hemolysate was estimated by the method of Fahey and McKelvey (11) to be 1.1 and 12.5%, respectively.

Figs. 6 and 7 show the frequency distributions for hemoglobin content of erythroid cells of various origins. It should be emphasized that the parameter measured for each cell was the diameter of the precipitate formed around each cell. These diameters were then converted into absolute amounts of hemoglobin as described in Materials and Methods. This conversion assumes that the diameter of each precipitate is proportional to the logarithm of the amount of hemoglobin in the cell. Although the assumption was tested and found valid for various concentrations of hemoglobin solutions (see Materials and Methods), this does not mean necessarily that it is also valid for the case of lysed cells. Therefore, the estimate of the absolute amounts of Hb per cell may not be accurate. The comparison, however, of the different populations of cells presented in Figs. 6 and 7 and Table IV is still valid.

The difference in tadpole hemoglobin content of circulating erythrocytes from premetamorphic

TABLE III

Animal No.	Source of sample	Days in thyroxine*	Tail/body	Hind leg/body	A % Cells reacting with mixtures of Abs to frog and tadpole Hbs	B % Cells reacting with antibodies to tadpole Hb	C % Cells reacting with antibodies to frog Hb	Overlap (B + C − A)
1	Blood	22	1.27	0.52	—	91.2 (68)‡	4.3 (94)	
	Liver	22	1.27	0.52	—	36.3 (80)	36.2 (174)	
2	Blood	23	—	—	—	82.0 (128)	8.6 (187)	
	Liver	23	—	—	—	51.1 (133)	14.9 (114)	
3	Blood	23	—	—	—	90.1 (142)	9.0 (166)	
	Liver	23	—	—	—	52.9 (198)	15.3 (268)	
4	Blood	24	1.1	0.73	—	91.8 (73)	0 (301)	
	Liver	24	1.1	0.73	—	88.1 (126)	2.3 (132)	
5	Blood	26	0.96	0.75	—	98.0 (102)	0 (276)	
	Liver	26	0.96	0.75	—	76.6 (94)	4.8 (124)	
6	Liver	24	—	—	55.7 (298)	34.6 (136)	21.7 (198)	+0.6 (SD 5.79)§
7	Blood	25	0.84	0.93	95.7 (346)	86.3 (344)	15.8 (514)	+6.4 (SD 2.79)
8	Blood	26	1.50	1.13	96.0 (448)	85.4 (520)	11.4 (554)	+0.8 (SD 2.25)
9	Blood	27	1.46	1.17	97.3 (586)	88.6 (385)	12.6 (688)	+3.9 (SD 2.16)
10	Blood	28	1.19	0.78	96.3 (270)	89.9 (335)	3.2 (621)	−3.2 (SD 2.02)
11	Blood	0	2.12	0.47	97.5 (236)	94.5 (435)	3.7 (591)	+0.7 (SD 1.68)
	Blood	27	0.92	0.94	96.5 (346)	81.8 (374)	15.3 (620)	+0.6 (SD 2.65)
12	Blood	0	2.19	0.50	—	86.1 (397)	15.5 (679)	
	Blood	27	1.9	1.17	95.0 (400)	73.1 (386)	24.1 (635)	+2.2 (SD 3.02)

* 2.5×10^{-8}M L-thyroxine.
‡ Numbers in parentheses indicate the number of cells on which percentage is based.
§ SD = standard deviation.

tadpoles and thyroxine-treated tadpoles (Fig. 7; Table IV) is surprising. If this difference is real, it can be explained in two ways. One explanation is that the blood of premetamorphic tadpoles has many red cells still synthesizing hemoglobin, while in the thyroxine-treated animals all of the circulating tadpole red cells have reached maturity. The finding, however, that in premetamorphic tadpoles less than 5% of the circulating red blood cells incorporated labeled amino acids (31) seems to rule this out. An alternative explanation could be that thyroxine stimulates the immature noncirculating erythroid cells to synthesize more tadpole hemoglobin than normal and that at the time of the measurements these cells were in circulation, causing a shift of the distribution curve towards higher hemoglobin content.

DISCUSSION

The above observations indicate that during metamorphosis tadpole and frog hemoglobins, at least their major components, do not coexist within a single erythrocyte in detectable amounts.

The microprecipitation method can detect 0.5% of the average amount of frog hemoglobin per adult erythrocyte. Our results are in agreement with those of Rosenberg (34) which were obtained with a considerably less sensitive method. The concentrations of the labeled immunoglobulins used for the immunofluorescent staining were

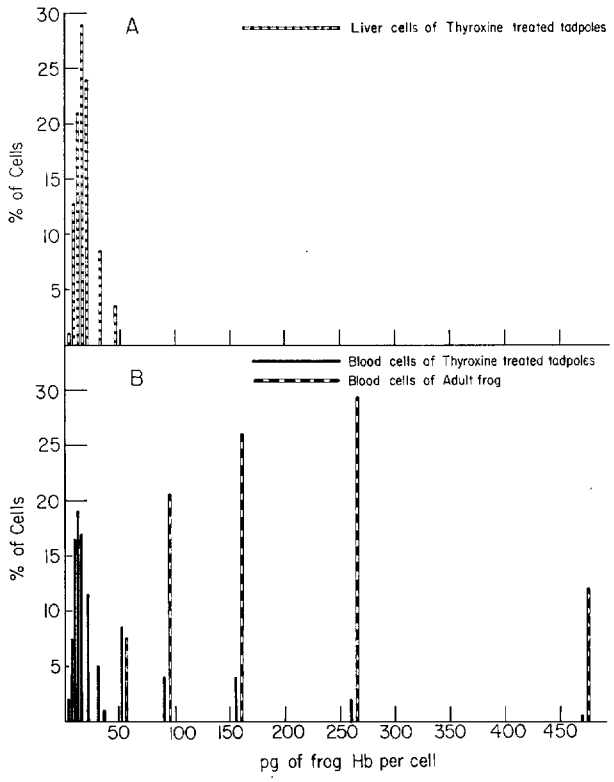

FIGURE 6 Histogram showing the frequency distribution of erythroid cells according to the amount of frog hemoglobin per cell.

TABLE IV

Estimated Amount of Hemoglobin per Cell (10^{-12} g)

	Frog hemoglobin			Tadpole hemoglobin		
	Mean	No. cells counted	Standard deviation	Mean	No. of cells counted	Standard deviation
Adult frog red blood cells	235.4	350	220			
Red blood cells of thyroxine-treated tadpoles	28.5	593	49.3			
Liver cells of thyroxine-treated tadpoles	8.5	211	6.3			
Red blood cells of premetamorphic tadpoles				97.5	217	49.5
Red blood cells of thyroxine-treated tadpoles				189.3	1323	96.0

those which gave no staining with the heterologous erythrocytes, i.e. with red cells of premetamorphic tadpoles for the antifrog-Hb antibodies and with frog erythrocytes for the antitadpole-Hb antibodies, since heterologous hemoglobins were not detected in vivo by any of the methods used (electrophoresis, precipitation, immunodiffusion, C fixation).

The different morphologic characteristics of the new red cells appearing during both natural and thyroxine-induced metamorphosis have been described (10, 18). Also, in the developing chick

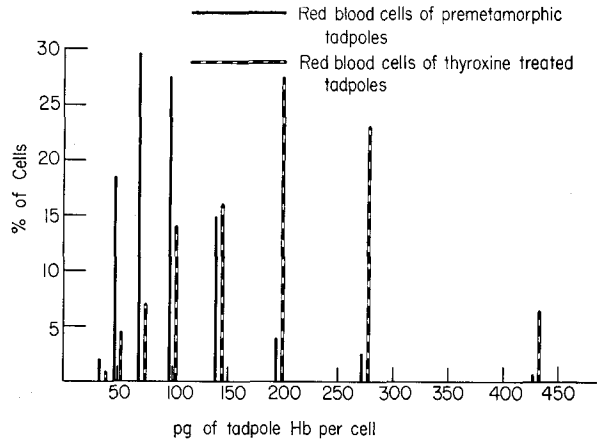

FIGURE 7 Histogram showing the frequency distribution of erythroid cells according to the amount of tadpole hemoglobin per cell.

embryo the primitive erythrocyte line begins to disappear by the fifth or sixth day of incubation and the morphologically distinct definitive cell line begins to predominate (7, 24). This change coincides grossly with changes in the types of hemoglobin (40), but proof that the new cell line produces adult hemoglobins exclusively is lacking. In the fetal mouse, Craig and Russel (6) and Kovach et al. (23) described two populations of erythroid cells. The early erythrocytes are nucleated and presumably are derived from the yolk sac blood islands, whereas the cells appearing in later stages are nonnucleated and are produced in the liver. The populations are associated with different hemoglobins. For human fetal and adult hemoglobins, however, the evidence is all in favor of the simultaneous presence of both in a single cell.

What, then, is the significance of the observation that during metamorphosis the erythroid cells carry either frog or tadpole hemoglobin? First, it indicates that regarding the *R. catesbeiana* erythroid cell the two differentiated states are mutually exclusive. On the reasonable assumption that no selective destruction of tadpole hemoglobin in the cell takes place and that basophilic erythroblasts divide, it also indicates that cell division is associated with the initiation of the new specialized cellular function, i.e., the production of frog hemoglobin. It does not help, however, to decide whether or not frog hemoglobin is produced by erythrocytes derived from a cellular clone different from the one which produces erythrocytes with tadpole hemoglobin. Our observation is compatible with two hypotheses: either that thyroxine directly or indirectly causes proliferation of a *new* cell line, or that it affects the same undifferentiated stem cell through some unspecified mechanism. If the clonal hypothesis were true, this would mean that there is a basic difference between animals in which fetal and adult hemoglobins are produced by different cells and those in which fetal and adult hemoglobins are produced by the same erythroid cell. However, coexistence of the two hemoglobin types in a single cell may be only the result of cell fusion, for which there is some evidence (37, 39). The change in red cell type and in hemoglobin synthesis during amphibian metamorphosis might correspond to the change from yolk sac to liver red cells and hemoglobins in the mouse, rather than to the much later developmental change from human fetal to adult hemoglobin. The latter change may have no corresponding changes in the bullfrog.

The mechanism which blocks polyspermy (9) can be used as a superficial analogy for another hypothesis. This alternative hypothesis, which does not imply a fundamental difference between the above two cases of erythroid differentiation, is the following. We assume that normally the two differentiated states (fetal and adult or larval and adult hemoglobin synthesis) are mutually exclusive and that a stem cell may differentiate in either direction, depending on the stimulus it receives. This means that the cell has a control mechanism such that once the cell starts differentiating in one direction, it is prevented from differentiating, at the same time, in the other direction. There is,

however, a time lag between the arrival of the stimulus for differentiation in one direction and the activation of the control mechanism. During this lag, the cell is receptive to both stimuli, i.e., to produce both adult and fetal hemoglobins. The difference, then, between animals in which no cells with both hemoglobins have been found (*R. catesbeiana*) and those which have cells with fetal and adult hemoglobins (human) might only be in the magnitude of this lag.

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