ERYTHROPOIESIS DURING AMPHIBIAN METAMORPHOSIS

II. Immunochemical Study of Larval and Adult

Hemoglobins of *Rana catesbeiana*

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ABSTRACT

Rabbit antibodies were prepared against the major hemoglobin components of the larval and adult stages of *R. catesbeiana*. The properties of the antisera were studied by double immunodiffusion, precipitation, and complement fixation. The antisera to tadpole and frog hemoglobins did not cross-react with either hemoglobin or apohemoglobin. The antiserum against frog hemoglobin was used for the detection of frog hemoglobin in tadpoles undergoing either natural or thyroxine-induced metamorphosis. It was shown that frog hemoglobin is detectable first in the liver, indicating that the liver is the site of erythrocyte maturation during metamorphosis.

INTRODUCTION

Antibodies to various hemoglobins have been extensively used as a sensitive tool for the study of conformational differences and changes in various hemoglobin forms (4, 25–27, 29), of structural and phylogenetic relations between different hemoglobins (11, 26, 34, 40), or for the study of the ontogeny of the hemoglobin (8, 10, 13, 37, 41). Use of immunological methods in the study of the developmental course of specific proteins requires a complete characterization of the antibody-antigen system. The present report deals with the preparation and properties of rabbit antibodies against larval and adult hemoglobins of the american bullfrog (*R. catesbeiana*). Since the antibodies were to be used for the intracellular detection of the hemoglobins,[1] it was considered necessary to examine the reactivity and cross-reaction of the antibodies with both the hemoglobins and the apohemoglobins. Ideally, the cross-reactivity of the

individual polypeptide chains should be checked, but, since there are no satisfactory preparative methods for the separation of the chains of these hemoglobins (1), the corresponding globins were used instead. It is conceivable that changes in the conformation of the proteins, when the heme is removed, could expose new antigenic sites common to both the frog and the tadpole hemoglobins.

MATERIALS AND METHODS

The source and maintenance of animals as well as the method for collecting the blood have been previously described (18).

Preparation of Hemoglobin Solutions

The erythrocytes were washed five times with amphibian Ringer's solution (32), the buffy coat was removed, and the erythrocytes were lysed in $\frac{1}{3}$ diluted Ringer's by freezing and thawing three times. The hemolysate was treated with 0.2 vol of CCl_4.

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[1] Maniatis, G. M., and V. M. Ingram. 1971. *J. Cell Biol.* **49**:390.

The lysis was complete as judged from the appearance of the stroma. The aqueous layer was separated in the clinical centrifuge, and the hemoglobin solution was finally centrifuged at 25,000 g for 30 min. All steps were carried out at 2°–4°C.

Determination of Hemoglobin Concentration

The concentration of hemoglobin in hemolysates was determined by diluting the hemoglobin in Drabkin's solution (7) and using the millimolar extinction coefficient of human cyanmethemoglobin of 44.0 at 540 nm (43).

The hemoglobin concentration in organ extracts and in very dilute hemolysates was determined from the difference spectrum of ferrous hemoglobin with respect to CO-ferrous hemoglobin as described in reference 16. Spectra were taken with a Cary 14 recording spectrophotometer. Protein was estimated by Lowry's method (17).

Frog and tadpole hemoglobins were precipitated by dialysis against 2.8 M phosphate buffer, pH 6.8, as described in reference 6. Dialysis of protein solutions was carried out in 8/32 Visking tubing (Union Carbide Corp., Food Products Div., Chicago, Ill.), boiled for 5 min, and washed thoroughly with distilled water. Vacuum dialysis was performed with the tubing tied at one end and with the other end fitted to glass tubing or a glass pipette through a rubber stopper. The stopper was fitted to a filtering flask half filled with the buffer. A vacuum of 150 mm Hg was applied with a water pump.

Preparation of Globin

Hemoglobin was first dialyzed against 0.001 M Na_2HPO_4 or, in the case of dilute solutions, concentrated by vacuum dialysis against the same solution. Globin was then prepared with the acid-acetone method (31). The precipitated globin was resuspended in water and lyophilized. An $E_{1cm}^{\%}$ of 20.9 was used for the estimation of globin concentration (19).

Hemin Preparation

Hemin was prepared according to reference 14.

Analytical Polyacrylamide Disc Gel Electrophoresis of Hemoglobin and Globin

Discontinuous polyacrylamide gel electrophoresis, pH 10.3, 2°C, was performed as described in references 20 and 21. The hemoglobin samples were first converted to the cyanmethemoglobin form by adding 1/10 vol of a solution of 2% $K_3Fe(CN)_6$, 0.5% KCN, 0.1% $NaHCO_3$. The gels were stained for hemoglobin with 0.2% benzidine-HCl (Calbiochem, Los

Angeles, Calif.) in 0.5 M acetic acid. Immediately before use 0.2 ml of 30% H_2O_2 was added to 100 ml of the above solution. The gels were kept in the benzidine-hydrogen peroxide solution until a blue color developed and then were stored in a solution of 0.5 M acetic acid, 0.5 M sodium acetate.

Preparative Polyacrylamide Gel Electrophoresis

A commercial apparatus, "Polyprep," (Buchler Instruments, Inc., Fort Lee, N. J.) was used. The procedure described in reference 21 was followed. In later experiments, hemoglobins were fractionated by polyacrylamide gel electrophoresis in wide tubes, 10 cm $\times$ 13 mm (I.D.); the gels were then cut and the hemoglobin was eluted. To facilitate elution the gels were dispersed by passage through hypodermic needles of decreasing diameter.

Gel Filtration of Hemoglobin

This was performed as described in reference 21.

Immunization of Rabbits

All protein solutions used as immunogens were dialyzed against 0.15 M NaCl. Male, New Zealand, white rabbits, weighing 3–4 kg each, were used for immunization. Once a week, for 3 wk the animals were given 2 mg of the immunogen in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) in a total volume of 1 ml. The immunogen was distributed among the toepads of the rear feet and two intramuscular sites. 7 days after the last injection, 2 mg of the immunogen were given intravenously. One week later, 100 ml of blood were collected from the marginal ear vein in 2 days. 1 wk after bleeding, a booster injection was given intravenously and, after another week, 100 ml of blood were drawn in 2 days. Only sera obtained on two consecutive days were pooled; otherwise they were kept separately. Two rabbits were immunized with the polyacrylamide gel serving as adjuvant (39). The band containing the fraction to be used as immunogen was cut out and the gel was homogenized by passing it successively through hypodermic needles of decreasing diameter (G-12 to G-20). The suspension was then injected subcutaneously at several sites.

Two-Dimensional Immunodiffusion

Double diffusion in agar was carried out essentially as described in reference 35.

Precipitin Reaction

The reaction was carried out in 3-ml conical centrifuge tubes. In each tube, 0.4 ml of antiserum

was mixed with 0.2 ml of antigen solution; the mixture was incubated for 1 hr in a 37°C water-bath and for 16 additional hours at 2°C. After incubation, the tubes were centrifuged for 30 min in a clinical centrifuge (top speed) at 2°C. The supernatant was decanted and the precipitate was washed twice with 2 ml of cold 0.15 M NaCl. The precipitates were dissolved in 0.5 ml of 0.1 M NaOH solution and the optical density was measured at 280 nm. The concentration of antibody in the dissolved precipitate was calculated as follows:

(a) A_{280} for immunoglobulin is 1.4 for 1 mg/ml concentration.

(b) A_{390} for immunoglobulin is essentially zero

(c) $\dfrac{A_{280}}{A_{390}}$ for hemoglobin is 1.06.

$$\text{Antibody (mg/ml} = \frac{A_{280} - 1.06\ (A_{390})}{1.4}$$

Quantitative Micro-Complement Fixation

Complement fixation was carried out essentially as described in reference 38.

OBSERVATIONS

Selection of the Immunogens

Comparison of the electrophoretic patterns of larval and adult hemoglobins of *R. catesbeiana* suggests the presence of some common minor bands (Fig. 1). In order to exclude from the immunogens the suspected common bands, the following procedures were used:

FROG HEMOGLOBIN: The major frog hemoglobin of *R. catesbeiana*, when stored at −20°C, forms dimers (eight-chain molecules) and higher polymers (30) which can be separated by gel filtration on Sephadex G-100 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). The central fractions of a symmetrical, well-separated peak, corresponding to the dimerized hemoglobin, were used as immunogen. Since the dimer runs ahead of the monomer on gel filtration, there is less danger of residual contamination with the minor frog hemo-
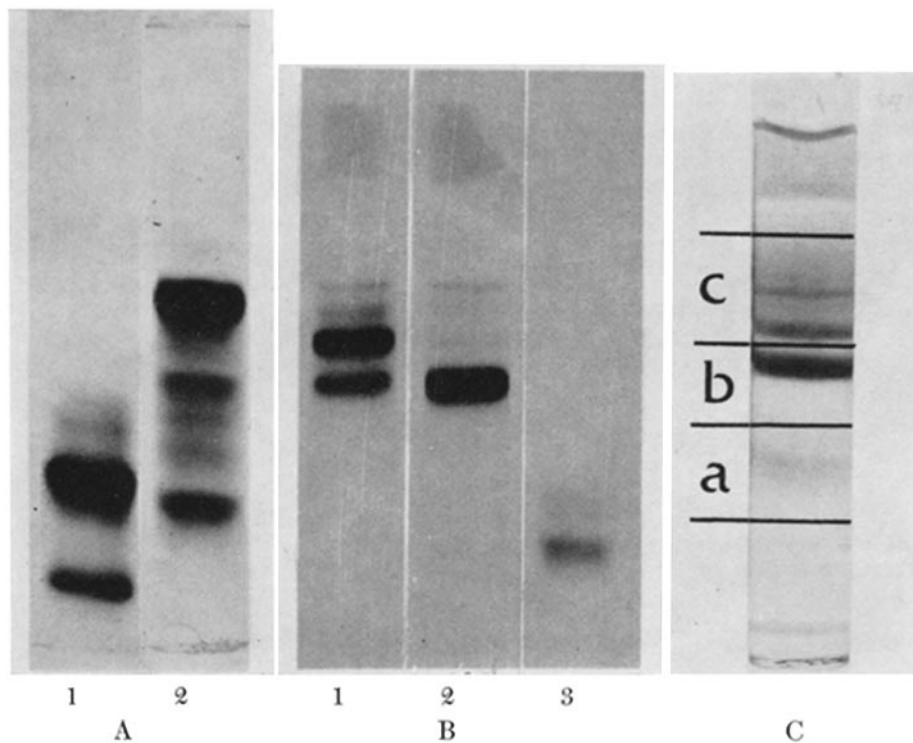

FIGURE 1 Polyacrylamide discontinuous gel electrophoresis, pH 10.3, 2°C. Fig. 1 A shows tadpole hemoglobin (at *1*) and frog hemoglobin (at *2*); benzidine stain was used. In Fig. 1 B frog hemoglobin fractionated on Sephadex G-100 is shown. This fractionation gives three hemoglobin peaks, *1*, *2*, a dimer of mol wt 130,000 (immunogen), and *3*; stained with benzidine. Fig. 1 C shows frog hemoglobin purified by crystallization and gel filtration before electrophoresis; it is unstained. Tubes of 13 mm I.D. were used.
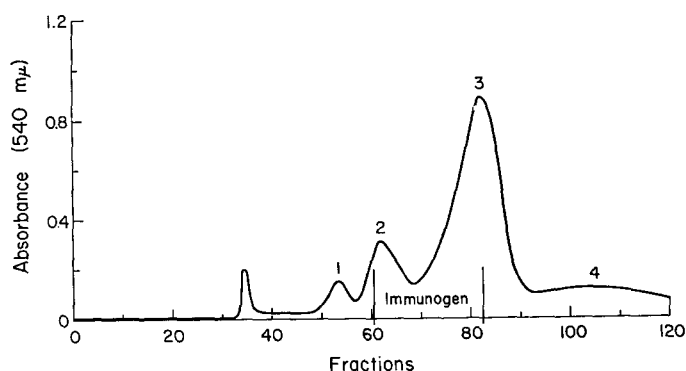
FIGURE 2 Elution pattern of preparative polyacrylamide gel electrophoresis of tadpole hemoglobin carried out with the Buchler apparatus. The fractions used as the immunogen are shown. The main purpose of this fractionation was to exclude peak no. 4.

globin components and any tadpole hemoglobins which do not polymerize.

TADPOLE HEMOGLOBIN: The tadpole hemolysate was fractionated by preparative acrylamide gel electrophoresis (Fig. 2). The two major hemoglobin components were used as immunogen. The main purpose of this fractionation was to exclude component no. 4 which could correspond to some minor components of the adult hemoglobin. Again, since the immunogen migrates ahead of peak no. 4, residual contamination is not likely. Some animals were immunized with tadpole hemoglobin fractionated by gel filtration on Sephadex G-100.

Characterization of Antisera

Two immune systems were used in these studies: frog hemoglobin (Hb) vs. rabbit antifrog Hb antibodies, and tadpole hemoglobin vs. rabbit antitadpole Hb antibodies. The immune systems were characterized by (a) gel diffusion analysis, (b) precipitation, and (c) complement fixation.

Gel Diffusion Analysis

FROG HEMOGLOBIN: Unfractionated frog hemoglobin, when reacted with rabbit antisera to frog hemoglobin fractionated by gel filtration or by electrophoresis, developed only one precipitin band in all cases (seven antisera tested). The concentration of the antigen varied from 7 μg to 100 mg/ml. Tadpole hemoglobin did not give any precipitin line with these antisera (Figs. 3 A and 3 B). The precipitin line stained with the benzidine reagent for heme proteins (Figs. 3 B and 3 D). Globin derived from frog hemoglobin reacted with the antihemoglobin (frog) antibodies and gave a

reaction of partial identity when compared with the hemoglobin (Fig. 3 C). The globin precipitin line did not stain with benzidine, as expected (Fig. 3 D). All the antisera formed a precipitin line with the frog hemoglobin purified successively by crystallization, gel filtration, and electrophoresis.

The results obtained by immunodiffusion indicate that the antisera from animals immunized with the dimer of frog hemoglobin are monospecific for the frog hemoglobins.

TADPOLE HEMOGLOBIN: All of the antisera prepared against tadpole hemoglobin, purified either by gel filtration or by electrophoresis (Fig. 2), developed two precipitin bands upon immunodiffusion (Figs. 3 A and 3 E). Even the antiserum prepared against the minor, fast-moving component (rabbit immunized with the homogenized polyacrylamide gel band) gave two precipitin bands. Two lines were also observed when the antigen was tadpole hemoglobin successively purified by crystallization, gel filtration, and gel electrophoresis.

The following observations were made with regard to the two precipitin lines of this immune system: (a) of the two lines, only one stained with benzidine (Fig. 3 B); (b) tadpole apohemoglobin gives only one precipitin line which merges completely with the second line of the hemoglobin (Fig. 3 E) (the one which does not stain with benzidine); (c) when a mixture of tadpole hemoglobin and globin (concn globin 0.25 mg/ml) is placed in the antigen well, the distance between the two precipitin lines increased (Fig. 3 E); (d) when hematin is added to the agar, tadpole hemoglobin gives only one precipitin line (Fig.
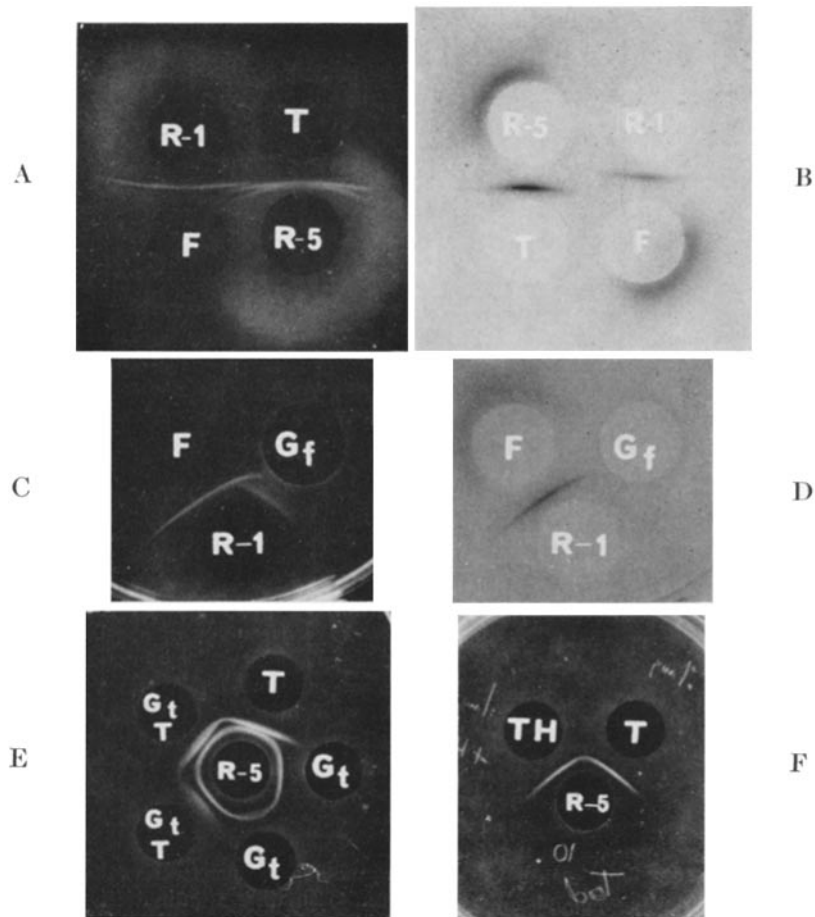
FIGURE 3   Two-dimensional immunodiffusion in agar (1% Ionagar No. 2, Consolidated Laboratories, Chicago Heights, Ill. in 0.15 M NaCl, 0.2% merthiolate, pH 8.0). *R-1*, antiserum to frog hemoglobin; *R-5*, antiserum to tadpole hemoglobin; *T*, tadpole hemoglobin; *F*, frog hemoglobin; G_f, frog apohemoglobin; G_t, tadpole apohemoglobin. Fig. 3 A is unstained and fixed with 7% acetic acid; Fig. 3 B, benzidine stained; Fig. 3 C was fixed and unstained; Fig. 3 D, benzidine stained, the same plate as Fig. 3 C; Fig. 3 E was unfixed and unstained; Fig. 3 F, hematin is incorporated into the agar. Note the absence of the second precipitin band. Hematin was added in amounts sufficient to color the agar green.

3 F); (*e*) antiserum absorbed with tadpole globin gives only one precipitin band with tadpole hemoglobin.

From these observations it is suggested that the immune system is not monospecific but is composed of two systems, one specific for hemoglobin and another for globin. This could be explained if we assume that, in the tadpole, hemoglobin is in equilibrium with its globin. Moss (19), on the basis of molecular weight determination by gel filtration, concluded that tadpole hemoglobin undergoes rapid, reversible dissociation of the polypeptide chains. Theil (36) also suggested the presence

of 9% globin in tadpole red cells. It has been shown that, when human hemoglobin is dissociated into polypeptide chains, new antigenic sites are exposed which are not present in the intact four-chain molecule (24). It seems, therefore, that the tadpole hemoglobin antisera, although not monospecific, are specific for the tadpole hemoglobin.

The above antisera did not give any precipitin band with frog hemoglobin (Figs. 3 A and 3 B).

One rabbit (R-3) was immunized with the fractions of frog hemoglobin corresponding to both the first and the second peaks of the Sephadex G-100 fractionation instead of only the second one

as for the rest of the rabbits. This antiserum also gave one precipitin band on double diffusion with frog hemoglobin. However, when this antiserum was tested with various fractions of tadpole hemolysate fractionated by gel filtration on Sephadex G-100, it was observed that a weak precipitin line developed with the fraction corresponding to the first, nonhemoglobin, peak. No other fraction of the tadpole hemolysate reacted. It was established that the observed cross-reaction was not due to either presence of frog hemoglobin in the tadpole or to reaction of antibodies against frog hemoglobin with tadpole hemoglobin. Most likely it was due to nucleoproteins eluted in the first peak, and it shows that attention should be paid to the careful preparation of the hemoglobins in order to avoid cross-reactivity.

SENSITIVITY OF DOUBLE DIFFUSION ANALYSIS: It was of interest for later experiments to estimate, approximately, the sensitivity of the Ouchterlony assay in the system used here, i.e., to determine the lowest concentration at which frog hemoglobin would give a precipitin line in the presence of a large excess of tadpole hemoglobin. It was found that in the presence of a 1000-fold excess of tadpole hemoglobin the lowest concentration of frog hemoglobin which would give a visible precipitin band was 10 μg/ml.

QUANTITATIVE PRECIPITIN REACTION

Precipitin curves were obtained for the antisera against fractionated frog or tadpole hemoglobins to gain some further information about the characteristics of the antigen-antibody reaction and the relative titer of individual sera. Fig. 4 shows some of the precipitin curves obtained.

COMPLEMENT (C) FIXATION

Fig. 5 shows the complement fixation curves for the frog Hb–antifrog-Hb immune system. The dilution of the antiserum for the optimal C fixation was 1:10,000, indicating that frog hemoglobin is a potent immunogen for the rabbit (compare references 23 and 25). The antibodies react mainly with the major hemoglobin component and only poorly with the minor, fast-moving component of the frog hemoglobin. Fig. 6 shows the complement curves for the tadpole hemoglobin–antitadpole hemoglobin immune system. The antiserum was diluted 1:2550 for optimal fixation. The antibodies react mainly with the slow-moving, major tadpole hemoglobin. It should be noted that, in all rabbits
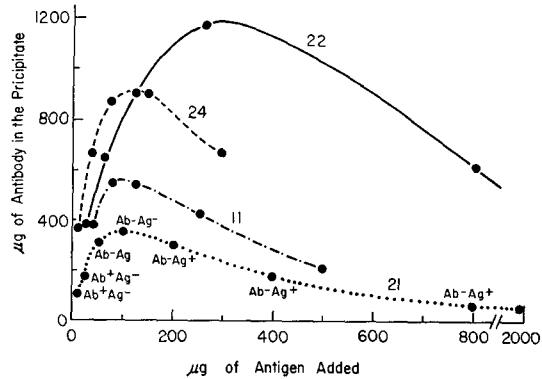

FIGURE 4 Precipitin reactions were carried out as described in Materials and Methods. Unfractionated hemoglobins were used as antigens. ———, whole serum from R-22 (antifrog) reacting with frog Hb; ----, whole serum from R-24 (antitadpole) reacting with tadpole Hb; —·—·—·, whole serum from R-11 (antitadpole) reacting with tadpole Hb; ·······, immunoglobulins from R-21 (antifrog) purified by precipitation with 40% ammonium sulfate and subsequent passage through diethylaminoethyl (DEAE) cellulose equilibrated with 0.02 M sodium phosphate, pH 7.5, reacting with frog Hb. Ag ± and Ab ± indicate the presence or absence of antigen or antibody, respectively, in the supernatant tested by double diffusion.

immunized, frog hemoglobin was a stronger antigen than the tadpole hemoglobin.

CROSS-REACTIVITY—INHIBITION

In order to test the cross-reactivity of the two immune systems, the concentrations of both the antigen and the antiserum were varied over a wide range. At the dilutions of antisera used for optimal complement fixation, there were no cross-reactions between the two systems over an antigen concentration range from 0.01 to 10 μg (i.e., 100-fold the optimal concentration for the homologous antigen) (Figs. 6 and 7). No cross-reaction was detected when the concentration of the antiserum to frog hemoglobin was raised 100-fold. For the antiserum to tadpole hemoglobin there was no cross-reactivity over a range of heterologous antigen concentration from 0.01 to 3 μg/ml and a 51-fold increase of antiserum concentration.

There are cases in which a substance fails to give complement fixation with an antiserum, although it reacts with the antibodies (28). This is shown by inhibition effects, that is, the presence of the weakly reacting substance inhibits the fixation of complement by the homologous system. We, there-
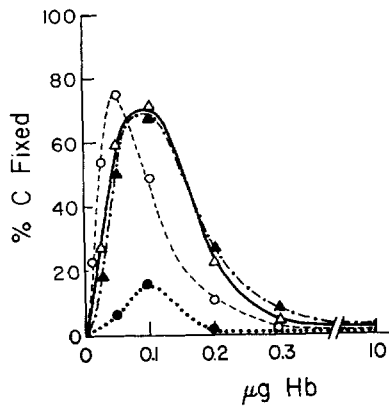
Figure 5 Complement fixation by the frog hemo-globin–antifrog hemoglobin system. The antiserum dilution was 1:10,000. ——, unfractionated frog hemo-globin; ......, fraction a (Fig. 1 C) of frog hemoglobin; -----, fraction b (Fig. 1 C) of frog hemoglobin; —·—·—·, fraction c (Fig. 1 C) of frog hemoglobin.
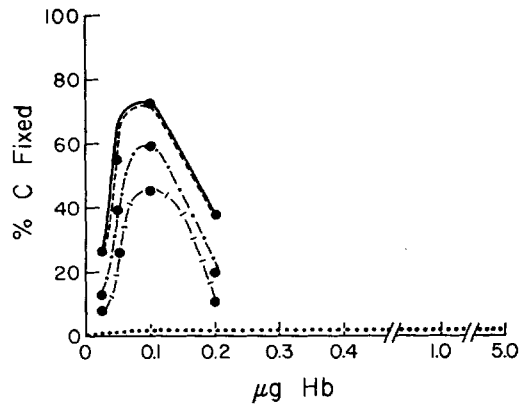

Figure 7 Inhibition of complement fixation by tad-pole hemoglobin in the frog hemoglobin system. The antiserum dilution was 1:10,000. ——, frog hemo-globin; ----, frog hemoglobin + 100-fold excess tad-pole hemoglobin; —·—·—·, frog hemoglobin + 200-fold excess tadpole hemoglobin; -|-|-|, frog hemoglobin + 400-fold excess tadpole hemoglobin; ......, tadpole hemoglobin.
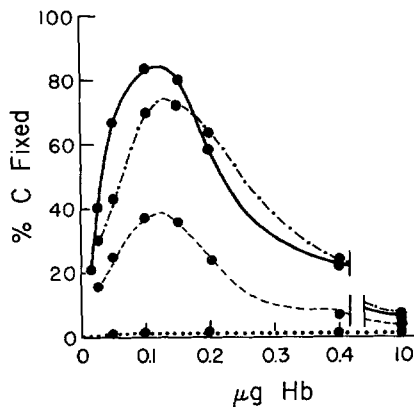

Figure 6 Complement fixation by the tadpole hemo-globin–antitadpole hemoglobin system. The antiserum dilution was 1:2550. ——, whole tadpole hemoglobins; -----, fast moving, minor component of tadpole hemo-globin; —·—·—·, major component of tadpole hemoglo-bin; ......, whole frog hemoglobins.

fore, studied the effect of the presence of tadpole hemoglobin on the fixation of complement by the frog Hb–antifrog Hb system. Fig. 7 shows that tadpole hemoglobin is not inhibiting the frog system when present in excess of up to 100-fold over the homologous antigen. In 200-fold excess there is an 18% inhibition while in 400-fold excess the inhibition of C fixation is approximately 40%.

The reactivity of the antihemoglobin antisera

with frog apohemoglobin was also studied. In order to obtain complement fixation comparable to that of hemoglobin, the concentration of the antiserum had to be raised more than fivefold (dilution of antiserum 1:1800), and the concentra-tion of the antigen 20-fold. Tadpole globin reacting with the antifrog-hemoglobin serum failed to fix C in concentrations up to 125 μg/ml. Tadpole globin, however, present in 150-fold excess over the homologous globin, inhibited the C fixation by 18%, whereas 10-fold excess of tadpole globin did not affect the frog system. In comparison to the frog globin, tadpole globin is a more effective antigen when reacted with the antitadpole hemo-globin sera. In order to get 90% C fixation the concentration of the antiserum was raised only 2.5-fold, and the concentration of antigen at maxi-mum fixation was 0.3 μg/ml, only three times higher than that of the hemoglobin. There was no C fixation when frog globin was added up to 3.2 μg/ml; nor was there inhibition of the homologous system when frog globin was present in amounts equal to those of tadpole globin.

IMMUNOLOGICAL DETECTION OF FROG HEMOGLOBIN IN METAMORPHOSING TAD-POLES: It was previously determined that a mix-ture of frog and tadpole hemoglobins gives a precipitin line with antisera to frog hemoglobin when frog hemoglobin is present in concentrations

higher than 10 μg/ml in the presence of a 1000-fold excess of tadpole hemoglobin. The total concentration of hemoglobin was determined by the difference spectrum method in blood hemolysate and in liver homogenate from each metamorphosing animal. The lysate was clarified by centrifugation at 25,000 g. Two- or threefold dilutions of the above hemoglobin solutions were made and each dilution was tested for the presence of detectable amounts of frog hemoglobin by double diffusion. The concentration of total hemoglobin at the highest dilution giving a precipitin band with the antiserum to frog hemoglobin was determined. Assuming that the concentration of frog hemoglobin in this dilution was of the order of 10 μg/ml in both the blood hemolysate and the liver homogenate (or, in any case, that it was the same in both), the total concentrations of hemoglobin in the blood and the liver preparation were compared. The lower the concentration of total hemoglobin at the highest dilution reacting with the antifrog-Hb serum, the higher the content of frog hemoglobin (Tables I and II).

DISCUSSION

Both frog and tadpole hemoglobins were found to be relatively strong immunogens for the rabbit. Immunization with frog hemoglobin, however, results always in more potent antisera than immunization with tadpole hemoglobin. It was a

TABLE I

Tadpoles from a Natural Population

Hind leg/tail	Frog-Hb-Liver/ Frog-Hb-Blood	Lowest concentration of total Hb at which frog Hb was detectable	
		Blood	Liver
		mg/ml	mg/ml
0.04	—	>10.00	>10.00
0.11	—	>10.00	>10.00
0.32	—	>10.00	>10.00
0.34	>1	>8.00	8.00
0.38	>1	>8.00	8.00
0.42	8	3.30	0.41
0.44	>1	>5.60	5.60
0.53	>2	>5.00	2.50
0.59	12	1.17	0.10
0.60	>1	>1.80	1.80
0.63	>43	>3.05	0.07
0.74	9	0.875	0.10
0.76	24	0.48	0.02
0.80	19	1.16	0.06
6.70	2	0.07	0.03

TABLE II

Thyroxine*-Treated Tadpoles

Days in thyroxine*	Hind leg/tail	Frog-Hb-Liver/Frog-Hb-Blood	Lowest concentration of total Hb at which frog Hb was detectable	
			Blood	Liver
			mg/ml	mg/ml
13	0.27	74	>7.40	0.10
15	0.32	26	>2.40	0.09
17	0.27	50	5.00	0.10
18	0.38	28	2.00	0.07
24	0.62	30	3.00	0.10
25	0.61	15	1.55	0.10
27	0.85	1	0.08	0.08
28	0.93	1	0.08	0.08

* 2.5×10^{-8} M L-thyroxine.

rather surprising finding that larval and adult hemoglobins of the same species do not cross-react to any detectable extent, particularly in view of previous studies which have shown significant interspecies cross-reactivity of proteins with a similar biological function. Landsteiner (15) and Boyden (5) have summarized some of the early studies, showing that the degree of immunological resemblance between homologous proteins of different species is often a function of how closely related the species are. More recently, Wilson et al. (33, 40) studied the serum albumins of primates, and Arnheim and Wilson (2) studied the bird lysozymes. All homologous proteins cross-reacted and the extent of cross-reactivity was consistent with the accepted phylogenetic position of the animals. After we noticed the lack of cross-reactivity between the major tadpole and frog hemoglobins, Wise (42) also reported that antisera to bullfrog tadpole and adult hemoglobins did not cross-react. Kurata and Okada (13) made similar observations for the hemoglobins of *Bufo vulgaris*. It is interesting that despite this lack of cross-reactivity, the same antisera cross-reacted to a significant extent with the hemoglobins of *R. pipiens* and *R. clamitans* but only with the hemoglobin of the corresponding developmental stage, i.e., the antifrog-Hb serum with frog Hb and the anti-tadpole Hb with the tadpole Hb. It should be noted, however, that although valid conclusions can be drawn from the comparison of the relative reactivities of a number of substances with an antiserum, the lack of cross-reactivity between two antigens does not give an absolute measure of

their dissimilarity, because it depends on the methods used and on the properties of the particular antibodies, which, in turn, are a function of the immunized animal, length of immunization, etc. It is relevant to mention in this respect that Askonas and Smyth (3) reported that only the β-chains of human hemoglobin A are immunogenic in the rabbit, while Reichlin (22) found that both the α- and β-chains were equally immunogenic. The lack of cross-reactivity between tadpole and frog hemoglobins may be taken as an indication that the major frog and tadpole hemoglobins do not share a common polypeptide chain. One could argue that the two hemoglobins, although they have a common chain, do not cross-react because all of the "immunodominant" groups are located in the noncommon chain. This is theoretically possible, but rather improbable. Reichlin et al. (27) have shown that antisera to human hemoglobin A_1 are directed against both α- and β-chains. And all hemoglobins, so far examined, which possess a common chain, cross-react (e.g., human Hb's A, F, and A_2). Also, the lack of cross-reactivity does not, necessarily, indicate extensive differences in the primary structure of the two molecules. Antibodies against the separated human hemoglobin chains α and β, for example, do not cross-react (24), although there are several regions of homology between the two chains (9). The number of antigenic determinants per molecule of hemoglobin may be small. For human hemoglobin the number is, probably, five (12, 23, 28). Although no cross-reactivity could be detected when tadpole hemoglobin reacted directly with antifrog Hb serum, and vice versa, a partial inhibition of C fixation occurred when the heterologous antigen was present in large excess (Fig. 7).

Tables I and II show the results of two series of experiments designed to detect immunologically the appearance of frog hemoglobin in the liver and the blood. One table includes animals taken from a population of naturally metamorphosing tadpoles, and the other includes thyroxine-treated animals. The results indicate that in both series frog hemoglobin was detectable first in the liver homogenate, or that the liver homogenate contained a larger proportion of frog hemoglobin than the blood hemolysate. Clearly, the liver is the most suitable place to search for cells containing both tadpole and frog hemoglobins. No significance should be attached to the absolute value of the ratio "frog Hb in the liver/frog Hb in the blood,"

other than that it is greater than 1, because the amount of circulating blood left in the liver after exsanguination varied from animal to animal. The results from these experiments are in agreement with the morphological evidence that, during metamorphosis, the maturing erythroid cells reside in the liver.

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