THE TRANSITION FROM TADPOLE TO FROG HAEMOGLOBIN DURING NATURAL AMPHIBIAN METAMORPHOSIS

I. PROTEIN SYNTHESIS BY PERIPHERAL BLOOD CELLS IN VITRO

J. BENBASSAT
Department of Medicine A, Hadassah University Hospital, Jerusalem, Israel

SUMMARY

The transition from larval to adult haemoglobin during natural metamorphosis of Rana catesbeiana tadpoles was studied by polyacrylamide gel electrophoresis of either untreated or reduced and alkylated haemolsates in 8 M urea. The capacity of the peripheral red cells to synthesize haemoglobin in vitro declined in the course of the development of tadpoles prior to metamorphosis. The beginning of the metamorphosis climax, characterized by emergence of the front legs, was associated with an abrupt increase in the capacity of the circulating cells to synthesize haemoglobin. The protein synthesized at this stage consisted mainly of frog haemoglobin and of smaller amounts of tadpole haemoglobin.

The observed changes in haemoglobin synthesis during metamorphosis are compatible either with the appearance of a new cell line synthesizing adult haemoglobin, or with the renewed proliferation of the tadpole erythroid cell line which eventually produces cells capable of synthesis of frog haemoglobin.

INTRODUCTION

Rana catesbeiana tadpole and frog haemoglobins are different in their affinity for oxygen (McCutchion, 1936), electrophoretic or chromatographic mobility (Herner & Frieden, 1961; Hamada, Sakai, Shukuya & Kaziro, 1964; Baglioni & Sparks, 1963; Moss & Ingram, 1965), 'fingerprint' peptide pattern (Baglioni & Sparks, 1963), polypeptide subunits (Moss & Ingram, 1968a; Aggarwal & Riggs, 1969) and antigenic determinants (Maniatis & Ingram, 1971). The transition from tadpole to frog haemoglobin occurs during metamorphosis (Herner & Frieden, 1961). It has been suggested, that the repression of tadpole haemoglobin synthesis and the stimulation of frog haemoglobin occur in a sequential manner: induction of metamorphosis by thyroid hormone has been shown to result in an early decline in tadpole haemoglobin synthesis followed by a subsequent stimulation of frog haemoglobin production. These observations have been interpreted as consistent with the possibility that tadpole and frog erythroid cells are derived from clonally distinctive stem cells (Moss & Ingram, 1965, 1968b).

The present report deals with the electrophoretic analysis of protein synthesized by circulating erythrocytes of R. catesbeiana tadpoles at different stages of development.
and natural metamorphosis. The results show that frog and tadpole haemoglobins are produced simultaneously by metamorphosing tadpoles. This finding is discussed in the light of the possibility that larval and adult haemoglobins are synthesized by the same erythroid cell line.

**MATERIALS AND METHODS**

**Animals**

*Rana catesbeiana* frogs and tadpoles at different stages of natural metamorphosis were obtained from a local pond and bled as described previously (Benbassat, 1970). The eggs of this species are fertilized during the summer and the tadpoles remain in the *premetamorphic period* (up to stage XI of Taylor & Kollros, 1946) for at least a year. The *metamorphic period* (Taylor and Kollros' stages XII–XIX), characterized by a rapid growth of the hind legs, begins at the following summer and lasts about 3 weeks. When the hind legs reach the length of the body, abruptly the front legs emerge and the *metamorphic climax* (Taylor & Kollros' stages XX–XXV) begins. This latter period lasts 7–14 days; it is characterized by the reabsorption of the tail and other anatomical changes resulting in the transformation of the tadpole into a froglet, which subsequently grows to reach the adult size (Etkin, 1963).

Experiments were performed during the summer on blood cells from: (a) early premetamorphic tadpoles (total length, 35 mm, estimated age 2–4 weeks); (b) early prometamorphic tadpoles (total length, 85 mm, hind legs, 2–5 mm, estimated age, at least 1 year); (c) late prometamorphic tadpoles (total length, 90 mm, hind legs, 40 mm); (d) animals at the beginning of the metamorphic climax (total length, 98 mm, hind legs, 45 mm, 2–3 days after emergence of the front legs); (e) juvenile froglets kept in laboratory for at least 4 weeks; and (f) adult frogs.

**Preparation of labelled haemolysates**

Between 10 and 20 x 10⁶ blood cells were incubated at 29°C in 5 ml Amphibian Culture Medium (Grand Island Biological Co.) with 25 µCi ¹⁴C-amino acid mixture (New England Nuclear Co.). After 16 h the cells were chilled, pelleted, washed in amphibian Ringer’s solution and lysed in 0.5 ml 0.05 % K₃Fe(CN)₆ in Tris-glycine buffer, pH 8.3, by freezing and thawing. The obtained methaemoglobin was converted to cyanmethaemoglobin by the addition of a drop of 5 % neutralized NaCN and the heavy particles and ribosomes were sedimented by centrifugation at 104,000 g for 90 min. The supernatant haemoglobin solution was dialysed overnight against 1 l. Tris-glycine buffer, pH 8.3, supplemented with 100 mg KCN and kept frozen until used as detailed below.

**Electrophoresis of haemoglobin on polyacrylamide gel**

Polyacrylamide gel electrophoresis was carried out in a discontinuous system in a standard EC 470 Vertical Gel Electrophoresis Cell (EC Apparatus Corporation, University City, Philadelphia, Pa.) according to the instructions of the manufacturer (technical bulletin No. 141). The electrode buffer, pH 8.3, contained 0.6 g Tris and 2.9 g glycine per l. distilled water. The gel was prepared by dissolving 7 g Cyanogum-41 (EC Apparatus Co.), 0.1 ml tetramethylene-diamine (TEMED) and 0.1 g ammonium persulphate (AP) in 100 ml 0.75 % Tris buffered to pH 8.9 with HCl. A 5-mm gel slab was used and 100 µg haemoglobin solution prepared as detailed in the preceding section in a volume of 25–50 µ1 were applied in 10-mm slots. Electrophoresis was carried out for 2–3 h at 300 V and 75 mA. Addition of KCN to the electrode buffer, or prolonged storage of the haemoglobin solution, had no effect upon the pattern of its electrophoretic resolution. After electrophoresis, the individual strips were separated and sliced into 0.05-in. (1.27-mm) fractions, which were transferred to planchettes, crushed by a glass rod in a small amount of water, dried and counted in a low background Nuclear Chicago Gas Flow Counter.
Reduction and alkylation of globin

Either unpurified haemolysates or eluted haemoglobin fractions after separation on polyacrylamide gels were used. The haemoglobin solutions were dissolved 1:10 (v/v) in freshly deionized 8 M urea and incubated under nitrogen with 0.1 M 2-mercaptoethanol at 37 °C for 1 h. Iodoacetamide, recrystallized from haptene, was then added to final concentration of 0.2 M. The samples were incubated for 30 min and dialysed overnight against 2 l. of electrode buffer of the 8 M urea polyacrylamide gel system to be employed. Subsequently, between 0.2 and 0.4 ml of the reduced and alkylated globin containing 0.2-0.4 mg protein and between 4000 and 10000 TCA-precipitable cpm were applied with sucrose in 20-mm slots of polyacrylamide gel slabs prepared in 8 M urea as detailed below.

Electrophoresis of reduced and alkylated globin in 8 M urea

Electrophoresis was carried out either in the continuous acid or in the discontinuous alkaline 8 M urea polyacrylamide gel systems described by Moss & Ingram (1968a) with the following modifications: an EC 470 Vertical Gel Electrophoresis Cell and 7 % polyacrylamide (Cyanogum-41) gels were used throughout. The running buffer of the acid continuous system contained 59 ml formic acid and 6 ml 2 M NaOH per l. of 8 M urea. The gel was prepared by dissolving 7 g Cyanogum-14, 0.6 ml TEMED and 0.3 g AP in 100 ml buffer. Excess persulphate was removed by preliminary application of 100 V and 70 mA for 2 h. Electrophoresis was carried out at the same current for 16 h.

The electrode buffer of the alkaline discontinuous system contained 0.6 Tris and 2.6 g glycine per l. of 8 M urea. The running buffer contained 4/6 % Tris buffered to pH 8.9 with HCl. The gel was prepared by dissolving 7 g Cyanogum-41, 0.1 ml TEMED and 0.1 g AP in 100 ml running buffer. Electrophoresis was carried out at 300 V and 75 mA for 16 h.

After electrophoresis the gels were sliced, each fraction was eluted in water and the 5 % TCA-precipitable radioactivity of the eluates was determined in a model 3375 Tricarb Scintillation Spectrometer (Packard).

RESULTS

Preparation and fractionation of labelled haemolysates

Preliminary experiments revealed that the amount of amino acids incorporated per cell was not affected by variations in the erythrocyte concentration between 0.1 and 4 x 10⁶ cells per ml incubation medium (Fig. 1). Pulse labelling after different periods of preincubation of frog or tadpole red cells showed that the rate of amino acid uptake declined slightly during incubation and after 24 h a pulse of label revealed only half of the initial level of synthesis (Fig. 2). There were no differences in the pattern of resolution of radioactivity of haemolysates prepared from freshly drawn cells and those prepared from erythrocytes which had been preincubated for up to 24 h before labelling. Therefore, in order to prepare haemolysates of high specific activity, red cells were incubated for 16 h with ¹⁴C-amino acids, pelleted, washed and lysed as detailed in Methods.

The specific activity of haemolysates of frogs and tadpoles at different stages of metamorphosis is shown in the first column of Table 1 (p. 351). The capacity of red cells of premetamorphic tadpoles to incorporate amino acids was roughly 18 times higher than that of late prometamorphic tadpoles. After emergence of the front legs the animals had red cells which incorporated about 7-8 times more amino acids than red cells of late prometamorphic tadpoles. The specific activity of the haemolysates
Fig. 1. Effect of cell concentration on the capacity of incubated tadpole erythrocytes to incorporate amino acids. Each incubation mixture contained the indicated number of cells and 2 μCi [14C]leucine per ml amphibian culture medium. After 2 h incubation at 29 °C at constant shaking under 5% CO₂, the cells were pelleted, washed and the 5% TCA-insoluble radioactivity was determined in a low background Nuclear Chicago Gas Flow Counter.

Fig. 2. Effect of preincubation at 29 °C on the capacity of frog (solid bars) and tadpole (hatched bars) red cells to incorporate amino acids. The preincubation mixture contained 0.5 x 10⁸ cells per ml amphibian culture medium. After different periods of preincubation, red cell aliquots were pelleted, resuspended in fresh culture medium and reincubated with [14C]leucine for 2 h as in Fig. 1.

decayed after metamorphosis, and the capacity of frog erythrocytes to incorporate amino acids was similar to that of prometamorphic tadpoles.

The electrophoretic mobility and the pattern of resolution of tadpole and frog haemoglobins on polyacrylamide gel are shown in the inset of Fig. 3. They were identical with those reported previously in animals obtained from the same pond (Benbassat, 1970). Both larval and adult haemoglobins were resolved into several fractions, which were designated arbitrarily as T₁, T₂ and T₃ for tadpole haemoglobin and F₁, F₂, F₃ and F₄ for adult frog haemoglobin. Fractions F₁ and T₁ had an identical electrophoretic mobility on polyacrylamide in the system employed, even though they
Table 1. Incorporation of $^{14}$C-amino acids into blood cells and haemoglobin of R. catesbeiana frogs and tadpoles at different stages of metamorphosis.

<table>
<thead>
<tr>
<th>Stage of metamorphosis</th>
<th>Unpurified haemolysates (cpm/o.D. 540)</th>
<th>Pooled purified haemoglobin fractions (cpm/o.D. 540)</th>
<th>Specific activity (cpm/o.D. 410) of main haemoglobins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F$_3$+F$_4$</td>
<td>F$_2$</td>
</tr>
<tr>
<td>Early premetamorphic tadpoles</td>
<td>199 500</td>
<td>92 500</td>
<td>—</td>
</tr>
<tr>
<td>Early prometamorphic tadpoles</td>
<td>29 000</td>
<td>20 000</td>
<td>—</td>
</tr>
<tr>
<td>Late prometamorphic tadpoles</td>
<td>11 200</td>
<td>Not done</td>
<td>—</td>
</tr>
<tr>
<td>Metamorphic climax</td>
<td>84 300</td>
<td>Not done</td>
<td>129 000</td>
</tr>
<tr>
<td>Froglets, 4 weeks after emergence</td>
<td>77 700</td>
<td>76 400</td>
<td>5 700</td>
</tr>
<tr>
<td>of front legs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult frogs</td>
<td>34 000</td>
<td>32 000</td>
<td>2 780</td>
</tr>
</tbody>
</table>

*F$_1$ and T$_1$ had an identical electrophoretic mobility on polyacrylamide gel in the system employed.

The conditions of incubation and purification of haemoglobin are detailed in the text. No carrier haemoglobin was added to the labelled haemolysates.
have been shown to be different in their antigenic determinants and in their globin subunits (J. Benbassat, unpublished observations). Red cells of premetamorphic and prometamorphic tadpoles contained only larval haemoglobin. Haemolysates of animals during the metamorphic climax contained predominantly larval haemoglobin. Frog haemoglobin was first detected at emergence of the front legs and its relative amount gradually increased during the metamorphic climax. The transition to frog haemoglobin was complete in juvenile froglets about 4 weeks after emergence of the front legs.

Polyacrylamide gel electrophoresis of labelled haemolysates of premetamorphic tadpoles revealed that 46% of the radioactivity was incorporated into the tadpole haemoglobin fractions, while the remaining 54% formed several slower moving peaks.
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(Fig. 3A). A similar distribution of the radioactivity among the haemoglobin and non-haemoglobin fractions was observed with haemolysates of prometamorphic tadpoles (Fig. 3B). In these experiments 76 and 88% of the TCA-precipitable radioactivity applied to the gels could be recovered after electrophoresis. The pattern of radioactivity observed after fractionation of labelled haemolysates of froglets 4 weeks after emergence of the front legs was identical with that of adult frogs (Fig. 3D, E). At these stages

Fig. 4. Electrophoresis of reduced and alkylated globin on polyacrylamide gels prepared in acid 8 M urea. Globin was prepared from: A, unpurified haemolysate of a prometamorphic tadpole (total length, 85 mm; hind legs, 2 mm); B, unpurified haemolysate of a tadpole at the beginning of metamorphic climax (total length, 98 mm; hind legs, 45 mm; 2 days after emergence of front legs); C, unpurified haemolysate of an adult frog; A', major haemoglobin fraction (T1) of a prometamorphic tadpole; B', major haemoglobin fraction (T2) of a tadpole at the beginning of the metamorphic climax; C', major frog haemoglobin (F3).

Preparation of labelled haemolysates, reduction, alkylation and electrophoresis as detailed in Methods. The separation of the haemoglobin fractions in A', B' and C' was carried out by polyacrylamide gel electrophoresis as shown in Fig. 3 and elution of the major haemoglobin fractions from the gels. Origin to the left, migration towards the cathode. Solid lines, radioactivity; dashed lines, densitometric tracing of the photographed amido black-stained gels (inset).
79 and 84% of the radioactivity were confined to the haemoglobin fractions. Recoveries were 84 and 82%, respectively.

Electrophoresis of labelled haemolysates of animals at the metamorphic climax showed that 83% of the radioactivity was incorporated into the haemoglobin fractions. Most of the radioactivity co-electrophoresed with added unlabelled fresh frog haemoglobin; some of the radioactivity, however, migrated with the tadpole haemoglobin fractions (Fig. 3c). These observations confirmed earlier findings (Herner & Frieden, 1961; Benbassat, 1970) that the newly synthesized red cell proteins during the metamorphic climax are resolved into an 'intermediate' electrophoretic pattern, different from that of frogs or tadpoles at earlier developmental stages.

In some experiments the specific activity of the haemoglobin fractions was determined after their elution from the gels. The results of a representative experiment are shown in Table 1. The specific activity of the haemoglobin fractions of early premetamorphic tadpoles was roughly 10 times higher than that in late prometamorphic tadpoles. Animals at the metamorphic climax were found to produce, in addition to frog haemoglobin (fractions F1, F3 and F4), red cell proteins which migrated with tadpole haemoglobins (T2 and T3). At this stage of metamorphosis the specific activity of fractions T2 and T3 was higher than that of the same fractions during the preceding (late prometamorphic) stage. The specific activity of the minor haemoglobin fractions (T2, T3 and F1, F2) was higher than that of the major haemoglobins (T1 and F3, F4).

Electrophoresis and fractionation of reduced and alkylated labelled globin in 8 M urea

Reduced and alkylated frog haemoglobin subunits can be separated from tadpole globin chains by electrophoresis on polyacrylamide gels prepared in 8 M urea (Moss & Ingram, 1968a). Therefore, in further experiments the labelled haemolysates were dissolved in 8 M urea, reduced and alkylated as detailed in Methods, and applied on polyacrylamide prepared in 8 M urea. After electrophoresis, the TCA-insoluble radioactivity was determined in the eluates of the gel fractions.

Inspection of the amido black-stained gels after electrophoresis in acid 8 M urea buffer revealed that tadpole globin was resolved under these conditions into one major and two minor faster bands (inset, Fig. 4). Frog globin separated into 1 major and a slightly faster moving minor band. Fractionation of labelled reduced and alkylated globin revealed several peaks of radioactivity which migrated together with the different protein fractions (Fig. 4). The pattern of resolution of globin of animals at the metamorphic climax indicated that red cells of tadpoles at this stage of metamorphosis synthesize predominantly frog globin and small amounts of tadpole globin subunits. Similar results and conclusions have been reported by Moss & Ingram (1968b) in tadpoles treated for 13 days with $5 \times 10^{-8}$ M thyroxine.

Fig. 5 depicts the results of electrophoresis of reduced and alkylated frog and tadpole globin in 8 M urea alkaline gels. Tadpole globin was resolved into 2 major relatively fast-moving bands and several fainter bands. This pattern differed from that of frog haemolysates, which were resolved into 1 major, relatively slow-moving band, and several minor bands. Fractionation of labelled reduced and alkylated globin of animals at the metamorphic climax confirmed that red cells of tadpoles at this stage
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Fig. 5. Electrophoresis of reduced and alkylated globin on polyacrylamide gels prepared in alkaline 8 M urea. Globin prepared from: A, unpurified haemolysates of a prometamorphic tadpole (total length, 85 mm; hind legs, 2 mm); B, unpurified haemolysates of a tadpole at the beginning of the metamorphic climax; C, unpurified haemolysates of an adult frog; A', purified fraction T₁ from a prometamorphic tadpole. The methods for preparation of haemolysates, reduction, alkylation and electrophoresis are detailed in the text. Origin to the left, migration towards the anode. Inset, amido black-stained gels.

of metamorphosis produce mainly frog haemoglobin subunits together with smaller amounts of tadpole globin.

DISCUSSION

The present results suggest the following sequence of events leading to the replacement of tadpole haemoglobin by frog haemoglobin during natural amphibian
metamorphosis: the first stage, occurring during the prometamorphic period, is characterized by a gradual decline in the capacity of the circulating cells to synthesize tadpole haemoglobin. Secondly, a sharp increase in haemoglobin synthesis occurs during the metamorphic climax. The circulating cells of tadpoles at this stage of metamorphosis produce mainly frog haemoglobin and relatively small amounts of tadpole haemoglobin. The third stage consists in the gradual decline in haemoglobin production by the erythrocytes in the course of the maturation of the juvenile froglet.

The observed synthesis of frog and tadpole haemoglobins during the metamorphic climax is in agreement with the findings by Herner & Frieden (1961), who reported a simultaneous labelling of larval and adult haemoglobins with $^{59}$Fe in blood samples of metamorphosing tadpoles. It is in agreement also with the findings by Moss & Ingram (1968), who detected tadpole globin synthesis in addition to that of frog globin during thyroxine-induced metamorphosis. It may therefore be concluded that induction of frog haemoglobin during metamorphosis is not preceded by a complete suppression of tadpole haemoglobin production: the experiments reported in Fig. 3 and Table 1 even suggest a stimulation of tadpole haemoglobin synthesis concomitant with the appearance of frog haemoglobin.

These findings do not answer the question whether frog haemoglobin is produced by the same cell line which synthesizes tadpole haemoglobin, or by a clonally distinct erythroid cell line which is released into the circulation at the metamorphic climax. The clonal theory is supported by the observation that during metamorphosis some $10\%$ of the circulating red cells are morphologically distinct by their immature appearance (Moss & Ingram, 1968; Benbassat, 1970) and contain frog haemoglobin (DeWitt, 1968). It may be claimed that frog haemoglobin synthesis is restricted to these newly formed cells, while the small amounts of tadpole haemoglobin produced during the metamorphic climax are synthesized by the remaining erythrocytes. This assumption is further supported by the findings of Maniatis & Ingram (1971) that the tadpole haemoglobin content of single cells is higher during metamorphosis than during the prometamorphic stage, and that red cells of metamorphosing animals contain either frog or tadpole haemoglobin, but not both.

An alternative hypothesis is that the type of haemoglobin produced by the erythroid cells is related to the number of preceding cell divisions. Different types of proteins have been shown to be produced by successive generations of the same cell line in the crystalline lens and have been assumed to be synthesized by different generations of human erythroid cells (Baglioni, 1966). According to this hypothesis, the same cell line, which in the tadpole is characterized by a maturation arrest and which consequently forms end cells producing tadpole haemoglobin only, is stimulated at metamorphosis to a renewed proliferation and further maturation, thereby producing end cells capable of frog haemoglobin synthesis. Thus, tadpole and frog haemoglobins could be produced by different generations of the same erythroid cell line. In normal adults only ‘mature’, frog haemoglobin-producing cells are released into the circulation. Under conditions of erythropoietic stress, such as those occurring during the metamorphic climax, younger generations of red cells gain access into the circulation, and they produce tadpole haemoglobin in detectable amounts. This hypothesis is
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consistent with the suggested differences in maturity between tadpole and frog erythrocytes (Benbassat, 1970) and with the present findings which show a transient increase in tadpole haemoglobin synthesis concomitant with the induction of frog haemoglobin during the metamorphic climax. Further supporting evidence has been recently presented by Maclean & Jurd (1971), who showed that during anaemia *Xenopus laevis* frogs start to resynthesize one of the tadpole haemoglobins.

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