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THE REPRESSION AND INDUCTION BY THYROXIN OF HEMOGLOBIN SYNTHESIS DURING AMPHIBIAN METAMORPHOSIS

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The tadpole and adult stages of the bullfrog (*Rana catesbeiana*) have different hemoglobins. Differences in oxygen binding between the larval and adult hemoglobins have been shown by McCutcheon[1] and Riggs.[2] Several laboratories have reported different electrophoretic patterns, chromatographic elution profiles, or sedimentation constants for hemoglobin from the tadpole and frog[3–8] and a change toward the adult pattern during metamorphosis.[4, 6, 7, 9] Baglioni and Sparks[6] and Sakai *et al.*[10] found differences in the primary structure of hemoglobins obtained from pre- and postmetamorphic animals. Although the hemoglobins have been shown to consist of subunits,[6] the number of different polypeptide chains has not been established. No studies, to our knowledge, have been reported on protein biosynthesis in tadpole or frog red blood cells.

The experiments to be reported here indicate that hemoglobin can be synthesized *in vitro* by circulating red blood cells of normal tadpoles. Soon after the administration of thyroxin, hemoglobin synthesis markedly declines. Following this, an

abrupt increase in the protein synthetic capacity of circulating red blood cells occurs. The major protein induced corresponds electrophoretically to the adult frog hemoglobin.

In a recent analogous report, Tatibana and Cohen[11] demonstrated the incorporation of C^{14}-leucine into the adult protein, carbamyl phosphate synthetase, in liver slices from thyroxin-treated tadpoles.

Methods and Materials.—Larval and adult stages of *Rana catesbeiana* were purchased from the Connecticut Valley Biological Supply Co., Southampton, Massachusetts. Tadpoles were bled into cold heparinized amphibian Ringer's solution[12] and the blood cells were routinely washed three times with large volumes of cold Ringer's solution. Amino acid incorporation experiments were carried out in Ringer's solution modified to contain 1 mg/ml of glucose and 5 μg/ml of $Fe(NH_4)_2(SO_4)_2 \cdot 6(H_2O)$. Either L-lysine-$C^{14}$ (146–198 mc/mmole) or mixed amino acids as algal C^{14}-protein hydrolysate were used (New England Nuclear). The incubations with L-lysine-C^{14} contained unlabeled amino acids in the concentrations used by Borsook.[13] No unlabeled amino acids were added to the incubation medium containing algal hydrolysate. Cells were lysed completely by adding aliquots of suspension to a 0.1 vol of 2% saponin or to 2 vol of cold distilled water followed by freezing and thawing. The lysate was centrifuged at 2,000 *g* for 15 min at 2°C after mixing with carbon tetrachloride. The supernatant was spun at approximately 15,000 *g* for 20 min. Globin, prepared according to Rossi-Fanelli *et al.*,[14] was resuspended in water and all of it lyophilized. The concentration of globin was determined by a microbiuret procedure.[15]

Polyacrylamide disk gel electrophoresis was performed by the method of Davis.[16] Pyrex tubes 0.6 × 8.5 cm were used. The electrophoresis of hemoglobin was carried out in a discontinuous buffer system in which the proteins concentrate in a 3% polyacrylamide gel at pH 8.3 and separate in a $7^1/_2$% polyacrylamide gel at pH 9.5.[17] The hemoglobin was converted to methemoglobin by the addition of a 0.1 vol of 5% potassium ferricyanide. After 5 min, the hemoglobin solution was diluted with an equal volume of pH 6.7 buffer consisting of 0.064 *M* H_3PO_4, 0.118 *M* Tris, 40% sucrose (and 0.003 *M* KCN when the methemoglobin cyanide form was desired), and layered over the upper gel. Potassium cyanide was added to the buffer to make a concentration of 100 mg per liter during electrophoresis of the hemoglobin in the methemoglobin cyanide form. Electrophoresis was carried out at 2°C with a constant current of 0.5 ma per tube until the hemoglobin entered the upper gel, and then at 3 ma per tube for an additional 3 hr.

Heme-free protein was reduced and alkylated by the method of Edelman *et al.*[18] in 8 *M* urea with mercaptoethanol and thrice-recrystallized iodoacetamide. Electrophoresis of globin was done on polyacrylamide gels using a discontinuous buffer system that was designed to concentrate proteins at pH 4.0 and separate them at pH 2.3.[17] All solutions were modified to contain urea. The lower gel was made of 7% acrylamide and initially contained 0.06 *M* KOH and 4.62 *M* acetic acid in 8 *M* urea. The upper gel was made of 3% acrylamide and initially contained 0.06 *M* KOH and 0.064 *M* acetic acid in 8 *M* urea. The reservoir buffer contained 3.74×10^{-2} *M* glycine and 5.32×10^{-3} *M* acetic acid in 8 *M* urea. All urea solutions were prepared fresh from reagent-grade urea. The reduced and alkylated protein was diluted with an equal volume of twice-concentrated upper gel buffer containing 20% sucrose. The electrophoresis took place at room temperature for 1 hr at 0.5 ma per tube and then at 3 ma per tube for another 6–8 hr.

Polyacrylamide gels were sliced longitudinally, dried, and exposed to X-ray film using methods similar to those of Fairbanks *et al.*[19] A quantitative method for liquid scintillation counting of C^{14}-radioactivity of bands from transversely sliced, stained gels was developed from the technique of Young and Fulhorst[20] for counting S^{35} in a gas-flow counter. Hydrogen peroxide (30%, 0.5 ml), prewarmed to 60°C, was added to a slice of gel in a glass vial for scintillation counting, which was then quickly sealed with a rubber stopper and incubated at 60°C for 8 hr. At the end of this time, the vial was placed on ice to reduce the internal pressure, and 1 ml of 1 *M* hyamine (Pilot Chemicals, Inc.) was added through the rubber diaphragm. The vial was allowed to stand overnight to trap small amounts of liberated $C^{14}O_2$. Three ml of distilled water, followed by 15 ml of Bray's scintillator,[21] modified to contain 6 gm of 2,5-diphenyloxazole per liter, were added. The samples were counted in a refrigerated liquid scintillation spectrometer (Ansitron, Inc.).

Efficiency, as determined with an internal C^{14} and an external Cs^{137} standard, varied from 35 to 40% for different samples.

Results.—Incorporation of C^{14}-lysine by tadpole blood cells into total soluble protein: When tadpole blood cells were incubated with C^{14}-lysine, a linear time course of incorporation into total soluble protein (purified by gel filtration) was usually observed for several hours. A severe temperature inhibition was found at 37°C or above (Fig. 1). Thereafter, incubations were routinely performed at 29°C. The addition of amino acids, glucose (0–10 mg/ml), and iron (0–50 mg/ml) during a 2-hr incubation had no effect on the specific activity of soluble protein.

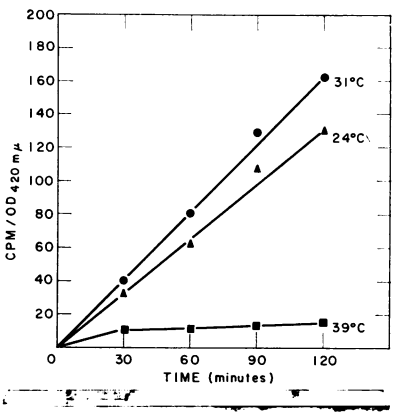

FIG. 1.—Time course of C^{14}-lysine incorporation into total soluble protein purified by thin-layer gel filtration on G-50 fine or superfine Sephadex equilibrated with 0.1 *M* $(NH_4)_2CO_3$, 0.005% KCN. Intact blood cells were incubated *in vitro* at 24, 31, and 39°C.

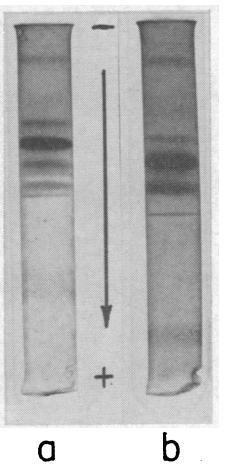

FIG. 2.—Amido black stain of polyacrylamide gels after electrophoresis with tadpole hemolysate. (*a*) Hemoglobin applied as methemoglobin cyanide complex; (*b*) hemoglobin applied as methemoglobin form.

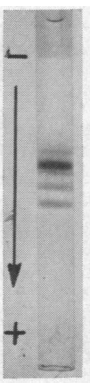

FIG. 3.—Photograph taken with a 550-mμ filter of an unstained polyacrylamide gel after electrophoresis of tadpole hemoglobin (methemoglobin cyanide).

Polyacrylamide gel electrophoresis: Gels of tadpole hemoglobin, stained with amido black (Fig. 2), show one major component and several minor ones. The very sharp leading minor component corresponds to the salt front. Five heme proteins were identified by their characteristic red color and photographed with a 550-mμ filter (Fig. 3). The minor band just trailing the major hemoglobin component produced a weaker benzidine reaction, compared to the intensity of its amido black stain, than other bands. Faint slow-moving nonheme proteins can be identified, if the gel is overloaded to the point where the hemoglobin bands are confluent. (Baglioni and Sparks[6] distinguished three tadpole hemoglobins on starch gel and four on Pevikon block electrophoresis but did not mention any nonheme proteins.)

Incorporation of C^{14}-amino acids by tadpole blood cells into hemoglobin: The incorporation of C^{14}-amino acids into different proteins separated by disk gel electrophoresis was studied both by radioautography of longitudinally sliced and dried gels and by scintillation counting of serial transverse slices. Figure 4 shows densitometer tracings of the amido black stain color and the X-ray film radioautogram made from the same gel loaded with hemolysate in the methemoglobin cyanide and

the methemoglobin form. The more intensely staining hemoglobin bands also show greater incorporation of radioactivity.

Incorporation of C^{14}-amino acids by tadpole blood cells into globin chains: Since most of the soluble protein in the carbon tetrachloride-extracted hemolysate is hemoglobin (compare Figs. 2 and 3), globin can be identified by its predominance even without the identifying heme group. The electrophoresis of reduced and alkylated heme-free protein at low pH in 8 *M* urea resulted in a simple pattern consisting of one minor fast band, a major band of intermediate mobility, and a minor slow band that sometimes appears, particularly in the densitometer tracing, only as a shoulder trailing the major band. Figure 5*a* is a densitometer tracing of the amido black-stained sliced polyacrylamide gel and its X-ray radioautogram. Most of the radioactivity corresponds to the major protein bands. Following electrophoresis, 96 per cent of the C^{14}-radioactivity applied to the gel was recovered. This value was obtained by counting an aliquot of protein as a trichloroacetic acid precipitate with albumin carrier on a gas-flow counter with corrections made for

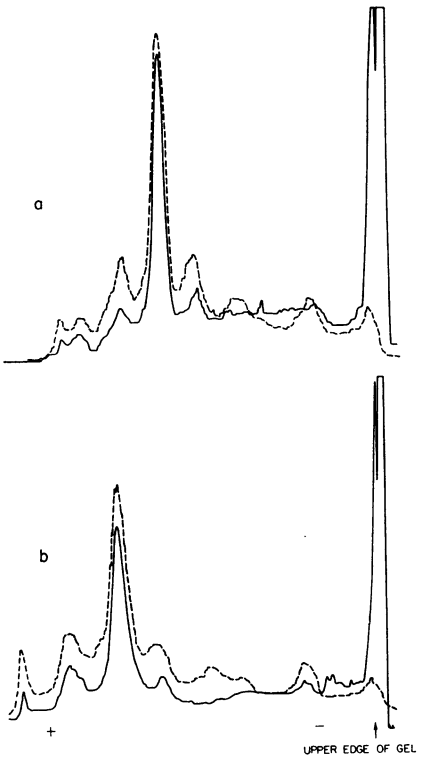

FIG. 4.—Densitometer tracings of amido black-stained polyacrylamide gels (solid lines) and X-ray radioautograms (interrupted lines). Initial solid line peak is an artifact of densitometry caused by the upper edge of the gel. (*a*) Hemoglobin applied as methemoglobin cyanide complex; (*b*) hemoglobin applied as methemoglobin form.

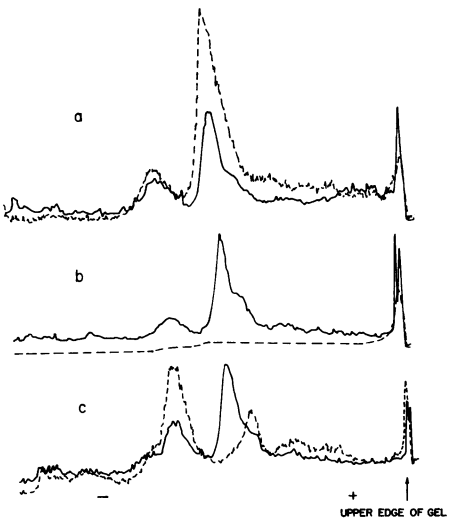

FIG. 5.—Densitometer tracings of amido black-stained polyacrylamide gels (solid lines) and X-ray radioautograms (interrupted lines). Initial solid line peak is partially an artifact of densitometry caused by the upper edge of the gel. Blood cells were incubated with C^{14}-algal hydrolysate for 2 hr at 29°C. Electrophoresis of reduced and alkylated globin on 8 *M* urea polyacrylamide gels. (*a*), (*b*), and (*c*) Incubations made with blood cells from control, 8-day thyroxin-treated and 15-day thyroxin-treated tadpoles, respectively.

self-absorption and efficiency and by counting the solubilized gels, which had been cut into 15 slices, in a scintillation counter with appropriate quench corrections; 11.8 per cent of the counts were in the upper gel and represent high molecular weight and/or slow-moving proteins or aggregates. It is believed that the protein bands in the lower gel represent globin subunits.

Qualitative effects of thyroid hormone: All tadpoles in an individual experiment came from the same shipment and were allowed at least 4 days' adjustment to a 23°C temperature. The tadpoles were immersed in 5×10^{-8} *M* L-thyroxin for varying lengths of time. A control group of tadpoles receiving no thyroxin was always included. Two hours' duplicate incubations with C^{14}-amino acids were made with pooled blood cells from 5–7 tadpoles. In the control experiment (Fig. 5*a*) the major portion of radioactivity corresponds to the major protein bands, believed to be the globin subunits. After exposure to thyroxin for 8 days, there is virtually a complete absence of incorporated C^{14}-amino acids into the globin chains (Fig. 5*b*). However, C^{14}-amino acids are still incorporated into protein of a very low mobility which has remained at the origin of the lower gel and which is probably not derived from hemoglobin. After 15 days of thyroxin treatment, an increased incorporation of radioactivity has occurred, but the pattern now is very different from the control group. Most of the radioactivity now corresponds exactly with the faster-moving globin band. A significant radioactive peak corresponds to a slow band, which appears on the densitometer tracing of the amido black stain only as a shoulder just trailing the major tadpole band. Very significantly, almost no radioactivity corresponds to the major tadpole band. TCA precipitates were made of aliquots of the protein solution prior to electrophoresis. The specific activities were 289,000 dpm/mg, 67,600 dpm/mg, and 271,000 dpm/mg for the control, 8-day thyroxin-treated, and 15-day thyroxin-treated groups, respectively. Of the radioactivity applied, 97 per cent was recovered from the gel showing thyroxin stimulation. Ten per cent of the radioactivity was in the upper gel. (The values were 96% total recovery and 11.8% in the upper gel for the control group.) No apparent qualitative difference in the protein (amido black stain) pattern was noted.

Electrophoretic comparison of tadpole and frog globin: Electrophoresis of reduced and alkylated tadpole and frog globins was done on 8 *M* urea gels (Fig. 6). It is emphasized that an individual band may represent one or more chains. The major frog band corresponds exactly with the fastest but minor tadpole band. Most of the radioactive protein induced by thyroid hormone corresponds in position to the major frog band by the highly discriminating technique of polyacrylamide disk gel electrophoresis. The slower peak of thyroid hormone-induced C^{14}-amino acid incorporation may correspond to a very faint band seen upon electrophoresis of frog globin.

Quantitative effects of thyroid hormone action: Tadpoles were treated with thyroid hormone, blood cells incubated with C^{14}-amino acids and globin prepared as before, but carrier frog hemoglobin was added prior to removing heme. In Figure 7 the exact correspondence of the major frog band with the fast tadpole band is apparent The repressive aspect of thyroxin action was studied in an experiment in which hormone was administered for up to 10 days. A measure of anatomical metamorphosis is provided by the change in the hind leg/tail ratio (Fig. 8*b*). A progressive

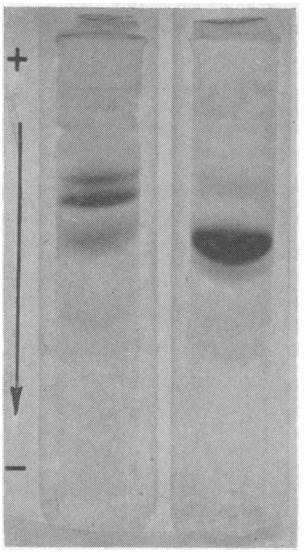

FIG. 6.—Amido black-stained 8 *M* urea polyacrylamide gels. *Left*, reduced and alkylated tadpole globin; *right*, reduced and alkylated frog globin.

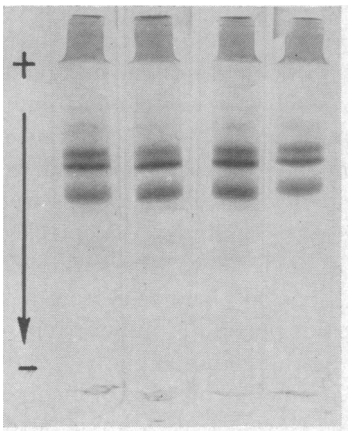

FIG. 7.—Amido black-stained 8 *M* urea polyacrylamide gels; electrophoresis with mixed tadpole and frog reduced and alkylated globin. Multiple gels are shown in order to indicate that the pattern is exactly reproduced for many samples.

fall in C^{14}-amino acid incorporation into total soluble protein purified by gel filtration occurs (Fig. 8*a*). The fall in amino acid incorporation involves all three of the major globin bands (Fig. 8*c*). The inductive aspect of thyroxin action can be seen in a separate experiment carried out for a longer period of time. Figure 8*a'* shows the fall and subsequent rise in C^{14}-amino acid incorporation into total soluble protein. The selective increase in incorporation, primarily into the major frog band, is well shown in Figure 8*c'*. The two slower and poorly separated tadpole bands were cut out of the gel as a single slice. A less dramatic rise occurs here; Figure 5*c* shows that the increase in radioactivity corresponds in electrophoretic position only to the slower band and not the major tadpole component, and therefore the line leading to this point (Fig. 8*c'*) has been dashed. The stimulation of protein synthesis occurs before the hind legs are half as long as the tail. In another single experiment, in which an average hind leg/tail ratio of 0.51 was obtained in 13 days, a more than 40-fold increase in C^{14}-amino acid incorporation into the major frog band occurred.

Discussion.—In preliminary experiments we studied the effects of thyroxin on the synthesis of whole hemoglobin. However, because of the numerous minor components (four in the tadpole and four in the frog[6]) but particularly because of the different states of aggregation of the hemoglobin[22, 7] (4.3*S*, 7.0*S*, and 10–11*S* in the frog, and 2.7*S* and 4.3*S* in the tadpole), it was decided to work with reduced and alkylated globin in 8 *M* urea.

Our results show thyroxin to exert both repressive and inductive regulatory actions. Over a 10-day period of hormone treatment, the circulating red cells lose practically all ability to synthesize hemoglobin. Although the hormone (or some metabolite) may act directly on the protein-synthesizing cells in the blood, another

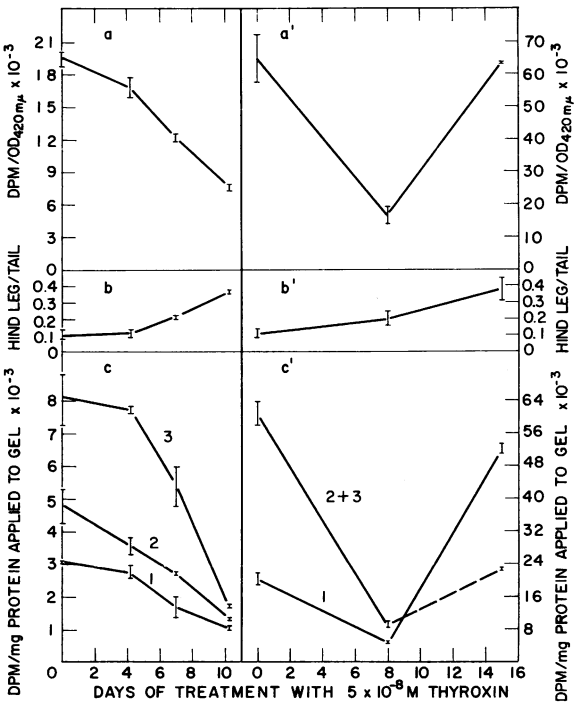

FIG. 8.—C^{14}-amino acid incorporation into tadpole and frog globins during thyroxin treatment. Unprimed and primed letters refer to two completely separate experiments. Blood cells from control and thyroxin-treated tadpoles were incubated with C^{14}-algal protein hydrolysate. (*a*) and (*a'*) Total soluble protein purified by G-50 Sephadex gel filtration. Bars indicate duplicate incubations. (*b*) and (*b'*) Hind leg to tail ratios of tadpoles. Bars indicate one standard deviation. (*c*) and (*c'*) Electrophoresis of reduced and alkylated globin on 8 *M* urea polyacrylamide gels. The bands stained with amido black were cut out, and radioactivity was measured in a scintillation counter as described in *Methods*. Bars indicate range of duplicate incubations. (*1*) Fast band corresponding to the major frog component; (*2*) major tadpole band; (*3*) slow minor tadpole band.

possibility appears more attractive. The assumption is made that despite the fact that the red cells remain nucleated, only the young cells have the capacity to synthesize hemoglobin. It has been observed that only immature chicken red blood cells can incorporate H^3-uridine or H^3-leucine.[23] A corollary of this assumption is that if new red cell production is stopped by the action of thyroxin, hemoglobin synthesis in existing red cells will decay as the cells mature. We believe that thyroid hormone acts not on the relatively mature circulating red cells, but on the precursor population. Furthermore, we suggest that during the first 10 days of hormone treatment, new red cells are not released into the circulation. By 13–15 days, a new line of cells may mature and be released into the blood. This new "adult" line of cells accounts for the new pattern of protein synthesis. The early histological observations of Jordan and Speidel[24] support this idea because they show that the kidney in the tadpole and the spleen in the frog are the major sites of erythropoiesis. During thyroid hormone-accelerated metamorphosis, increased cellular activity can be seen in the spleen.[24]

According to the model which we have presented, thyroxin, directly or indirectly, would cause the *inhibition* of proliferation of the old cell line making mostly tad-

pole hemoglobins, while simultaneously or subsequently *stimulating* the proliferation of a new cell line which synthesizes predominantly adult frog hemoglobins. We do not know whether this second cell line was already "programmed" for making frog hemoglobins before proliferation began or whether thyroxin, directly or indirectly, activated the adult structural hemoglobin genes selectively. It should be added that one must also consider the possibility that thyroxin causes red cell precursors from the kidney or elsewhere to colonize the spleen and that these cells now produce adult hemoglobin in response to thyroxin or to their new environment or both.

Summary.—The capability of normal circulating tadpole red blood cells to synthesize hemoglobin *in vitro* has been demonstrated. The rate of tadpole hemoglobin synthesis markedly decreased during the initial phase of treatment of tadpoles with L-thyroxin. Subsequently, the synthesis of adult frog hemoglobin is specifically induced. The effects of thyroxin may be direct or indirect.

We are indebted to Dr. Alexander Rich (M.I.T.) for the use of a Joyce-Loebl microdensitometer.

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