THE METAMORPHOSIS OF HEMOGLOBIN IN THE BULLFROG

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I

INTRODUCTION

The metamorphosis of the frog is at once a dramatic and subtle process, for the radical structural changes which take place are accompanied by equally striking biochemical alterations. For example, thyroxine is produced for the first time during metamorphosis. Indeed, it is well known that thyroxine administration speeds up and thyroidectomy blocks metamorphosis.

Two types of conjugated protein are now known to undergo a major change in their properties during metamorphosis: the pigments of rod vision, and hemoglobin. Wald (26) has shown that the retina of the bullfrog tadpole (*Rana catesbeiana*) contains primarily the purple light-sensitive pigment, porphyropsin, derived from vitamin A_2. During metamorphosis this is replaced by the red light-sensitive pigment, rhodopsin, derived from vitamin A_1. Porphyropsin is characteristic of fresh water fishes, rhodopsin of land vertebrates.

In 1936 McCutcheon (22) found equally striking changes in bullfrog hemoglobin, which, like the pigments of rod vision, undergoes a change from a specific tadpole hemoglobin to a quite different adult form. McCutcheon reported that during metamorphosis the hemoglobin of the tadpole undergoes three radical alterations: (1) the affinity for oxygen decreases; (2) the oxygen equilibrium function (percentage saturation *vs.* oxygen pressure) changes from a rectangular hyperbola to an S-shaped curve; (3) in adult hemoglobin the oxygen affinity increases with increased pH (the Bohr effect), whereas the opposite occurs prior to metamorphosis, increased pH lowering the oxygen affinity (reversed Bohr effect).

Many other studies have been reported dealing with differences between the adult and fetal hemoglobins of various animals. These include such diverse properties as the rate of denaturation by alkali (5), rate of spreading in mono-

* I am greatly indebted to Professor George Wald for suggesting this problem and for guidance in its investigation. The experiments were supported in part by a grant to Professor Wald by the Medical Sciences Division of the Office of Naval Research. I wish to express my indebtedness also to Professor Jeffries Wyman for many helpful suggestions and advice and to Mr. Karl F. Guthe for help with a number of problems.

molecular films (18), crystal form and solubility (19, 30), and molecular structure as revealed by x-rays (20).

Among hemoglobins, high resistance to alkali has been correlated with slow spreading in monomolecular films. It is believed that both spreading and alkaline denaturation involve the destruction of hydrogen bonds between adjacent polypeptide chains. In man, fetal hemoglobin has a higher resistance to alkali than adult; in contrast, the fetal form in the cow, sheep, and goat is less resistant than that of the adult (18).

These differences have in some instances been correlated with x-ray data on structure. Thus Kendrew and Perutz (20) have shown that adult sheep methemoglobin consists of a single asymmetric unit of molecular weight 68,000, which shows no tendency to dissociate upon dilution. On the other hand, fetal sheep methemoglobin consists of four identical or almost identical subunits of molecular weight 17,000, and there is a considerable tendency for this molecule to dissociate into quarters upon dilution.

This property of splitting has an important bearing upon the oxygen equilibrium. When horse hemoglobin is split into halves—by exposure to strong solutions of urea (7) or sodium chloride (2)—the oxygen affinity rises sharply and the oxygen equilibrium curve becomes more hyperbolic (29).

If a hemoglobin possesses a single heme, or if its hemes are wholly independent of one another, the reaction with oxygen should be described in terms of the simple equilibrium, $Hb + O_2 \rightleftharpoons HbO_2$, for which the mass action equation is

$$K = \frac{(HbO_2)}{(Hb)(pO_2)};$$

here the plot of fractional oxygenation,

$$\frac{(HbO_2)}{(HbO_2) + (Hb)}, \textit{ vs. } pO_2$$

is a hyperbola. However, mammalian blood hemoglobins have S-shaped, not hyperbolic, oxygen equilibrium curves. It is now well known that the existence of an S-shaped oxygen equilibrium curve requires that (1) the hemoglobin molecule must possess more than one heme; and that (2) the hemes must interact; *i.e.*, the attachment of oxygen to one heme must increase the likelihood that oxygen will attach to a neighboring heme.

Hill (17) first proposed that the S-shaped curve might be explained on the basis of the reaction $Hb + nO_2 \rightleftharpoons HbO_{2n}$ for which the mass action equation is

$$K = \frac{(HbO_{2n})}{(Hb)(pO_2)^n}.$$

This equation can be transformed into the form,

$$\frac{y}{100} = \frac{Kp^n}{1 + Kp^n},$$

in which y is the percentage saturation, and p is the oxygen pressure. However, mammalian hemoglobin measurements indicate that n is about 2.8, a number which can have no simple physical significance in a mass action equation. This constant has, however, retained significance as an empirical index of the degree of interaction between adjacent hemes. Since Hill's equation does not hold well at high or at low saturations, n is best evaluated in the neighborhood of half saturation. When n equals 1 there is no interaction, and the oxygen equilibrium curve is hyperbolic. A value of n greater than 1 results in an S-shaped curve, and indicates heme-heme interaction. Thus a 4-heme molecule might yield a hyperbolic curve if its hemes ceased to interact, or if three hemes were inactivated (as by oxidation), or if the molecule were split into quarters. Frequently, it might be difficult to decide among these alternatives. The subject of heme-heme interaction, first developed by Pauling (23), has been extended and discussed in detail by Wyman (29).

These considerations raise a number of questions about the nature of the change observed by McCutcheon (22) in bullfrog hemoglobin during metamorphosis. First, in analogy with myoglobin, if the tadpole oxygen equilibrium curve is hyperbolic the molecule might be suspected to possess a single heme and have the molecular weight 17,000; alternatively, it could have a molecular weight of 68,000 and possess wholly independent hemes. In the one case metamorphosis would have resulted in a quadrupling of the molecule, in the other in the establishment of interaction among the hemes. The first experiments described in this paper were carried out to decide between these possibilities.

II

The Molecular Weight of Tadpole Hemoglobin

The sedimentation rate of tadpole hemoglobin was determined in the ultracentrifuge with solutions prepared as described below.[1] The red cells, washed in 0.65 per cent NaCl, were hemolyzed with distilled water. After centrifugation the supernatant was diluted with phosphate buffer and the sedimentation rate of the hemoglobin was measured. The data are shown in Table I.

The sedimentation constant (S_{20}) of 4.9 units is virtually identical with the best values obtained for mammalian hemoglobins, and indicates a molecular weight of about 68,000. It entirely excludes the possibility that any large

[1] These measurements were made by Professor J. L. Oncley of the University Laboratory of Physical Chemistry related to Medicine and Public Health, Harvard University, to whom I am very grateful.

proportion of tadpole hemoglobin consists of molecules of molecular weight 17,000 (S_{20} about 2). These solutions had a small fraction (5 to 10 per cent) of other material, including two components with sedimentation constants of 7 and 2; the nature of these substances is obscure.

TABLE I

Sedimentation Constant of Tadpole Hemoglobin

Buffer: 0.1 M phosphate (Na_2HPO_4 + KH_2PO_4).

pH	Hb	Sedimentation constant
	per cent	
7.19	0.30	4.9
7.19	0.56	4.9
6.64	1.50	4.9
6.64	2.07	4.4

III

The Oxygen Equilibrium

A. Apparatus and General Procedure.—

A vessel was designed which contained an optical absorption cell at its base.[2] This cell was 0.17 mm. in depth, and was arranged to be examined in the Beckman spectrophotometer, Model DU, for which a special holder was designed. The vessel and holder are illustrated in Fig. 1.

Briefly, the technique is as follows: A solution of hemoglobin in the vessel is reduced by evacuation, and its absorption spectrum is measured. A small, known amount of air is injected into the vessel by syringe through the rubber plug, and the vessel is equilibrated by rotation in a water bath at constant temperature. Its spectrum is again measured. By repeating this process with increasing amounts of air, families of spectra are obtained. Fig. 2 shows such data recorded in initial measurements with cattle hemoglobin. The fractions of oxy- and reduced hemoglobin can readily be determined from such spectra. The oxygen pressure is calculated from the quantity of gas injected, and the known volume of the vessel.

Animals.—The tadpoles were obtained in Massachusetts, and the adults from Louisiana. Both were identified specifically as *Rana catesbeiana*. One adult is sufficient for one experiment, but a dozen tadpoles were generally needed for each experiment. The adults measured 160 to 180 mm. in body length. The tadpoles averaged 42 mm. in body length, 113 mm. in total length, and the hind legs averaged about 5 mm. No external forelegs were present in any tadpoles.

Preparation of Blood.—The blood of either tadpole or adult is removed from the

[2] I wish to acknowledge my indebtedness to Mr. Allyn P. Kidwell, with whom I designed this vessel. The absorption cell itself was constructed by the Pyrocell Manufacturing Company of New York.

ventricle of the heart with a hypodermic syringe containing 4 per cent sodium citrate. Enough citrate is placed in the syringe so that the ratio of citrate to blood is at least 1:10. About 5 ml. of blood are obtained from an adult frog, and 0.2 to 0.3 ml. from each tadpole. The citrated blood is immediately saturated at 0°C. with carbon monoxide, and the cells are washed at least three times with 0.65 per cent NaCl. Hemolysis of about 50 per cent of the cells is accomplished within 2 hours, by the addition of distilled water at 0°C. Longer periods of hemolysis were found to result in noticeable denaturation of tadpole hemoglobin. High speed centrifugation is necessary to remove a small amount of material remaining in suspension. A small fraction of this appears

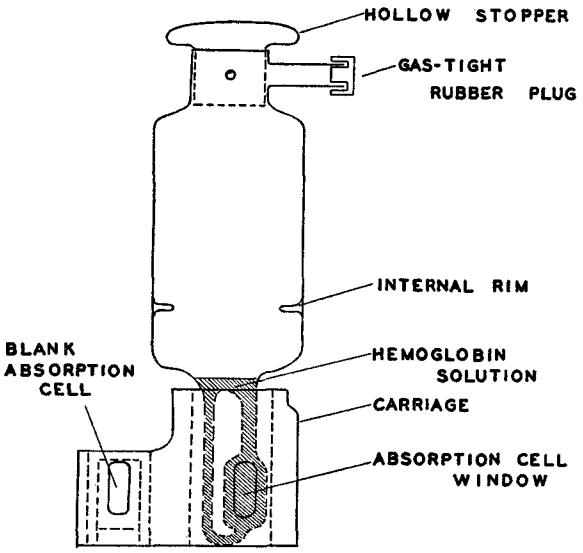

FIG. 1. The tonometer with cell holder for measurements in the Beckman spectrophotometer. The vessel is initially evacuated and measured amounts of air are injected through the rubber plug to obtain an array of oxygen pressures.

to be denatured hemoglobin; the rest is colorless. The supernatant is mixed with suitable buffer, and is centrifuged again to remove the last traces of cellular debris.

Buffers.—The following buffers were used. Phthalate buffer (50 ml. 0.2 M potassium acid phthalate + 23.7 ml. 0.2 N NaOH) was mixed with an equal volume of hemoglobin solution to give a pH of 5.0. At this pH oxidation takes place and results in inaccuracy in the oxygen equilibrium curves. It seems probable that the phthalate also has a more specific effect; the day following a phthalate experiment, the hemoglobin had partly precipitated. Between pH 5.5 and 7.4, 0.2 M K_2HPO_4-KH_2PO_4 or Na_2HPO_4-KH_2PO_4 buffers were mixed with equal volumes of hemoglobin. A borate-borax mixture was used at pH 8.4 (1 ml. Hb solution + 3 ml. of a buffer mixture of 4.5 ml. of 0.5 M $Na_2B_4O_7$ and 5.5 ml. of 0.2 M H_3BO_3). A glycine-sodium hydroxide buffer was used at pH 9.0 (70 ml. of 0.1 M glycine + 30 ml. of 0.1 N NaOH have a pH of 9.7; equal volumes of buffer and tadpole hemoglobin solution yield a final pH of 9.0).

Technique of Measurement.—Solutions prepared in this way are introduced into the tonometer (volume, 474 ml.), which is held in a horizontal position. The vessel is evacuated to a total pressure of about 25 mm., washed with nitrogen, and equilibrated at 20°C. by rotation at 20 R.P.M. in a water bath for 10 minutes. This process is repeated at least three times. The presence of a strong light facilitates the removal of the carbon monoxide. If the vessel is not kept in a horizontal position until evacuated the solution wets the absorption cell and evacuation causes large bubbles to form in the cell which are difficult to remove.[3]

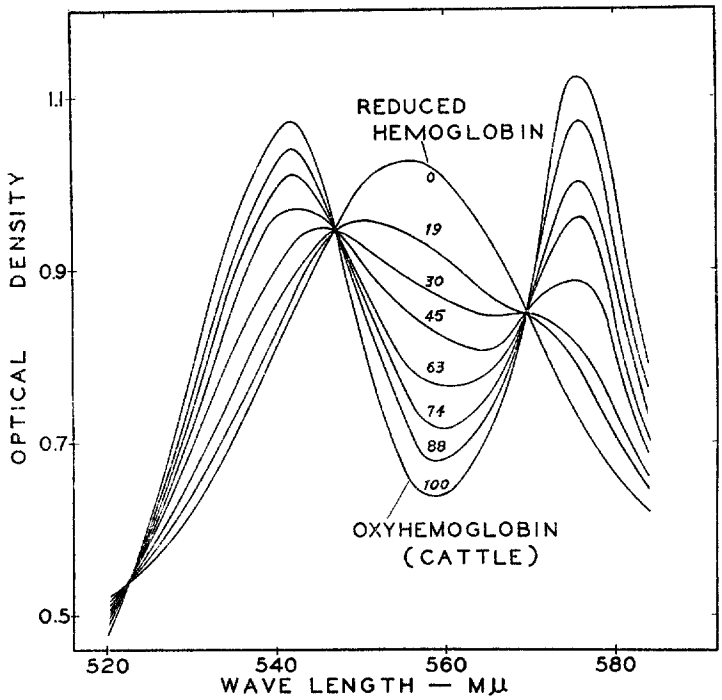

FIG. 2. A family of absorption spectra of cattle hemoglobin in various states of oxygenation. The numbers show the percentage oxygenation associated with each curve.

After complete reduction, the vessel is placed in the spectrophotometer, and an initial spectrum is run. For the determination of oxygen equilibria, a large change in absorption is desirable. Ideally, measurements might be made at two wave lengths at which the absorption changes in opposite directions upon oxygenation. However, in practice the advantage of using two wave lengths is often outweighed by a serious disadvantage. In a region of the spectrum in which the absorption changes rapidly

[3] Unless the cell is extremely clean, it does not fill completely. Cleaning is accomplished by running a succession of cleaning agents through the tube, using a rubber-tipped pipette connected with an aspirator to speed the passage of the solutions.

with wave length, a very small change in wave length setting can result in a large error in optical density. There is some advantage therefore in avoiding resetting the wave length by working at one wave length.

Large differences in extinction between oxy- and reduced hemoglobin occur at 410 and 430 mμ in the Soret band, at 558 mμ in the green, and at 700 mμ in the red. It is not possible to obtain reproducible readings in the Soret band unless a very narrow slit width is used and the wave length knob is never touched after the initial setting. Oxygen equilibrium curves measured at any of these wave lengths are identical with one another. The curves obtained with cattle hemoglobin in initial tests were also identical within experimental error with those obtained gasometrically on mammalian hemoglobin (12).

After determining the absorption spectrum of the reduced hemoglobin, a calibrated syringe with a No. 27 needle is used to introduce a known amount of air into the tonometer. Simultaneous measurements of barometric pressure, temperature, and humidity permit the calculation of the oxygen pressure within the vessel. For concentrated solutions a correction must be made for oxygen absorbed by the hemoglobin. However, the volume of the tonometer is so much greater than that of the hemoglobin that this correction can usually be neglected.

The percentage saturation, y, can be obtained by using the formula,

$$\frac{y}{100} = \frac{K_r - K}{K_r - K_o},$$

in which K is the optical density measured and K_r and K_o are the optical densities of reduced and oxygenated hemoglobin at the same wave length. The oxygen pressure may be determined by the relation,

$$pO_2 = \frac{T}{V}\left(\frac{P_0 V_0}{T_0} - nR\right);$$

pO_2 is the oxygen pressure, T the temperature in the tonometer, T_0 the room temperature, V the volume of gas in the tonometer, V_0 the volume of air injected, n the number of moles of oxygen combined with hemoglobin, and R the gas constant. $P_0 = 0.21\ (P - Hp)$, in which P is the barometric pressure, H the relative humidity, and p the vapor pressure of water. The amount of oxygen physically dissolved in the solution is negligible.

The concentration of hemoglobin was measured after the experiment by liberating the oxygen from oxyhemoglobin with potassium ferricyanide in standard Warburg manometric vessels. The concentration of hemoglobin corresponded to 6 to 12 $\times$ 10^{-7} moles O_2 per ml. solution. This is about 1 to 2 per cent hemoglobin on the basis of a molecular weight of 68,000, with 4 combining centers per molecule of hemoglobin. The molar extinction coefficient, ϵ,[4] is about 1.26 $\times$ 10^7 cm.^{2} per mole oxygen at 560

[4] The molar extinction coefficient $\epsilon = \frac{1}{cl}\log_{10}\frac{I_0}{I}$, in which c is the concentration, here on the basis of combined oxygen; l is the depth of absorbing layer; and I_0 and I are the light intensities transmitted through the blank and through the solution.

$m\mu$ for reduced tadpole hemoglobin, and 0.705×10^7 for tadpole oxyhemoglobin. The figures are essentially the same in the adult bullfrog.

B. The Shape of the Oxygen Equilibrium Curve.—The procedure outlined above was the result of a gradual evolution. Initially, reproducible curves were not obtained. Oxygen equilibrium curves for tadpole hemoglobin yielded curves of variable shape and position; the value of n varied between 1.0 and 2.3. These

TABLE II

Oxygen Equilibrium of Tadpole Hemoglobin

Barometric pressure	765 mm. Hg.
Relative humidity	0.70.
Room temperature	23°C.
Bath temperature	20°C.
Oxygen capacity	6.88×10^{-7} moles O_2/ml. solution.
Volume of solution	4 ml.
Buffer	0.1 M K_2HPO_4 – KH_2PO_4.
pH	7.32.

Air added	Optical density (440 mμ)	pO_2	y (per cent HbO_2)
ml.		*mm. Hg*	
0	0.655	0.0	0.0
2	0.652	0.66	0.9
4	0.646	1.33	2.7
6	0.618	1.99	11.1
8	0.592	2.65	18.9
10	0.562	3.32	27.9
12	0.510	3.98	43.5
16	0.458	5.30	59.2
20	0.412	6.62	73.0
30	0.365	9.95	87.2
40	0.345	13.3	93.2
56	0.333	18.6	96.7
Room air	0.322	157.6	100.0

variations were found to be associated with the following conditions: (1) exposure to atmospheric oxygen during preparation resulted in a drop in the value of n, the magnitude of which was a function of the duration of exposure; (2) preparation at room temperature or shaking accelerates this process. Apparently adult hemoglobin is more stable than that of the tadpole; adult hemoglobin never gave hyperbolic curves under conditions which produced this result in tadpole hemoglobin.

This situation is of great importance for understanding the status of oxygen equilibrium curves of hemoglobin. Both the value of n and the pressure at half saturation, p_{50}, tend to decrease as hemoglobin deteriorates. The tendency

to deteriorate apparently differs widely from one animal to another. It is difficult to avoid some deterioration in tadpole hemoglobin, and a consequent drop in n and p_{50}. On the other hand, careful procedures give the highest n and p_{50} values, and these alone are significant. This situation may be compared with the forcing upward of a melting point on purification of a compound; here again only the highest value is significant.

TABLE III

Oxygen Equilibrium of Adult Bullfrog Hemoglobin

Barometric pressure	760 mm. Hg.
Relative humidity	0.50.
Room temperature	23°C.
Bath temperature	20°C.
Oxygen capacity	7.5×10^{-7} moles O_2/ml. solution.
Volume of solution	4.0 ml.
Buffer	Borate-borax mixture (see text).
pH	8.4.

Air added	Optical density		pO_2	y (per cent HbO_2)
	430 mμ	440 mμ		
ml.			*mm. Hg*	
0	1.65	0.718	0.0	0.0
4	1.62	0.708	1.33	2.3
8	1.62	0.699	2.66	3.4
13	1.55	0.653	4.3	14.0
18	1.38	0.602	6.0	28.3
23	1.26	0.542	7.6	42.0
28	1.11	0.490	9.3	56.2
33	0.973	0.441	10.9	69.0
38	0.890	0.402	12.6	78.5
43	0.840	0.382	14.3	83.5
53	0.788	0.354	17.6	89.8
Room air	0.692	0.311	157.4	100.0

By carrying out the preparation of hemoglobin rapidly in an atmosphere of carbon monoxide at 0°C., consistent results were obtained in which n was about 2.3 for the tadpole and 2.8 to 2.9 for the adult. It was thought for a time that this difference is real. When, however, it was observed that one group of tadpoles possessed somewhat brownish blood, suspicion was cast on all specimens. The diseased tadpoles had been kept, apparently healthy, for 3 to 4 months, and had been fed boiled spinach, lettuce, and wheat flakes. Spectral examination of the blood revealed that about 2/3 to 3/4 of the hemoglobin was apparently in the form of methemoglobin. It is possible that the diseased condition was due to an ascorbic acid deficiency, for it has been reported that methemoglobinemia is associated with this condition (3).

Fresh tadpoles yielded quite different results; no significant differences in n remained between them and adults. Tadpoles were collected in Woods Hole, Massachusetts, and the blood was removed from them within 1 to 2 days after capture. This blood was immediately saturated with carbon monoxide in the cold, and the entire experiment, from the time of drawing the blood, was com-

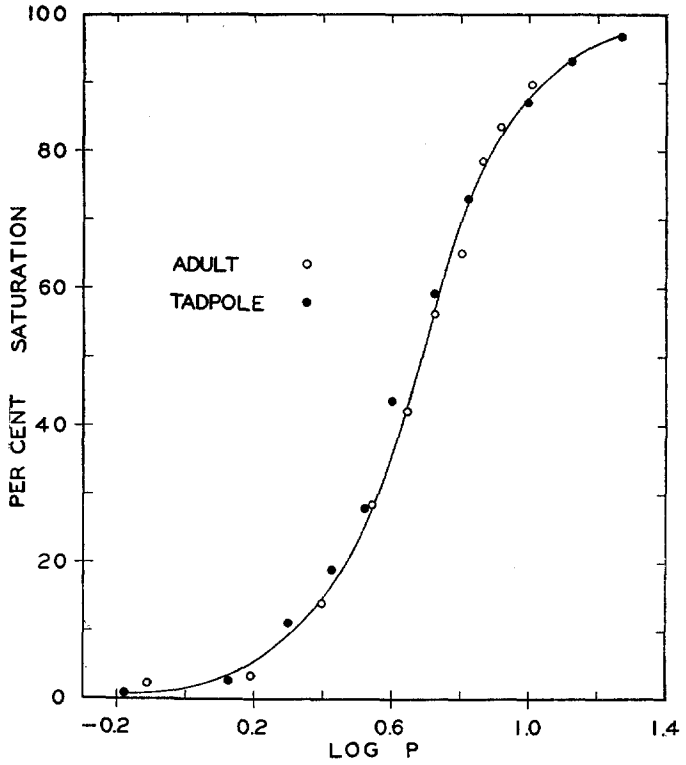

FIG. 3. A comparison of the oxygen equilibria of tadpole and adult hemoglobins. The adult curve has been moved along the abscissa (logarithm of the oxygen pressure) to coincide with the tadpole curve. It is clear that both sets of points lie on a single curve.

pleted within 12 hours. In these experiments, it became clear that n is about 2.8 in tadpoles as well as in adults.

The data for two experiments, one on tadpole hemoglobin, the other on adult hemoglobin, are shown in Tables II and III. These data may be compared in the following way. It has been shown repeatedly that oxygen equilibrium curves of mammalian hemoglobin obtained at different pH's can all be superimposed by simple displacement along the abscissa, when the fractional saturation is

TABLE IV

Oxygen Equilibria of Tadpole and Adult Hemoglobins at Various pH's

pO_2 is the oxygen pressure in mm. Hg; y is the percentage oxygenation. 20°C.

Tadpole

pH 6.28		pH 7.32		pH 9.0	
pO_2	y	pO_2	y	pO_2	y
mm. Hg		*mm. Hg*		*mm. Hg*	
0.67	0.27	0.66	0.9	0.67	3.2
1.34	7.0	1.33	2.7	1.34	8.0
2.01	18.6	1.99	11.1	2.02	16.0
2.67	31.3	2.65	18.9	2.69	24.0
3.34	44.7	3.32	27.9	3.36	37.2
4.02	56.9	3.98	43.5	4.04	48.1
5.35	71.7	5.30	59.2	5.37	60.8
6.68	83.3	6.62	73.0	6.71	72.1
8.69	90.0	9.95	87.2	10.1	87.5
12.1	96.0	13.3	93.2	13.4	93.3
16.0	98.4	18.6	96.7	18.1	96.2

Adult

pH 5.0		pH 5.89		pH 6.00		pH 6.18		pH 6.54		pH 6.94	
pO_2	y	pO_2	y	pO_2	y	pO_2	y	pO_2	y	pO_2	y
mm. Hg		*mm. Hg*		*mm. Hg*		*mm. Hg*		*mm. Hg*		*mm. Hg*	
10.1	32.8	13.1	33.0	13.1	19.6	13.3	13.4	10.2	10.6	5.0	0.0
16.8	68.9	19.6	50.2	26.2	53.6	26.6	47.4	17.0	34.8	11.7	9.9
23.3	89.9	26.2	71.0	32.8	72.2	33.3	61.4	23.7	46.9	21.7	44.0
				39.4	83.6	40.0	72.1	30.5	60.6	26.7	59.5
								37.3	72.0	33.4	73.8

pH 7.22		pH 7.40		pH 8.40	
pO_2	y	pO_2	y	pO_2	y
mm. Hg		*mm. Hg*		*mm. Hg*	
6.6	21.4	6.7	19.8	1.33	2.3
13.1	50.0	13.4	52.6	2.66	3.4
16.4	61.5	20.2	74.0	4.3	14.0
23.0	78.5			6.0	28.3
				7.6	42.0
				9.3	56.2
				10.9	69.0
				12.6	78.5
				14.3	83.5
				17.6	89.8

plotted against the logarithm of the pressure, rather than against the pressure itself (4, 6, 11, 1). In terms of Hill's equation, this means that n remains constant, though K varies with pH.

This procedure has been followed in Fig. 3. The results indicate clearly that the shape of the oxygen equilibrium curve is virtually identical in both adult

TABLE V

Summary of Data on the Relations between pH, Oxygen Pressure at 50 per cent Saturation (p_{50}), and the Value of n in Hill's Equation

Animal	pH	p_{50}	n
Aquarium tadpoles	6.28	2.8	2.24
	7.00	3.0	2.40
		3.4	2.14
		3.5	2.35
		3.6	2.32
	8.40	3.5	2.15
		3.5	2.30
Average		3.3	2.27
Fresh tadpoles	6.28	3.6	2.53
	7.32	4.6	2.77
	9.0	4.2	2.20
Average		4.1	2.50
Adults	(5.0	13.5	~4)*
	5.89	19.5	2.8
	6.0	25.0	3.4
	6.18	27.8	2.4
	6.54	24.5	2.4
	6.94	23.5	2.8
	7.22	13.2	2.4
	7.40	13.5	2.4
	8.4	8.5	2.8
Average		—	2.68

* These measurements made in phthalate buffer are suspect for reasons discussed in the text.

and tadpole. There is no suggestion that the tadpole has a hyperbolic curve. Its value of n, 2.8, is closely similar to the values between 2.8–3.0 obtained in the adult.

C. The Effect of pH upon the Oxygen Equilibrium.—The oxygen equilibrium function has been measured at different pH's in the tadpole and adult. The data for these experiments are summarized in Tables IV and V. In Table IV

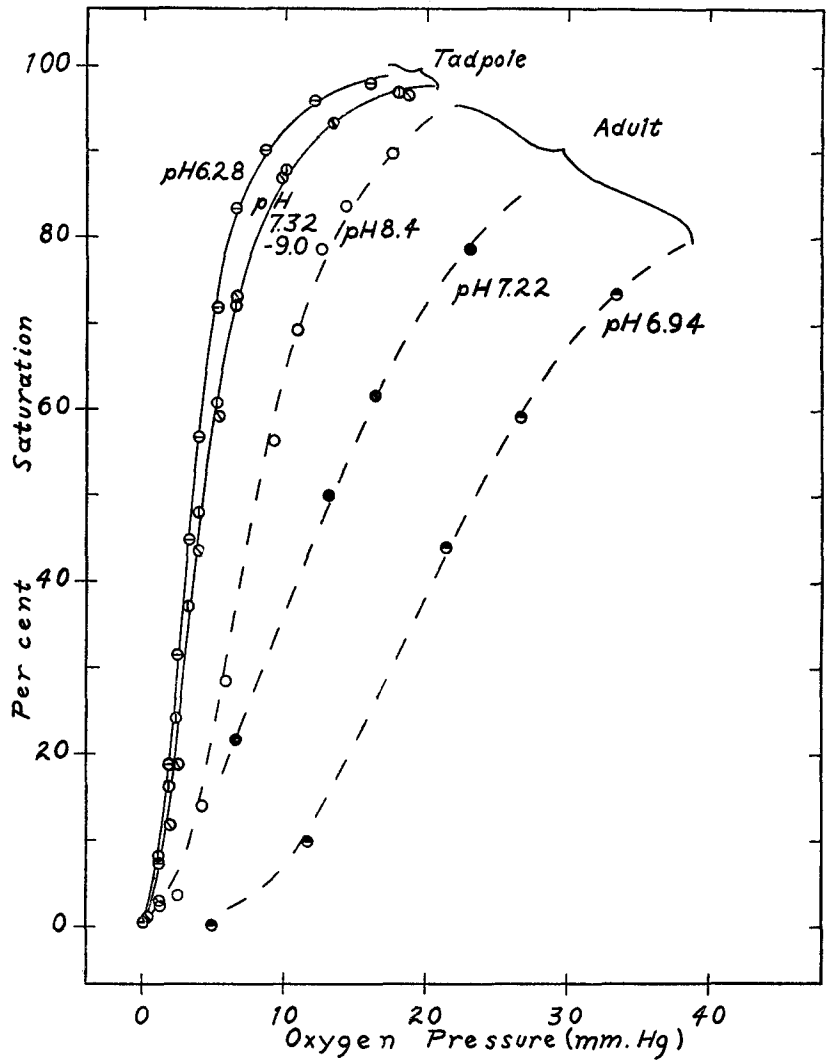

FIG. 4. The effect of pH upon the oxygen equilibrium of tadpole and adult frog hemoglobin. The hemoglobin of the tadpole, high in oxygen affinity and practically unaffected by pH, is replaced after metamorphosis by an adult hemoglobin with low oxygen affinity and a large pH, or Bohr, effect.

are given the data for the experiments performed with fresh material; Table V includes the early data obtained from tadpoles which were kept for 3 to 4 months. The interpretation of the differences between fresh tadpoles and those kept for long periods in the laboratory is discussed in the previous section.

The results of these experiments bring out two remarkable differences between adult and tadpole hemoglobins. First, there is an enormous difference in oxygen affinity, confirming the work of McCutcheon (22). This is clearly shown in Figs. 4 and 5. The adult hemoglobin is half saturated at oxygen pressures between 8 to 27 mm. Hg, depending upon the pH, whereas the tadpole hemoglobin is half saturated at about 3.5 to 4.5 mm. Hg. Second, the oxygen

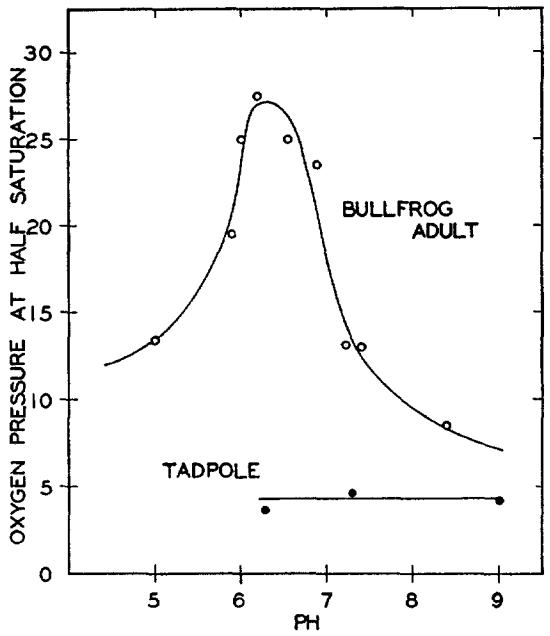

FIG. 5. The variation with pH of the oxygen pressure at half saturation (p_{50}). Change in pH has marked effect in the adult, but almost none in the tadpole. The adult hemoglobin has a minimum oxygen affinity at pH 6.2. Above this pH there is a large Bohr effect, while below it there is a reversed Bohr effect, as in mammalian hemoglobin. The measurements at pH 5.0 were made in phthalate buffer and are suspect for reasons discussed in the text.

equilibrium of tadpole hemoglobin appears to be affected very little if at all by variations in pH.

It is true that at pH 6.3 the data indicate a small drop in the oxygen pressure at half saturation—a reversed Bohr effect, such as McCutcheon's data also indicate. I believe however that in these experiments this effect is due to modification of the hemoglobin. The absorption spectrum of tadpole hemoglobin in even mildly acid solutions indicates the presence of an appreciable fraction (up to 10 per cent) of methemoglobin. This is sufficient to bring about a consider-

able increase in oxygen affinity and also a decrease in the value of n. For these reasons I believe the minor variations observed are probably not significant.

IV

DISCUSSION

The foregoing observations show that in tadpole hemoglobin the hemes interact with one another in exactly the same way as they do in adult bullfrog and mammalian hemoglobin. Since the oxygen equilibrium of tadpole hemoglobin is hardly affected by pH, whereas both adult bullfrog and mammalian hemoglobins are strongly affected, no relation appears to exist between heme-heme interaction and the Bohr or pH effect, at least between pH 6 and 9.

This conclusion has a number of interesting implications. Henderson first suggested an explanation of the pH or Bohr effect in mammalian hemoglobin (16). He supposed that there is an acid group linked with the heme in such a way that oxygenation strengthens its acidity. That is, oxygenation causes protons to dissociate more readily from the group. Conversely, it must be true that a decrease in hydrogen ion concentration is accompanied by increased oxygenation. Henderson estimated that the increased acid strength of oxygenated hemoglobin in the physiological range could be accounted for by a shift from 7.6 to 6.7 in the pK of the acid group. The existence of oxygen-linked acid groups is further supported by the work of Hastings *et al.* (14) and German and Wyman (13), and now seems to be well established.

The nature of these groups, however, is not so clear. It was first suggested by Conant in 1932 (9) that they might be imidazole residues of histidine. Thirty-three groups are known to be titrated between pH 5 and 9 in horse hemoglobin (8) and this is identical with the number of histidine residues determined analytically. Wyman has measured the heat of dissociation of the groups titrated between pH 5 and 9 by measuring the effect of temperature upon the titration curve of oxyhemoglobin, and has obtained the value 6200 cal./mole (27). By measuring the variation of the heat of oxygenation with pH, Wyman also determined the heat of dissociation of the oxygen-linked acid groups in horse hemoglobin to be 6500 cal./mole (28). Such heats of dissociation are characteristic of imidazoles.

Roughton (25), however, has pointed out a number of objections to the imidazole hypothesis. His more direct calorimetric measurements of the heat of dissociation of protons from the oxygen-linked groups in cattle hemoglobin yielded values some 2,000 calories higher than those obtained by Wyman. These higher values Roughton considers characteristic of amino groups. Roughton believes there is a direct carbamino combination of carbon dioxide with the oxygen-linked acid groups. So far as is known, imidazole does not form carbamino compounds, and therefore, if Roughton's view is correct, imidazole could not be identified with the oxygen-linked acid groups.

Coryell and Pauling (10) have proposed an ingenious electronic explanation of the Bohr effect. This theory is based upon their magnetic measurements (24), and assumes the validity of the imidazole hypothesis. The measurements indicate that reduced hemoglobin is paramagnetic whereas oxyhemoglobin is not, and that reduced hemoglobin contains four unpaired electrons per iron atom, as do ferrous ions. Oxyhemoglobin possesses no unpaired electrons. In completely covalent bonding there is no paramagnetism, and it is therefore concluded that the iron bonds of reduced hemoglobin are ionic, whereas those of oxyhemoglobin are covalent. With this information and the imidazole hypothesis, Coryell and Pauling showed that one of the important resonant structures of imidazole would bear a resemblance to the pyridinium cation, $= N - H^+$, which is acidic. On the other hand, in reduced hemoglobin, the most important resonating structure would have the characteristics of a basic amine, $-\ddot{N} - H$. Thus oxygenation would increase the dissociation of protons.

If such a formulation is accepted for mammals or for adult frogs, it cannot be correct for the tadpole, since in the tadpole oxygenation does not affect the acidity appreciably. Furthermore, if Lemberg's hypothesis (21) of heme-heme interaction *via* these same imidazole groups be correct for mammals, it would seem to imply that heme-heme interaction should be different in the tadpole. Actually tadpole and mammalian hemoglobins appear to have identical or almost identical heme-heme interactions, as judged from the value of n. This casts some doubt on Lemberg's hypothesis.[5]

Whatever linkage of heme to globin is postulated for the tadpole, involving imidazole, amino, or carboxyl groups, it seems clear that the nature of this linkage must undergo some fundamental change during metamorphosis. This change affects specifically the oxygen affinity and introduces a new sensitivity to pH, while leaving the heme-heme interaction unaltered. We may say in accordance with the above discussion that during metamorphosis heme becomes acid-linked, or that acid groups to which the heme is already bound become sensitive to oxygenation. The intimate mechanism of these changes is still obscure.

CONCLUSIONS

1. Tadpole and adult hemoglobin do not differ significantly in molecular weight. The molecular weight of both is in the neighborhood of 68,000.

2. Heme-heme interaction as measured by the value of n in Hill's equation is virtually the same—about 2.8—in both tadpole and adult.

[5] The present observations bear also upon Haurowitz's ideas (15) concerning the formation of aquo complexes in various types of hemoglobin, and their connection with the Bohr effect.

3. There appears to be no significant effect of pH upon the oxygen equilibrium of tadpole hemoglobin, in contrast to large Bohr and reverse Bohr effects in the adult. This is taken to mean that during metamorphosis acid groups of globin become sensitive to the oxygenation of heme by some change in the mode of linkage between heme and globin.

4. The oxygen affinity of tadpole hemoglobin is about seven times as great as that of the adult at pH 6 and twice as great at pH 9.

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