THE EFFECT OF ORGANIC PHOSPHATES ON THE ALLOSTERIC PROPERTY OF Rana catesbeiana HEMOGLOBINS*

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SUMMARY

Difference in the oxygen affinity between tadpole and frog hemoglobins of Rana catesbeiana was investigated in relation to the interaction of organic phosphates with the hemoglobin. The oxygen affinity of both hemoglobins increased remarkably by the stripping procedure. The addition of DPG, ATP and IHP which were proved to be present in the erythrocytes, to the stripped hemoglobins resulted in notable decrease of the oxygen affinity, except that IHP showed little effect on the frog hemoglobin. Affinity constants of organic phosphates for the hemoglobins were obtained from the oxygen equilibrium data. IHP was found to have the highest affinity for tadpole hemoglobin, while DPG was the highest for frog hemoglobin.

Functional and chemical differences between Rana catesbeiana tadpole and frog hemoglobins have been reported from several laboratories (1-5). One of the functional differences is that the tadpole hemoglobin shows higher affinity for oxygen than does the frog hemoglobin. On the other hand, it has been reported by Benesch and Benesch (6,7) that organic phosphates, such as DPG and ATP which are normally present in human erythrocytes, interact with the hemoglobin to regulate its functional property, thus decreasing the oxygen affinity significantly under physiological condition.

In view of these facts, it would be of particular interest to know whether the chemical difference of the tadpole and frog hemoglobins per se is responsible for the difference in oxygen affinity and how are these hemoglobins regulated by organic phosphates.

Abbreviations; ATP; adenosine triphosphate, DPG; 2,3-diphosphoglycerate, IHP; inositol hexaphosphate (phytic acid), P; phosphorous.

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We have now conducted an experiment to determine the content of three organic phosphates, DPG, ATP and IHP in the erythrocytes, and to examine the effect of these organic phosphates on the oxygen affinity of tadpole and frog hemoglobins. Results of experiments along this line are presented here.

MATERIALS AND METHODS

Hemolysates prepared from tadpole and frog erythrocytes by the method previously described (3), were passed through the column (2.5 x 30 cm) of Sephadex G-25 equilibrated with 0.1 M NaCl solution to strip the hemoglobins of endogenous organic phosphates and small molecules according to Benesch and Benesch (7). The hemoglobin fraction eluted from the column was further treated with Sephadex G-25 column equilibrated with 0.1 M Tris-HCl buffer, pH 7.0. The stripped hemoglobin thus obtained was proved to be completely free of phosphates. All procedures above mentioned were performed below 4°. Oxygen equilibrium curves were determined at 23° as previously reported (8). Oxygen affinity was expressed in terms of P_{50} which is assigned to the partial pressure of oxygen at half saturation and heme-heme interaction coefficient was represented by n in Hill equation, $Y = Kp^n / (1 + Kp^n)$.

DPG and ATP contents in the erythrocytes were enzymatically estimated by the method of Krimsky (9) and of Adams (10), respectively. IHP content was determined according to Ohshima et al.(11), except that phosphorous concentration was estimated by the method of Ames and Dubin (12).

RESULTS AND DISCUSSION

As can be seen in Figs. 1 and 2, the oxygenation curves measured with tadpole and frog hemolysates were each shifted to left by the Sephadex treatment without any change on the shape of the curve. P_{50} was found to decrease by the stripping process from 6.0 mmHg to 3.1 mmHg in the tadpole and from 33 mmHg to 11 mmHg in the frog hemoglobin. This suggests by analogy with human hemoglobin that some organic phosphates which facilitate unloading of oxygen might have been removed by the stripping process. Since the oxygen affinity of the tadpole hemoglobin was still higher than that of the frog hemoglobin even after stripping, it is most likely that the difference in the oxygen affinity between the tadpole and frog hemoglobins is essentially based on a difference between their structures.

The ability of DPG, ATP and IHP to reverse the increased oxygen affinity of both tadpole and frog hemoglobins was tested. It appears from Figs. 1 and 2 that DPG and ATP are each effective to decrease oxygen affinity of the stripped tadpole and frog hemoglobins, and it is also noteworthy that IHP which is known to be the major portion of the organic phosphates in bird and turtle bloods (7,

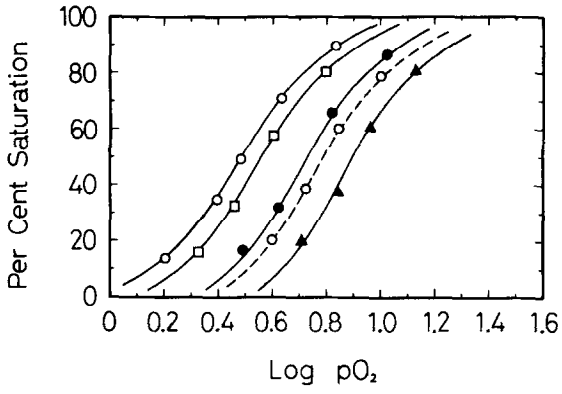

Fig. 1 Effect of organic phosphates on the oxygen equilibria of tadpole hemoglobin.
o———o, stripped hemoglobin; □———□ , ●———●, and ▲———▲ , ditto in the presence of 0.1 mM DPG, ATP and IHP, respectively; o------o, hemolysate. All the curves were drawn theoretically from Hill equation as n = 2.7. Hemoglobin concentration, 0.012 mM in 0.1 M Tris-HCl buffer, pH 7.0, 23°.

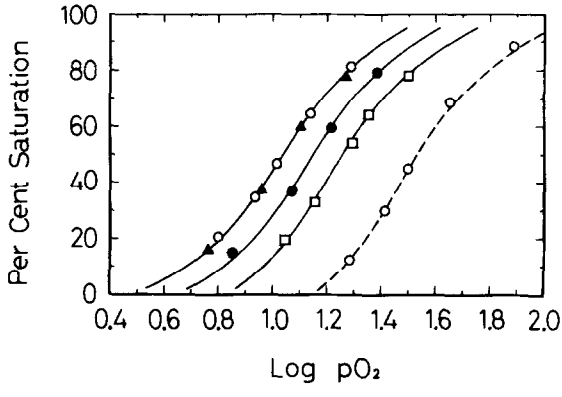

Fig. 2 Effect of organic phosphates on the oxygen equilibria of frog hemoglobin.
Symbols and conditions are the same as those in Fig. 1.

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13) has a profound effect on the oxygen affinity of the tadpole hemoglobin, whereas it does not reveal any effect on that of the frog hemoglobin.

The oxygen affinities (log P_{50}) of the tadpole and frog hemoglobins are shown as a function of the concentration of organic phosphates in upper part of Figs. 3 and 4. IHP was the most sensitive effector for the tadpole hemoglobin and enhanced log P_{50} about 2 fold at 0.5 mM. ATP followed next, giving a rise in log P_{50} to almost the same extent as IHP. DPG was the least sensitive, however, it still increased log P_{50} near by the hemolysate level at a concentration of about 2 mM.

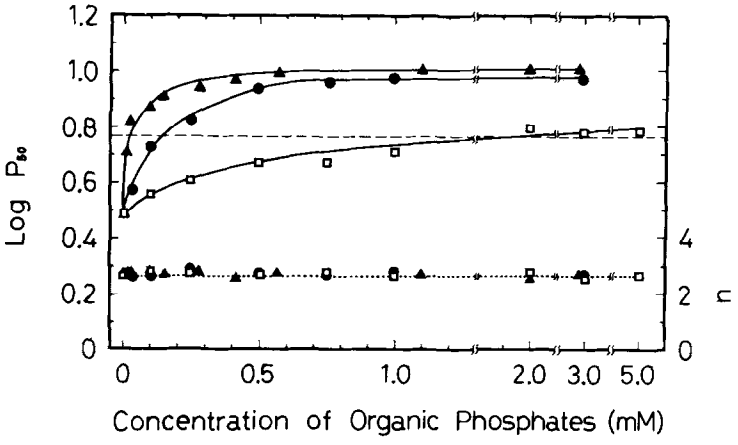

Fig. 3 Dependence of log P_{50} and n value of tadpole hemoglobin on the concentration of organic phosphates.
□, DPG; ●, ATP; ▲, IHP. Solid line and dotted line stand for log P_{50} and n value, respectively. Measurements were made with the same condition as in Fig. 1, except phosphate concentration. Broken line in the figure indicates log P_{50} level of tadpole hemolysate.

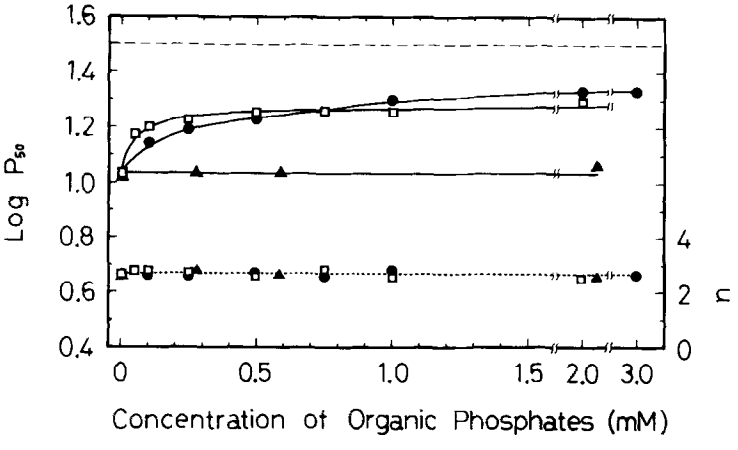

Fig. 4 Dependence of log P_{50} and n value of bullfrog hemoglobin on the concentration of organic phosphates. Symbols and conditions are the same to Fig. 3, except that broken line in the figure represents log P_{50} level of frog hemolysate.

On the other hand, log P_{50} of the frog hemoglobin was enhanced by DPG and ATP about 20 % at a concentration of approximately 0.75 mM, respectively. It is also characterized in Fig. 4 that the maximum values of log P_{50} of the frog hemoglobin in the presence of DPG and of ATP were found to be much lower than that of the frog hemolysate, whereas log P_{50} of the stripped tadpole hemoglobin in the presence of ATP and IHP strikingly increased to pass over its hemolysate level. The reason remains obscure. Unlike DPG and ATP, IHP which was the

most effective on the oxygen affinity of tadpole hemoglobin did not show any significant effect on the frog hemoglobin within the concentration range where DPG and ATP were effective. Interaction coefficient retained constant value (n = 2.6 - 2.8) independent of the change in the oxygen affinity of both tadpole and frog hemoglobins as shown in lower part of Figs. 3 and 4.

Aggarwal and Riggs (14) mentioned similar effect of DPG on the oxygen affinity of adult frog hemoglobin, in conformity with our results. However, little is said about the effect of DPG on the tadpole hemoglobin and the physiological significance of the phosphate in the bullfrog erythrocytes. Considering the striking effect of the phosphates mentioned above on the bullfrog and tadpole hemoglobins, an attempt was made to estimate the content of organic phosphates in their erythrocytes.

Distribution of acid soluble organic-P in the erythrocytes of tadpole and frog are listed in Table I. The concentration of the total organic-P and of ATP-P in the frog erythrocytes was consistent with the value reported by Rapoport and Guest (13), as shown in the parenthesis. In contrast to their report, however, a considerable amount of DPG and IHP was found to be present in both erythrocytes.

It is also noted from the Table I that ATP is a major organic phosphate amounting to 60 - 70 per cent of total organic phosphate in both tadpole and frog erythrocytes, although DPG constitutes the major portion of the organic phosphates of most mammalian erythrocytes (6,7), and that the concentration of each organic phosphate in the tadpole erythrocytes was as high as roughly two times

Table I. Distribution of acid soluble organic phosphates in the erythrocytes of Rana catesbeiana*

		Total Organic P	ATP-P	DPG-P	IHP-P
Frog	mg/dl**	40.3 (45.3)	28.0 (20.9)	9.3	5.4 (0)
	%	100	69.5	23.1	13.4
Tadpole	mg/dl**	97.6	55.8	19.4	8.9
	%	100	57.2	19.9	9.1

* Numbers in the parenthesis were cited from Reference (15)
** Volume of packed erythrocyte

of that in the frog erythrocytes. Molar concentration of each phosphate was calculated as follows; ATP, 6.02 mM; DPG, 3.13 mM; IHP, 0.48 mM for the tadpole erythrocytes, and ATP, 3.01 mM; DPG, 1.5 mM; IHP, 0.29 mM for the frog erythrocytes. Total amount of the organic phosphates as allosteric effectors which influence the oxygen affinity of the hemoglobins was calculated to be 9.6 mM and 4.5 mM in the tadpole and frog erythrocytes, respectively. Since the hemoglobin content in the erythrocyte was obtained to be 4.84 mM for tadpole and 3.5 mM for frog (15), amount of the effector in each erythrocyte which is required for the control of the oxygen affinity physiologically, appears to be sufficient, if all the phosphates act concertedly on the hemoglobin molecule.

According to Benesch and Benesch (7), affinity constants ($-\log K_2$) of the organic phosphates for the hemoglobins were obtained from the equation, $\log K_3 = \log K_1 + \log K_2$, where K_1 and K_3 are the equilibrium constants for the oxygenation reaction of the hemoglobin in the absence and presence of the organic phosphates, respectively. Calculations were made with the experimental value of $n = 2.7$ and of P_{50} (16,17). Results are summarized in Table II.

It can be seen from the table that IHP has the highest affinity to the tadpole hemoglobin followed by ATP and DPG in decreasing order, whereas DPG the highest to the frog hemoglobin followed by ATP. This remarkable difference in the responsibility to the phosphates between the tadpole and frog hemoglobins could primarily be ascribed to the molecular transition from tadpole form to frog form which occurs during metamorphosis.

It can also be pointed out that the affinity constants of the phosphates for both hemoglobins have somewhat lower values as compared with those for human hemoglobins, and that the affinity constant of organic phosphates decreases from tad-

Table II. Affinity constant ($-\log K_2$) of organic phosphates for hemoglobins

	DPG	ATP	IHP
Tadpole Hb	3.25	3.75	4.83
Frog Hb	3.01	2.97	—— *
Fetal Hb**	4.6	4.9	
Adult Hb**	5.5	5.8	

* not calculated because of little effect on the P_{50}
** Reference (9)

pole to frog by metamorphosis, whereas that for human hemoglobin receives a distinct increase ontogenically according to Tyuma and Shimizu (16,17).

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