Molecular Physiology, 2 (1982) 263–272
Elsevier Biomedical Press

CONTROL OF HEMOGLOBIN FUNCTION IN THE SALAMANDER, *AMBYSTOMA TIGRINUM*

STEPHEN C. WOOD[a], REED W. HOYT[a,b] and WARREN W. BURGGREN[c]

[a]*Department of Physiology, University of New Mexico, School of Medicine, Albuquerque, NM 87131;* [b]*Department of Hematology, Children's Hospital, Philadelphia, PA 19104 and* [c]*Department of Zoology, University of Massachusetts, Amherst, MA 01003, U.S.A.*

(Received 10 October 1981, accepted 10 February 1982)

Key words: Hemoglobin; Organic phosphate; Red cell pH; Metamorphosis; (Salamander)

Summary

The oxygen affinity of blood in the salamander, *Ambystoma tigrinum*, is quite low (P_{50} approx. 40 mmHg) and, unlike previously studied amphibians, does not change following metamorphosis from aquatic (gilled) forms to the terrestrial (gill-less) forms. In further contrast to previously studied poikilotherms, blood O_2 affinity increases (to approx. 25 mmHg) following cold (5°C) acclimation. In response to hypoxia, the nucleated red cells of this species, like those of fish, show a higher O_2 affinity (P_{50} approx. 25 mmHg) and lower organic phosphate concentration. The cellular mechanisms of these changes in O_2 affinity are partially explained by the allosteric effect of the major organic phosphates in *A. tigrinum* red cells (ATP and 2,3-diphosphoglycerate). The indirect effect of organic phosphates on the Donnan distribution of H^+ is pronounced (intraerythrocytic pH increases from 7.1 to 7.6 following organic phosphate depletion). However, due to the very low Bohr effect ($\Delta \log P_{50}/\Delta pH = -0.08$) this change in cell pH has a negligible effect on O_2 affinity of hemoglobin. Consequently, half of the decreased P_{50} of blood following hypoxia and cold acclimation is not explained by the effects of reduced organic phosphates.

Introduction

The cellular mechanisms that control hemoglobin (Hb) function are much better defined for mammalian red cells than for the nucleated red cells of other vertebrate groups. Although, organic phosphates in both types of red cells have, qualitatively, the same effect in reducing O_2 affinity (Benesch and Benesch, 1967; Gillen and Riggs, 1971;

0166-3178/82/0000–0000/$02.75 © 1982 Elsevier Biomedical Press

Wood, 1971; Wood and Johansen, 1972), the metabolic pathways producing the organic phosphates are not the same in nucleated and anucleated red cells (cf. Schweiger, 1962). One important difference is the oxygen dependence of organic phosphate production. The major organic phosphate of mammalian red cells, 2,3-diphosphoglycerate, is produced by anaerobic glycolysis while the dominant organic phosphates in nucleated red cells usually depend on oxidative phosphorylation for their synthesis (e.g., ATP). One important effect of this difference, relative to Hb function, is the opposite effects of hypoxia on organic phosphate levels.

The present study examines the control of Hb function in *Ambystoma tigrinum*. This salamander was selected because its Hb must function over a wide range of environmental and organismic conditions, and because its red cells, unlike those of most poikilotherms, contain, both 2,3-diphosphoglycerate and ATP as potential allosteric controllers of Hb. The variables we examined were metamorphosis, temperature acclimation and hypoxia. Our aim was to define the cellular mechanism(s) of changes in the respiratory properties of blood due to these variables.

Methods and Materials

Animals. Ambystoma tigrinum larvae ($n = 77$) were collected from ponds in southern and northern New Mexico. They were kept in the laboratory in filtered and aerated aquaria at 20°C for at least 3 weeks before being studied. Metamorphosis is facultative in this species and sexually mature animals of larval phenotype (neotenic adults, with gills and keeled tail) are common. The neotenic adults that underwent metamorphosis after collection provided the transformed adults ($n = 33$). Body mass ranged from 39 to 150 g in both neotenic and transformed adults.

Whole blood measurements. Blood was obtained in heparinized syringes from the exposed truncus arteriosus of animals killed by rapid pithing. The short time required to sample blood (30–45 s) resulted in blood gas and pH values that did not differ significantly from those obtained from cannulated animals in a previous study (Burggren and Wood, 1981).

Hemoglobin concentration was measured as cyanmethemoglobin. Hematocrit was measured after centrifugation in a micro-hematocrit centrifuge at 15 000 × g for 5 min. Oxygen capacity was measured as the O_2 content of blood after equilibration with 25% O_2 for 10 min (BMS-2 tonometer). O_2 content was measured with a fuel cell (Lex-O2-Con, Lexington Inst. Co., Waltham, MA).

Oxygen dissociation curves were measured using a continuous spectrophotometric method (Hem-O-Scan; American Inst. Co.). The instrument was modified to avoid certain inherent problems. A new sample holder was constructed to accomodate a micro-pH probe (MI-410; Microelectrodes, Inc.) with the tip positioned in a drop of blood adjacent to the the smear used for saturation measurements. The reliability of this technique was initially checked by simultaneous tonometry of blood (BMS-2) for construction of pH = f (pCO_2) curves. The sample chamber was found to have large air leaks during operation due to the presence of a faulty seal around the stirring fan. This was corrected by fitting a heavy duty O-ring gasket. The Bohr factor (Δ log P_{50}/Δ pH) was determined in individual blood samples by changing the pCO_2 in the matched set of equilibration gases.

Red cell pH was estimated by centrifuging blood in capillary tubes, freeze-thawing the tubes to lyse the red cells, and measuring the pH of the plasma and the hemolysate (cf. Battaglia et al., 1965). This was done with both fresh blood from normoxic animals and blood from hypoxic animals with depleted organic phosphate levels.

The concentrations of 2,3-diphosphoglycerate and ATP were measured using enzymatic methods (Sigma Chemical Co.). The ATP test actually measures all nucleoside triphosphates (NTP) including GTP, an important allosteric effector of Hb in some species. According to Bartlett (1976), the major component of NTP in *A. tigrinum* is ATP.

Hemoglobin solutions. Hemoglobin solutions were prepared by lysing (with distilled water and gentle sonication) red cells that had been washed twice in cold 0.6% NaCl. They were purified by gel chromatography (AcA 54 Ultragel). Analysis of the 'stripped' Hb for organic phosphates showed that about 98% of the organic phosphate was removed by this procedure.

The oxygen dissociation curve of hemoglobin solutions was measured as described above except that interference filters of 439 and 448 nm were substituted for those used with whole blood in order to increase sensitivity. The pH of the solutions was adjusted to desired values with bis-Tris, a buffer that has little effect, per se, on O_2 affinity over a 10-fold concentration range (Benesch and Benesch, 1967).

Changes in Hb composition following metamorphosis of enivronmental acclimation, a possible mechanism of blood O_2 affinity changes, was checked by electrophoresis (cellulose acetate strips).

Results

Effects of metamorphosis. The respiratory properties of blood in neotenic and transformed adults are almost identical. As described by Burggren and Wood (1981), the O_2 affinity of blood is quite low (P_{50} approx. 400 mmHg) in the neotenic adults and unaffected by transformation to the terrestrial adults. This result, while unexpected, is consistent with the lack of significant change in the total organic phosphate concentration following metamorphosis. There was also no change in the electrophoretic pattern of hemoglobin with metamorphosis.

Effects of temperature acclimation. Temperature acclimation had two unusual effects on the properties of *A. tigrinum* blood. In contrast to previous studies in poikilotherms (where cold acclimation resulted in a right-shifted oxygen density curve, cf. Wood, 1980), the blood of neotenic adults had a left-shifted oxygen density curve (P_{50} = mmHg, pH 7.7, 25°C) after acclimation to 5°C, while the blood of adults showed no effect of acclimation on O_2 affinity (P_{50} = 39.3 and 41.4 mmHg, pH 7.7, 25°C for cold- and warm-acclimated animals; cf. Burggren and Wood, 1981). These findings are also consistent with the lack of change in organic phosphate concentration in adult blood. For neotene blood, two possible mechanisms can account for the increased O_2 affinity, since there was a decrease in organic phosphate level (from 19.7 to 11.9 μmol NTP + diphosphoglycerate/ g Hb) and an increase in methemoglobin (to 16.5%) following cold acclimation.

Effects of hypoxia. Neotenic adults were exposed to a hypoxic environment in covered aquaria for 8 days. The pO_2 was about 30 mmHg in water and 90 mmHg in air.

As shown in Fig. 1, this resulted in a marked left-shift of the oxygen density curve, with P_{50} decreasing from 41 to 25 mmHg after 2 days.

An expected result, based on previous studies of hypoxia acclimation in animals with nucleated red cells, was the decrease in red cell ATP (from 10 to 6.6 μmol/g Hb) as a result of hypoxia. An unexpected result, based on previous studies of hypoxia acclimation in mammals, was the significant decrease in 2,3-diphosphoglycerate (from 13 to 6 μmol/g Hb). There was no hematopoietic response (or hemoconcentration) evident in this 8 day period.

Properties of hemoglobin solutions. The oxygen affinity of *A. tigrinum* blood increased substantially upon lysis and even further upon 'stripping' of organic phosphates.

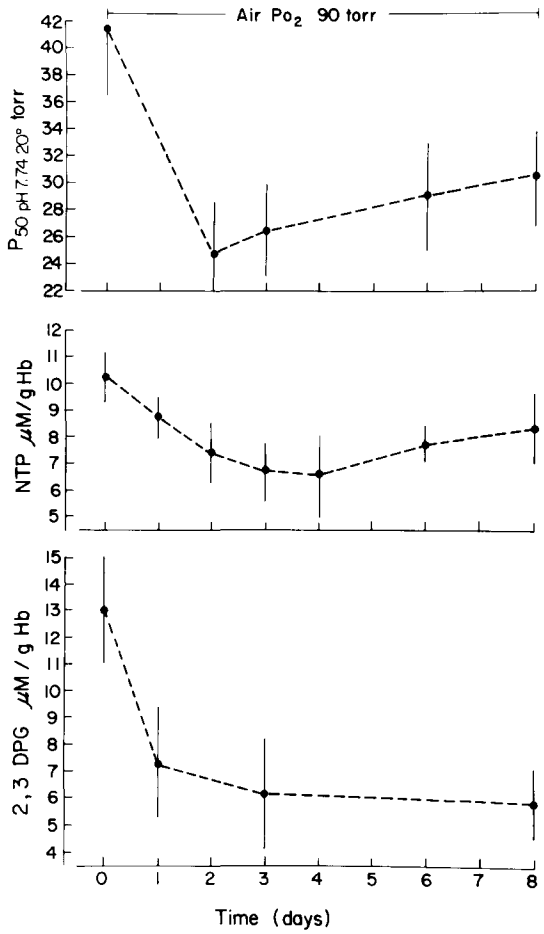

Fig. 1. Time course of changes in blood P_{50} (pH 7.74, 20°C) and red cell NTP and 2,3-diphosphoglycerate (2,3 DPG) concentrations during exposure to hypoxic air and water. Initial points are control (normoxic) values. Vertical bars are ± 1 S.D.; n = 30.

These findings, consistent with those reported by others, have several potential mechanisms, discussed below, that are also the potential mechanisms for the changes in the O_2 affinity of blood observed during hypoxia and cold acclimation of neotenes.

Allosteric control of O_2 affinity. Anions decrease the O_2 affinity of Hb by binding preferentially to deoxyHb thus stabilizing hemoglobin in the deoxy form and lowering the affinity for O_2. Organic phosphates compete with chloride, and other anions, for the O_2-linked binding sites. However, on a molar basis, organic phosphates are about 1000-fold more effective (Benesch and Benesch, 1967). Consequently, they can regulate O_2 affinity without significantly affecting the osmotic equilibrium of red cells.

A. tigrinum red cells have two major organic phosphates, 2,3-diphosphoglycerate and ATP. Both proved to be effective allosteric controllers of O_2 affinity. As shown in Fig. 2, addition of either ATP or 2,3-diphosphoglycerate to stripped Hb increased the P_{50}. The quantitative effect (maximum change in logP_{50}) was low compared with that reported for other hemoglobins (see Discussion) but the same for both ATP or 2,3-diphosphoglycerate. The maximum increase in P_{50} was from 15 to 19 mmHg at a ratio of 1 mol organic phosphate/1 mol Hb tetramer ($\Delta \log P_{50} = 0.088$). However, when a mixture of ATP and 2,3-diphosphoglycerate was added to stripped Hb, the effect on P_{50} (at the same molar ratio of total organic phosphate) was less then either ATP or 2,3-diphosphoglycerate alone. Further work is needed to explain the mechanism of this apparent negative interaction of ATP and diphosphoglycerate.

Hydrogen ions have almost no effect on O_2 binding. In *A. tigrinum* Hb, the Bohr

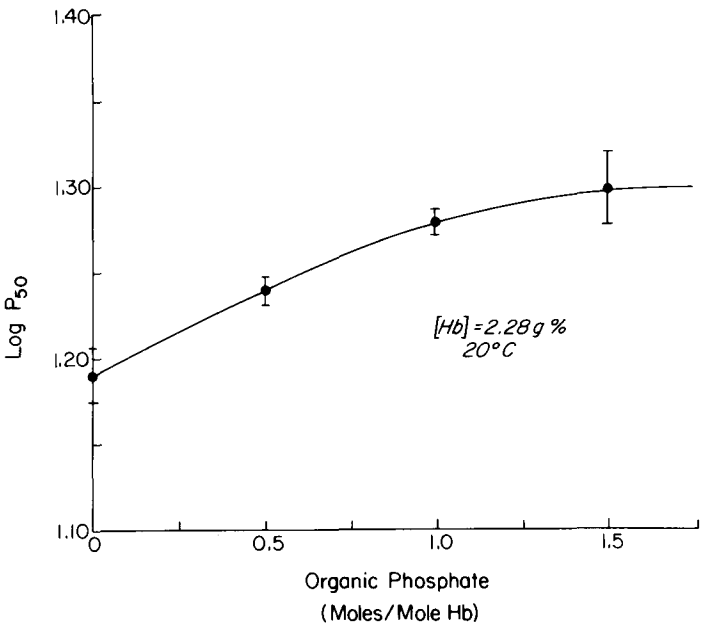

Fig. 2. Effects of added 2,3-diphosphoglycerate or ATP on O_2 affinity of *A. tigrinum* Hb. In bis-tris buffer at pH 7.08, 20° C. The P_{50} values of molar ratios of 1.0 and 1.5 are not significantly different.

effect ($\Delta \log P_{50}/\Delta$ pH) was only -0.083, not significantly different from that of whole blood (-0.078 for neotenes and -0.072 for transformed adults). To illustrate how miniscule this Bohr factor is, if *A. tigrinum* blood were acidified from pH 7.8 to 6.8 the P_{50} would increase from 40 to 48 mmHg. In contrast, blood with a Bohr factor of -0.48 (typical mammalian value) acidified by the same amount would have a P_{50} increase from 40 to 121 mmHg.

Donnan equilibrium of hydrogen ions. In addition to their allosteric (direct) effect on O_2 affinity, organic phosphates produce a Donnan effect on the distribution of hydrogen ions across the red cell membrane. This creates a lower pH inside red cells than in plasma (Funder and Weith, 1966) that alters O_2 affinity via the Bohr effect (cf. Steen and Turitzen, 1968). The pH gradient across the red cell membrane is proportional to the concentration of organic phosphates (Duhm, 1971; Wood and Johansen, 1973).

The effect of organic phosphate concentration on the red cell pH gradient in *A. tigrinum* blood is illustrated in Fig. 3. Red cells containing roughly normal concentrations of ATP and 2,3-diphosphoglycerate (10.3 and 13 μmol/g Hb) have an intracellular pH of about 7.1 when plasma pH is 7.8. Depletion of organic phosphate to roughly half these values (as occurred during hypoxia) increases the intracellular pH to 7.6. Also, as seen in Fig. 3, the magnitude of the pH gradient depends on plasma pH. Thus, the indirect effect of organic phosphate will increase with factors, e.g., lower temperature, that elevate plasma pH.

Discussion

Interpreting the significance of oxygen dissociation curves

Before discussing the results of this study it is appropriate to consider certain caveats

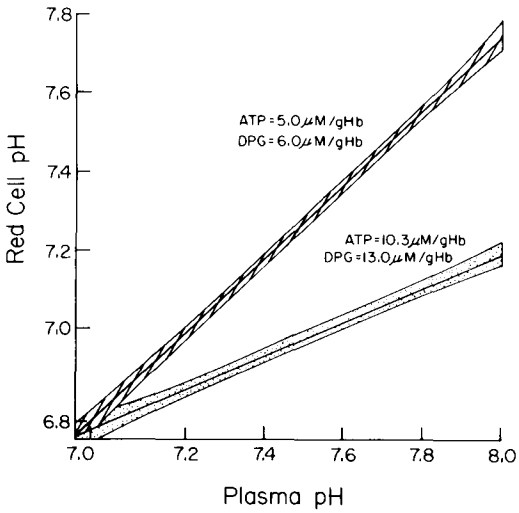

Fig. 3. Effect of organic phosphate concentration on the pH difference between plasma and red cells. Bands indicate 95% confidence limits for the following regression equations: High organic phosphate level, red cell pH = 3.4 + 0.48 plasma pH; $r = 0.97$, $n = 11$; Low organic phosphate level red cell pH = 0.078 + 0.96 plasma pH; $r = 0.99$, $n = 4$.

relevant to oxygen dissociation curves in general. Quantitative descriptions of oxygen binding by hemoglobin solutions or blood usually include P_{50}, Hill's coefficient or 'n', O_2 capacity, Bohr coefficient, temperature coefficient, etc. These data are informative and useful, per se. However, they often form the basis for physiological interpretations, i.e., 'adaptive' properties. This is unwarranted in most cases since, without data on arterial and mixed venous blood gases and pH, nothing can be said about the physiological significance of a particular oxygen dissociation curve. Likewise, whether a shift of the oxygen dissociation curve to the left or the right is adaptive or not is only determined by direct measurements of the effects of the shift on O_2 delivery to the tissues. Such data are difficult to obtain, particularly with small animals.

Oxygen dissociation curves in A. tigrinum blood

With the above caveat in mind it is possible to partially assess the significance of the oxygen density curve in *A. tigrinum*. Arterial blood gases and pH, but not mixed venous, have been measured in this species (Burggren and Wood, 1981). At 25°C, arterial pO_2 was 50–80 mmHg in neotenes kept in aerated water but with free access to air (they are obligate air-breathers at 25°C). The pO_2 in the lungs was approx. 100 mmHg under these conditions. The large (20–50 mmHg) difference in pO_2 between lung air and arterial blood in this species, and others with central vascular shunts, is an example of the difficulty in interpreting the significance of oxygen density curves.

Since the P_{50} of blood in neotenes (and transformed adults) is about 400 mmHg, an arterial pO_2 of 50 corresponds to a saturation of only 60%. Thus, at the lower end of the range of in vivo arterial pO_2 values, the upper end of the oxygen density curve is not used in the systemic circulation. At first glance, this could be interpreted as blood having a deleteriously low O_2 affinity. It appears reasonable to argue that this species, having arterial pO_2 as low as 50 mmHg due to shunting, would be better off with a left-shifted curve resulting in higher arterial saturation. Likewise, the left-shift of the oxygen density curve during hypoxia certainly seems adaptive in that 'loading' of O_2 would be improved.

However, the effect of a shift in the oxygen density curve in the presence of low arterial pO_2 depends on the source of the hypoxia. If the low arterial pO_2 were due only to environmental hypoxia then, indeed, a left-shifted oxygen density curve would be adaptive in promoting O_2 'loading' in the lungs. However, in this case the arterial hypoxemia is due to shunting of venous blood into the arterial circulation as well as low environmental pO_2. When deoxygenated and oxygenated bloods mix in a closed system (syringe, in vitro; central vascular shunt, in vivo) the pO_2 of the resulting mix is a dependent variable of the % saturation. This, of course, is opposite to the situation in open systems where % saturation is the dependent variable of pO_2. Consequently, a higher O_2 affinity would not alleviate the problem of low arterial saturation in *A. tigrinum* blood. For a given degree of shunt, a left-shifted oxygen density curve would, in theory, lower arterial pO_2 and worsen the problem. This hypothesis was recently tested and supported by computer models of gas exchange and by direct measurements in other species with central vascular shunts (Turek and Kreuzer, 1981; Wood, 1982).

Control of red cell function

Metamorphosis had no effect on the O_2 affinity of blood. This was unexpected in the light of previous studies of metamorphosis in both anuran and neotenic urodele amphibians. The ontogeny of Hb function in the bullfrog is well-known (cf. Riggs, 1951). In both the axolotyl (*Ambystoma mexicanum*) and the ambystomid salamander, *Dicamptodon ensatus*, the oxygen density curve is also right-shifted after metamorphosis (Gahlenbeck and Bartels, 1970; Wood, 1971). As discussed below, two factors may account for the apparent absence of the 'fetal-maternal' shift.

One basis for the similar oxygen density curves of neotenes and transformed adults is that *A. tigrinum* larvae undergo two metamorphoses. The first is a 'biochemical' metamorphosis occurring at age 3–5 months. At this point, there is a change in hemoglobin composition from larval to adult type (Ducibella, 1974). We studied the second, morphological, metamorphosis which, in this neotenic species, may not occur for years (if ever). A related, but more teleological, basis for the absence of a change in O_2 affinity is that fact that the neotenic adults, although aquatic and possessing well-developed gills, are obligate air-breathers at higher temperatures (Burggren and Wood, 1981). Thus, the external metamorphosis, though striking in the complete loss of gills and thickening of skin, does not significantly alter the already established pattern of air-breathing.

Two factors that did alter red cell function in *A. tigrinum* were acclimation to the cold and hypoxia. As discussed below, our attempt to define the cellular mechanisms of these alterations was only partially successful.

Methemoglobin formation did occur in the cold-acclimated neotenic adults. This could provide a partial explanation for the left-shifted oxygen density curve since the presence of methemoglobin increases the O_2-affinity of normal Hb (Darling and Roughton, 1942) but is unlikely to account for the observed 15 mmHg decrease in P_{50}.

Role of organic phosphates

The fall in organic phosphate concentration following exposure to hypoxia and cold acclimation provides a dual mechanism to explain the observed left-shift of the oxygen density curve, i.e., reduced allosteric binding and increased red cell pH. The decrease in red cell ATP is consistent with previous reports of hypoxia acclimation in species with nucleated red cells (cf. Wood, 1980) and consistent with the presence of oxidative phosphorylation providing ATP in such red cells (Schweiger, 1962). The decreased level of 2,3-diphosphoglycerate during hypoxia is opposite to the results of previous studies of mammals. However, in mammals exposed to environmental hypoxia the increase in 2,3-diphosphoglycerate results in large part from the increased red cell pH secondary to hyperventilation alkalosis. Decreased red cell pH causes 2,3-diphosphoglycerate depletion in mammalian red cell and this could account for the present results if lactic acidosis occurred during hypoxia.

As shown in Fig. 4, only about 50% of the observed decrease in P_{50} of whole blood is explicable on the basis of the direct and indirect effects of organic phosphate depletion. This is due to the relatively mild allosteric effect of both organic phosphates and hydrogen ions on Hb-O_2 affinity in this species. Since the potency of the indirect mechanism for controlling O_2 affinity when organic phosphate levels decline depends

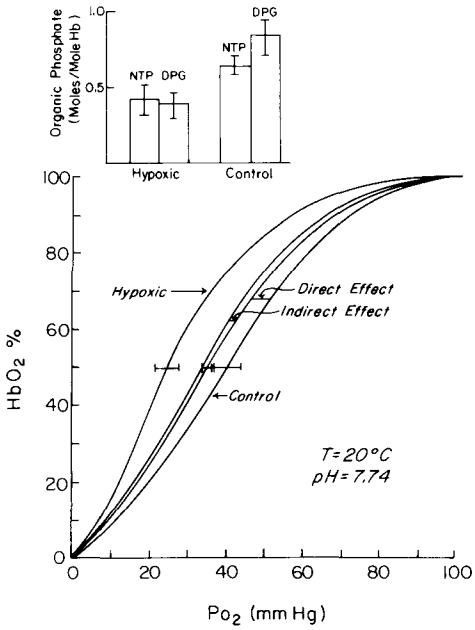

Fig. 4. Effect of hypoxia on the O_2 affinity and organic phosphate concentrations of *A. tigrinum* blood. The curve labeled 'direct effect' indicates the amount of the whole blood shift that can be ascribed to the allosteric effect of the reduced organic phosphate levels. The curve labeled 'indirect effect' indicates the additional amount of the whole blood shift that can be ascribed to the indirect effect of reduced organic phosphates on red cell pH. The remaining amount of shift remains unexplained.

on the magnitude of the Bohr effect, it has little influence on O_2 affinity of *A. tigrinum* red cells. Consequently, the large increase in red cell pH accompanying the organic phosphate depletion with hypoxia and cold acclimations has only a minor effect on O_2 affinity. If the Hb of this species had a Bohr effect in the range of -0.3 to -0.4 ($\Delta\log P_{50}/\Delta$pH) the indirect effect on P_{50} via increased red cell pH would entirely account for the left-shift of the oxygen density curve during hypoxia and cold acclimation. However, with the low Bohr effect of *A. tigrinum* Hb, 50% of the observed decrease in blood P_{50} remains to be explained. Some intracellular factor we did not measure may be operating as an allosteric controller, or alternatively, may be interfering with organic phosphate binding.

Summary

Whole blood of the salamander, *A. tigrinum,* differs from that of most previously studied poikilotherms in two ways. First, there is no change in O_2 affinity following metamorphosis from gill to lung forms. Also, O_2 affinity increases in neotenic adults following cold acclimation. The effect of hypoxia on red cell function is similar to that described for fishes, i.e., O_2 affinity increases. The cellular mechanisms for the changes in O_2 affinity that we examined, i.e., the direct (allosteric) and the indirect (Donnan)

effect of organic phosphates, do not appear to account for the magnitude of the change in blood O_2 affinity. This results primarily from the extremely low Bohr effect in this species.

Acknowledgement

This research was supported by NSF Grant, PCM 77-24246.

References

Bartlett, G. (1976): Phosphate compounds in red cells of reptiles, amphibians, and fish. Comp. Biochem. Physiol. 55A, 245–256.

Battaglia, F.C., Behrman, R., Hellegers, A.E. and Battaglia, J.D. (1965): Intracellular hydrogen ion changes during acute respiratory acidosis and alkalosis. J. Pediatr. 66, 737–742.

Benesch, R. and Benesch, R.E. (1967): The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochem. Biophys. Res. Commun. 26, 162–167.

Burggren, W.W. and Wood, S.C. (1981): Respiration and acid-base balance in the tiger salamander, *Ambystoma tigrinum*: Influence of temperature acclimation and metamorphosis. J. Comp. Physiol. 144, 241–246.

Darling, D.C. and Roughton, F.J.W. (1942): The effect of methemoglobin on the equilibrium between oxygen and hemoglobin. Am. J. Physiol. 137, 56–68.

Ducibella, T. (1974): The occurrence of biochemical metamorphic events without metamorphosis in the axolotyl. Dev. Biol. 38, 175–186.

Duhm, J. (1971): Effects of 2,3-diphosphoglycerate and other organic compounds on oxygen affinity and intracellular pH of human erythrocytes. Pflugers Arch. 326, 341–356.

Funder, J. and Weith, J.O. (1966): Chloride and hydrogen ion distribution between human red cells and plasma. Acta Physiol. Scand. 68, 234–245.

Gahlenbeck, H. and Bartels, H. (1970: Blood gas transport properties in gill and lung forms of the axolotyl (*Ambystoma mexicanum*). Respir. Physiol. 9, 175–182.

Gillen, R.G. and Riggs, A. (1971): The hemoglobins of a fresh water teleost, *Cichlasoma cyanoguttatum* (Baird and Girard) I. The effects of phosphorylated organic compounds upon the oxygen equilibria. Comp. Biochem. Physiol. 38B, 585–595.

Rahn, H. (1966): Evolution of the gas transport system in vertebrates. Proc. Roy. Soc. Med. 59, 493–494.

Riggs, A. (1951): The metamorphosis of haemoglobin in the bullfrog. J. Gen. Physiol. 35, 23–40.

Schweiger, H.G. (1962): Pathways of metabolism in nucleate and anucleate erythrocytes. Int. Rev. Cytol. 13, 135–201.

Steen, J.B. and Turitzen, N. (1968): The nature and biological significance of the pH difference across red cell membranes. Respir. Physiol. 5, 234–242.

Turek, Z and Kreuzer, F. (1981): Effects of shifts of the O_2 dissociation curve upon alveolar-arterial O_2 gradients in computer models of the lung with ventilation-perfusion mismatching. Respir. Physiol. 45, 133–139.

Wood, S.C. (1971): Effects of metamorphosis on blood respiratory properties and erythrocyte ATP level of the salamander, *Dicamptodon ensatus*. Respir. Physiol. 12, 53–65.

Wood, S.C. and Johansen, K. (1972): Adaptation to hypoxia by increased Hb-O_2 affinity and decreased red cell ATP concentration. Nature, New Biol. 237, 278–279.

Wood, S.C. (1980): Adaptation of red blood cell function to hypoxia and temperature in ectothermic vertebrates. Am. Zool. 20, 163–172.

Wood, S.C. (1982): Effect of blood oxygen affinity on arterial pO_2 in animals with central vascular shunts. J. Appl. Physiol., in the press.