Thermal and Hypoxic Induced Alterations of Selected Red Cell Organophosphates in the Female Fresh-Water Turtle, Pseudymys scripta elegans

Daniel Culking
Loyola University Chicago

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THERMAL AND HYPOXIC INDUCED ALTERATIONS
OF SELECTED RED CELL ORGANOPHOSPHATES IN
THE FEMALE FRESH-WATER TURTLE, PSEUDYMYS
SCRIPTA ELEGANS

by
Daniel Culkin

A Thesis Submitted to the Faculty of the
Graduate School of Loyola University of
Chicago in Partial Fulfillment of
the Requirements for the
Degree of
Master of Science

May
1975
Daniel J. Culkin
Loyola University of Chicago

THERMAL AND HYPOXIC INDUCED ALTERATIONS
OF SELECTED RED CELL ORGANOPHOSPHATES
OF THE FEMALE FRESH-WATER TURTLE, PSEUDYMYS SCRIPTA ELEGANS

Groups of female fresh-water turtles were maintained and acclimated to room temperature (22° C), cold (10° C), and cold-hypoxia (10° C) for three to five weeks. Animals were fed ad libitum twice weekly. At the appropriate times, turtles were killed by decapitation and exsanguinated into 10 mM EDTA--10 U/ml heparin--0.8% NaCl. Erythrocytes were isolated by centrifugation, hemolyzed in 10 mM Tris, and boiled for 15 minutes. Content of Adenosine 5'-triphosphate was assayed fluorometrically while Adenosine 5'-diphosphate, Adenosine 5'-monophosphate, and 2,3-diphosphoglycerate were estimated spectrophotometrically. Hemoglobin estimates and hematocrits were also taken on each animal. The room temperature mean levels of ATP, ADP, AMP, and 2,3-DPG (expressed in micromoles/gm of hemoglobin) were $3.8 \times 10^{-4}$, $3 \times 10^{-3}$, $2 \times 10^{-3}$ and 2.3, respectively. The cold group experienced a 1/5 and 1/3 reduction in ATP and ADP respectively, and a four-fold rise in AMP, while the 2,3-DPG and Hb/HCT values remained constant. The cold-hypoxic group experienced a 2/3 reduction of ATP with a concomitant rise in Hb/HCT values, indicating an increase in the intracellular concentration of hemoglobin. There was also a 1/3 decline in ADP and 2,3-DPG and a 3.5-fold rise in AMP. The cold exposure may favor glycolysis with little or no modulation of hemo-
globin function, while exposure of this poikilothermic organism to hypoxia results in increased intracellular hemoglobin concentrations as well as the possible enhancement of glycolytic activity (Belkin, 1968; Clark and Miller, 1973; Penney, 1974).
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VITA

The author, Daniel Joseph Culkin, is the son of Lawrence Culkin and Madeline (Joyce) Culkin. He was born October 3, 1950 in Chicago, Illinois.

His elementary education was obtained at St. Francis Xavier in Wilmette, Illinois, and secondary education at Loyola Academy also in Wilmette, Illinois, where he was graduated in May, 1968.

In September, 1968, he entered Creighton University, and in June, 1972, received the degree of Bachelor of Science with a major in biology and a minor in chemistry.

In September of 1973, he became a candidate for a Master of Science degree in biology.
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
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</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>A®</td>
<td>Angstrom</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
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<tr>
<td>cAMP</td>
<td>3',5'-Cyclic adenylic acid</td>
</tr>
<tr>
<td>2,3-DPG</td>
<td>2,3-Diphosphoglycerate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetate</td>
</tr>
<tr>
<td>FDPase</td>
<td>Fructose diphosphatase</td>
</tr>
<tr>
<td>glucose-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>gm %</td>
<td>Gram percent (gm/100 ml)</td>
</tr>
<tr>
<td>gm % Hb/HCT</td>
<td>Gram percent of hemoglobin/hematocrit</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>IHP</td>
<td>Inositol hexaphosphate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>Mg2+</td>
<td>Magnesium ion</td>
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<tr>
<td>NADH, NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>pCO2</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>pO2</td>
<td>Partial pressure of oxygen</td>
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<tr>
<td>PEP</td>
<td>Phosphoenol pyruvate</td>
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<tr>
<td>PK</td>
<td>Phosphofructokinase</td>
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<tr>
<td>PGA</td>
<td>Phosphoglycerate</td>
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<td>Phosphoglyceromutase</td>
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<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<td>UDP-glucose</td>
<td>Uridine diphosphate-glucose</td>
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INTRODUCTION

This study undertakes an analysis of red cell metabolism and hemoglobin function undergoing hypothermic and hypoxic conditioning. Both of these factors occur in the natural environment and, thus, are important to the understanding of the adaptation of the North American fresh-water turtle, *Pseudemys scripta elegans*.

In the fall, the North American painted turtle encounters hypothermia as the ambient temperature decreases. In the winter the ambient temperature drops further, freezing the lake surfaces and restricting the organism to a lake bottom habitat. In order to survive, the turtle must then adapt to a cold-hypoxic situation by depending solely on its cutaneous and cloacal respiratory mechanisms. These breathing mechanisms are 6% as efficient as the air breathing one in *Pseudemys scripta elegans* at 20 °C (Robin, 1964). Thus the animal assumes a hypoxic condition in the winter months.

Two integrally related systems are being considered in this investigation, respiration and cellular metabolism. This is accomplished by an examination of the red cell concentrations of adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), and 2,3-diphosphoglycerate (2,3-DPG), as well as a discussion of their relative influences upon red cells' energy state and hemoglobin function.
External respiration is the mechanism by which oxygen enters the body. The circulatory system then picks up and transports this oxygen by way of the hemoglobin in the red blood cell to the peripheral tissues. Within erythrocytes, organophosphates, which act as both substrates and products in biochemical pathways, alter the activities of key enzymes involved in biosynthesis and/or biodegradation (Atkinson, 1966). These intermediates also exert influences upon the ability of hemoglobin to combine with oxygen (Brewer, 1970).

**Interaction of Hemoglobin and Oxygen:** One essential role of the blood is to transport agents to and from tissues and organs. Less than half of the volume of the blood is cellular, with the most prevalent component being the red blood cell or erythrocyte. The respiratory pigment hemoglobin exists within the erythrocytes of higher vertebrates and is responsible for the transport of molecular oxygen \((O_2)\) as well as the removal of carbon dioxide \((CO_2)\) from the tissues.

This macromolecule is a tetrameric protein which, in its normal state, consists of two alpha and two beta chains (Boyes-Watson and Perutz, 1943). Each chain is in turn attached to a heme group, the position of attachment for \(O_2\) (Boyes-Watson and Perutz, 1943).

Hemoglobin combines with oxygen in four steps: 1) \(Hb_4 + O_2 \rightarrow Hb_4O_2\); 2) \(Hb_4O_2 + O_2 \rightarrow Hb_4O_4\); 3) \(Hb_4O_4 + O_2 \rightarrow Hb_4O_6\); 4) \(Hb_4O_6 + O_2 \rightarrow Hb_4O_8\). Each step involves an iron atom and
each reaction has a different equilibrium constant (Adair, 1925). The amount of hemoglobin oxygenation is related to the partial pressure of oxygen in the blood (Figure 1). The characteristic shape of this curve is sigmoidal and is largely due to the increased affinity of the fourth oxygenation reaction at the higher partial pressure of oxygen (pO₂) (Atkinson et al., 1965). The amount of oxygen liberated from hemoglobin per unit decrease in pO₂ is rather small until the pO₂ falls to a certain point (Figure 1). That is, below this level large quantities of oxygen are liberated linearly with only a slight decrease in the pO₂. Thus a diffusion gradient is maintained.

In 1913, Hill described the hemoglobin-oxygen interaction in terms of an equation \( y = \frac{Kx^n}{1 + Kx^n} \), where \( y \) is the fraction of hemoglobin saturated with oxygen, \( x \) is the partial pressure of oxygen, \( n \) is the number of O₂ molecules bound per hemoglobin molecule and \( K \) is a constant. Hill's work demonstrated that the experimental sigmoid curve is dependent on cooperative binding (i.e. each combination of molecular oxygen to one of the four heme groups increases the affinity of the protein for the next). The Hill equation presupposes an ideal case of infinite cooperativity. Thus the log plot of product formed (i.e. log \( y \)) versus the concentration of substrate (i.e. pO₂) will have a slope of \( n \) and will be linear within a certain range. The stronger the cooperative effect the more closely the hemoglobin-oxygen saturation kinetics resemble Hill's ideal curve.
Figure 1

Dissociation curve for adult human hemoglobin.
(Modified in part from K. Schmidt-Nielsen, Animal Physiology @ 1964, Prentice-Hall, Inc., Englewood Cliffs, New Jersey.)
HEMOGLOBIN — OXYGEN SATURATION CURVE

% HEMOGLOBIN SATURATION

\( pO_2 \) (mm Hg)
The ability of hemoglobin to transport $O_2$ from the lungs to the tissues depends on two properties: 1) the ability to change from the deoxygenated form to the oxygenated form at an extremely fast rate; and 2) the ability to release this $O_2$ at a variable rate dependent upon local metabolic requirements.

A number of studies have demonstrated the in vitro affects of such parameters as pH, temperature, ionic strength and the concentration of individual organophosphates on the oxygenation of hemoglobin (Benesch and Benesch, 1967; Chanutin and Curnish, 1967; Benesch et al., 1968; Engel and Duc, 1968; Chanutin and Herman, 1969; Gillen and Riggs, 1971; Tan and Noble, 1973; Tyuma et al., 1973). For example, a dramatic decrease in hemoglobin-oxygen affinity was effected by the addition of ATP and 2,3-DPG to dilute hemoglobin solutions (Benesch and Benesch, 1967; Chanutin and Curnish, 1967). In 1969, Benesch and Benesch observed that the beta chains but not the alpha chains bind 2,3-DPG. Murihead (1967) demonstrated that the beta chains move apart by some 6 Å during deoxygenation which makes binding to the beta chains highly attractive. His observation supports the theory that these organophosphates bind more to the deoxy- than oxyhemoglobin (Murihead, 1967; Benesch et al., 1968; Garby et al., 1969). These ideas have been further verified by Woods in 1972 and Benesch and Benesch in 1974.

The findings in this area have led to the recognition of a feedback system for the regulation of the oxygen affinity to
hemoglobin. A close inverse relationship has been observed between oxygen affinity and the concentrations of ATP and 2,3-DPG in intact human erythrocytes (Akerblom et al., 1968; Engel and Duc, 1968; Lenfant et al., 1970). Recently, even more support has been given to the theory that the affects of these phosphocompounds are due to their direct binding to the hemoglobin molecule and more specifically, to the beta chains (Anderson et al., 1973; Jensen et al., 1973; Gibson and Nagel, 1974). In 1974, Benesch and Benesch reported that isolated beta chains retained the sigmoidal characteristics of binding although homogeneous alpha chains did not.

The position of the dissociation curve is also considerably altered by changes in the partial pressure of CO₂ (pCO₂) and acidity. An increase in the concentration of hydrogen ions causes a rightward shift in this curve (Figure 1). The Bohr effect represents an interaction between oxygenation equilibrium and proton dissociation (Prosser, 1973). The normal Bohr effect is a rightward shift in the curve as CO₂ enters the blood from the tissues and the affinity of hemoglobin for oxygen is reduced. The converse of the Bohr effect or a leftward shift in the curve is the Haldane effect. The Haldane effect is the facilitation of CO₂ loss from the blood upon hemoglobin oxygenation (Prosser, 1973). There is another effect of acidic groups upon oxygenation. The Root effect is the reduction of the maximal amount of Hb hemoglobin saturation attained at high pO₂'s (Prosser, 1973).
The Bohr effect is negligible or absent at the uppermost part of the curve where hemoglobin is 75% and 100% saturated (i.e. Hb$_{406}$ and Hb$_{408}$, respectively).

Important components of red blood cells that modify hemoglobin's affinity for oxygen are 2,3-DPG, ATP, ADP, AMP and inositol hexaphosphate (IHP). The relative concentrations of these intermediates vary considerably in the erythrocytes of different organisms (Rapoport and Guest, 1941; Bartlett, 1970).

It is presumed that the oxygenation of hemoglobin in the microenvironment influences the homeostatic state at the cellular as well as organismic level. In like manner, hemoglobin function will fluctuate according to metabolic factors which include the availability of oxygen, the acidity (pH), and the temperature (Benesch and Benesch, 1968; Lambersten, 1968; Tyuma et al., 1973).

The majority of studies of red cell metabolism have been focused upon mammalian organisms, especially the human (Benesch and Benesch, 1967; Chanutin and Curnish, 1967; Garby et al., 1967; Galton and Brewer, 1968; Engel and Duc, 1968; Lenfant et al., 1970; Benesch and Benesch, 1969; Benesch and Benesch, 1974; Gibsen and Nagel, 1974). Fewer studies of a comparative nature have been made in relation to blood changes within the poikilothermic organism (Rapoport and Guest, 1941; Grigg, 1969; Bartlett, 1970; Gillen and Riggs, 1971; Anderson et al., 1973; Tan and Noble, 1973). It is hoped that this investigation will help describe the role of hemoglobin kinetics within the poi-
kilothermic organism, particularly *Pseudymys scripta elegans*, upon hypothermic and/or hypoxic exposures.

**Modification of Cellular Metabolism:** Various investigations support the idea of the existence of a relationship between the activities of certain key glycolytic enzymes and the relative concentrations of the adenylates (Atkinson, 1966). The overall pattern of cellular metabolism (catabolic or anabolic) is controlled by an integrated feedback system of regulatory enzymes which allows the organism to adapt its energy needs to a changing environment. The presence of regulatory enzymes within the cell drives important reactions which lead to the storage or degradation of materials necessary to sustain the life of the cell (Atkinson, 1966).

In the past two decades, the adenylates have been found to exert a profound control over many enzymes involved in the metabolism of the cell (Mansour, 1963; Hathaway and Atkinson, 1963; McGilvery and Pogell, 1964; Rosell-Perez and Larner, 1964). These low molecular-weight metabolites (i.e. ATP, ADP, AMP) are often involved in the same or related metabolic pathways (Atkinson, 1966).

Englehart and Sakov (1943) proposed that phosphofructokinase is a key enzyme regulated by the 5'-adenylates because hexose-phosphates occupy a metabolic branchpoint in glycolysis. Since this proposal, their hypothesis has undergone much investigation. In 1962, Mansour and Mansour demonstrated that liver fluke PFK
is stimulated by 3', 5'-cyclic AMP while other workers showed mammalian heart and skeletal muscle PFK to be positively modulated by 5'-AMP as well as the cyclic isomer (Passonneau and Lowry, 1962; Mansour, 1963). Lardy and Parks (1965) showed that ATP inhibition of PFK is overcome by 5'- and/or 3',5'-AMP. This ATP inhibition of PFK and its reversal by AMP was the first recognized direct regulatory feedback link between the energy state of the cell and glycolysis (Atkinson, 1966).

Fructose diphosphatase, especially important in gluconeogenesis, acts as a bypass in the biochemically irreversible step catalyzed by PFK (McGilvery and Pogell, 1964). FDPases from liver and kidney were inhibited by 5' and/or 3', 5'-AMP (Taketa and Pogell, 1963; Mendecino and Vasarhely, 1963; Nosholme, 1963). This inhibition of the FDPase enzyme links the energy level of the cell with glyconeogenesis and demonstrates the special role of AMP isomers in the regulation of glycolysis and glyconeogenesis.

Glycogen phosphorylase exists in two forms, b and a (Cori et al., 1938). Phosphorylase b requires AMP (5' and 3',5') for activation while the a form appears to need much less or no AMP, with the amount necessary varying with the tissues or organism. Cori and his co-workers discovered an enzyme that catalyzes the conversion of the a form to the b form by the hydrolysis of phosphoserene. Fischer and Krebbs in 1955, found the enzyme responsible for the reverse transformation, a phosphorylase kinase. Subsequent to these studies, adenosine - 3', 5' monophosphate was identified as a factor in the enhancement of
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phosphorylase activity (Sutherland and Rall, 1958; Rall and Sutherland, 1958). In skeletal muscle (Drummond et al., 1965) and heart (Hammermeister et al., 1965; Drummond et al., 1965), 3', 5'-AMP facilitates the conversion of phosphorylase $b$ kinase to a more active form which exhibits a much higher affinity for phosphorylase $b$ (Krebs et al., 1964). Morgan and Parmeggiani (1964) showed that both ATP and glucose-6-phosphate inhibit the action of phosphorylase $b$. The forgoing research demonstrated the reversibility of glycogen breakdown as well as its position as a metabolic control point (Atkinson, 1966).

Glycogen synthetase is an enzyme that catalyzes the conversion of UDP-glucose to glycogen. It exists in two forms: I, a form that is less sensitive to its substrate (UDP-glucose), and D, a more sensitive form (Atkinson, 1966). Here, also, a conversion occurs from one form to the other which involves the phosphorylation of glycogen synthetase I at the expense of an ATP molecule. This interaction is enhanced by 3', 5'-AMP, while the reverse process (i.e. conversion of the D form to the I form) results in the loss of a phosphate from the enzyme (Rosell-Perez and Larner, 1964). Glucose-6-P increases the affinity of UDP glucose for both forms of glycogen synthetase (Atkinson, 1966).

The analysis of hemoglobin function via the proposed physiological system (i.e. the modulation of hemoglobin kinetics by the organophosphates) (Brewer and Eaton, 1970) and the red cells' energy state via the adenylate control hypothesis (Atkinson, 1966) should help to clarify the alteration of the physio-
logical state of the *Pseudymys scripta elegans* during hypothermia and/or hypoxia.
MATERIALS AND METHODS

Care of Animals: Since significant sexual differences have been found in whole blood desaturation rates (Eaton, 1969), the question of sexual variation was eliminated by employing only female animals for this study. Female, fresh-water turtles, *Pseudymys scripta elegans*, of shell length 4-6 inches, were obtained from the Mogul-Ed Company of Oshkosh, Wisconsin. The animals were fed Hill's horsemeat twice weekly until no further food consumption was observed. These turtles were maintained under three different conditions: 1) the control group at 20\(^\circ\)C; 2) the cold group at 9\(^\circ\)C (at this temperature, the turtle becomes torpid and feeding is greatly reduced); and 3) the cold hypoxic group at 9\(^\circ\)C. The control and cold animals were placed in tubs of water (48" x 24" x 20") and allowed to surface freely. The cold hypoxic group was forcibly submerged with a sheet of glass after a 3-6 day exposure to hypothermia. The water of each group was changed at least once every four days to minimize area accumulation. All groups were acclimated for three to five weeks during the summer months (i.e. June through August) with a 12:12 photoperiod.

Preparation of Erythrocytes: In isolating the red blood cells the procedure of Beutler (1971) was modified as here in presented. After acclimation, the animals were killed by de-
capitation and exsanguinated. Blood was collected in chilled centrifuge tubes containing an anticoagulant mixture of 10 mM EDTA - 10 U/ml of heparin in 0.8 % NaCl. This concentration of saline is isotonic for *Pseudymys scripta elegans* (Penney and Shemerdiak, 1973). Both red and white blood cells were separated from the plasma by centrifugation at 1500 x g for 15 minutes at 7°C in a Sorvall RCZ-B refrigerated centrifuge. The plasma and the buffy coat were removed by aspiration, leaving only a cellular pellet in the tube. The thin layer of white blood cells on the top of the red cell pellet was removed by gently swabbing with a Q-tip. The erythrocytes were then resuspended in five volumes of 0.8 % NaCl, and the centrifugation and wash steps were repeated twice. No noticeable hemolysis occurred using this procedure, which results in a 10-fold reduction in the number of white blood cells (Beutler, 1971).

**Preparation of Hemolyzates:** The final red cell pellet was hemolyzed with ten volumes of 10mM Tris-HCl, pH 7.4. The resulting hemolyzate was immediately boiled for 15 minutes in order to inactivate all the enzyme systems. The change in volume of hemolyzate due to boiling was noted and compensations made for the calculation of dilution factors. The inactivated mixture was then centrifuged at 10,000 x g for 10 minutes at 7°C in a Sorvall RC2-B chilled centrifuge. The supernatant, containing the red cell intermediates, was frozen for 4 - 7 days prior to analysis.
Estimation of Hemoglobin: Concentrations of hemoglobin were measured by adding 0.02 ml of the unboiled hemolyzate to 10 ml of ferricyanide-cyanide reagent (Beutler, 1971). This converts the hemoglobin to cyanmethemoglobin. Five ml's of each unknown solution were then placed in cuvettes for spectrophotometric measurement of its optical density at 540 nm. A Bausch and Lomb Spectronic 20 was used for such measurements, which were then compared with hemoglobin values obtained from a standard curve of various concentrations of hemoglobin.

Assay of ATP: The concentration of ATP was measured by the procedure of Beutler and Blauda (1964) with modifications herein presented. A luciferase extract was prepared by grinding 50 mg of firefly tails (obtained from the Sigma Chemical Company, St. Louis, Missouri) in a chilled mortar and pestle containing 5 ml of 0.1 M Arsenate buffer, pH 7.4. After the particulate had settled out, the supernatant containing luciferase was pipetted into a chilled test tube. Further settling of particulate matter was allowed to continue during subsequent transfer into test tubes for approximately 3 minutes. Exactly 50 mg of MgSO₄ was mixed with the final extract, which was then diluted 1:5 with chilled, distilled, deionized water. This diluted luciferase extract was now ready to react with solutions containing ATP. Such interactions produce a flash whose luminescence is directly proportional to the concentration of ATP.

A volume of 0.8 ml of luciferase extract was added on a timed bases to fluorometer tubes containing 0.2 ml of ATP.
standard. A series of such standards had been prepared in 0.04 M Tris-Borate buffer, pH 9.2, containing bovine serum albumin. Flashes were detected exactly 30 seconds after the interaction had begun with a Farrand Model A Fluorometer equipped with a Coleman Model 22 galvanometer. Using a similar procedure, the amount of ATP present in extracts of hemolyzates was then compared with that present in the standards.

**Assay of 2,3,-DPG:** The technique of Krimsky (1965) was used for the assay of 2,3,-DPG. The basis of this assay is that 2,3,-DPG is stoichiometrically required as a cofactor for the phosphoglycerate mutase reaction sequence is

\[
\text{PEP} \xrightleftharpoons{\text{enolase}} 2 \text{PGA} \\
2 \text{PGA} \xrightarrow{\text{PGM}} 3 \text{PGA} \\
\]

The initial rate of reaction is proportional to the concentration of 2,3,-DPG, provided that it is present in a limiting amount. The rate of reaction was measured by following the decrease in optical density at 240 nm as PEP was consumed. The reaction mixture contained 0.02 ml of 1 M Tris-HCl, pH 7.4; 0.01 ml of 0.5 M MgCl₂; 0.03 ml of 0.025 M PEP; 0.01 ml of 1 mg protein/ml enolase; a standard or sample volume of 2,3-DPG; and distilled, deionized water to bring the final volume to 1 ml. After allowing the reaction mixture to stabilize for two minutes, 0.01 ml of PGM was added to initiate the reaction. The response was followed
for a minimum of three minutes and measured as an increase in % Transmittance with a Beckman DB-GT spectrophotometer equipped with a ten inch chart recorder plotting at a rate of 0.5 inches/minute.

**Assay for Adenosine 5'-monophosphate and Adenosine 5'-diphosphate:** The amounts of 5'-ADP and 5'-AMP were quantitated using the method of Beutler (1971) with the following modifications. The ADP present in the red cell hemolyzates were measured through the following sequence of reactions:

1) \[ \text{PEP} + \text{ADP} \xrightarrow{\text{PK}, \text{Mg}^{2+}} \text{Pyruvate} + \text{ATP} \]

2) \[ \text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+ \]

It is clear that the amount of pyruvate formed in the pyruvate kinase reaction bears a stoichiometric relationship to the amount of ADP in the system. The ADP content of the hemolyzates was estimated by measuring the total NADH-NAD\(^+\) transition at 340 nm with a Beckman DB-GT spectrophotometer and comparing these values with those of the ADP standards.

AMP was measured by making use of the adenylate kinase reaction, which converts AMP and ATP to two molecules of ADP, thereby resupplying the pyruvate kinase reaction in reaction 1. This conversion continues until all the AMP present is consumed. With the adenylate kinase system in operation, additional NADH is oxidized to NAD\(^+\), as evidenced by a further decrease in optical
density. This decrease is stoichiometrically related to the content of AMP within the system.

All results were expressed as the average concentration of selected organic phosphates within the red blood cells of *Pseudymys scripta elegans*, acclimated to different experimental conditions. These values were calculated in units of micromoles/gram of hemoglobin.

The calculated ration of gm% Hb/HCT was used to estimate the amount of hemoglobin present in the red blood cells of the various control and experimental groups.

The Fischer Student-t test was used to estimate the significances of differences from the room temperature values (Sokal, 1964).
RESULTS

Effects of Cold Acclimation: Certain organic phosphates which were assayed demonstrate significant changes upon acclimation to the cold (Figures 2 - 5). Using room temperature turtles as the control group with respect to the cold-acclimated animals, a comparison of the hemoglobins (gm%) with the hematocrits (Figure 8) revealed no significant changes in the amounts of hemoglobin, assuming no change in the volume of the erythrocytes due to acclimation conditions.

The levels of ADP (Figure 3) and ATP (Figure 2) showed a $1/3 \ (p < 0.05)$ and $1/5 \ (p < 0.05)$ reduction, respectively, while AMP levels (Figure 4) rose four-fold ($p < 0.001$). The ration of ATP/AMP (Figure 6) was reduced six-fold ($p < 0.05$), while the ATP/ADP ratio was unaffected (Figure 7) by cold acclimation.

Effect of Acclimation to Cold-Hypoxia: Acclimation to cold-hypoxia, as described in the materials and methods, produced a significant $2/3$ reduction ($p < 0.02$) in the red cell's ATP (Figure 2) and a $1/3$ reduction ($p < 0.05$) in the red cell's 2,3-DPG (Figure 5). A comparison of the hemoglobins (gm%) and the hematocrits of the cold-hypoxic animals with the room temperature animals showed a $1/3$ rise ($p < 0.001$) in the amount of hemoglobin within cold-hypoxic erythrocytes (Figure 7), assuming there is no intracellular volume change due to acclimation conditions.
Figure 2

This is a graphical representation of red cell ATP concentrations of room temperature, cold, and cold-hypoxic acclimated turtles. The respective means and standard deviations are expressed in micro­moles of ATP/gram of hemoglobin. The number of animals used was 10 for the room temperature group, 10 for the cold group, and 6 for the cold-hypoxic group.
Thermal and Hypoxic influences upon ATP

ACCLIMATION CONDITIONS

\( \mu \text{ moles ATP/g Hemoglobin (X 10^2)} \)

ROOM TEMPERATURE
COLD
COLD HYPOXIC
Figure 3

This is a graphical representation of red cell ADP concentrations of room temperature, cold, and cold-hypoxic acclimated turtles. The respective means and standard deviations are expressed in micromoles of ADP/gram of hemoglobin. The number of animals used was 12 for the room temperature group, 12 for the cold group, and 7 for the cold-hypoxic group.
Thermal and Hypoxic influences upon ADP

μ moles ADP / g Hemoglobin

ROOM TEMPERATURE  COLD  COLD HYPOXIC

ACCLIMATION CONDITIONS
This is a graphical representation of red cell AMP concentrations of room temperature, cold, cold-hypoxic acclimated turtles. The respective means and standard deviations are expressed in micromoles of AMP/gram of hemoglobin. The number of animals used was 10 for the room temperature group, 13 for the cold group, and 7 for the cold-hypoxic group.
Thermal and Hypoxic influences upon AMP

\[ \text{\mu moles AMP / g Hemoglobin} \]

ACCLIMATION CONDITIONS

ROOM TEMPERATURE

COLD

COLD HYPOXIC
This is a graphical representation of red cell 2,3-DPG concentrations of room temperature, cold, and cold-hypoxic acclimated turtles. The respective means and standard deviations are expressed in micromoles of 2,3-DPG/gram of hemoglobin. The number of animals used was 10 for the room temperature group, 10 for the cold group and 6 for the cold-hypoxic group.
Thermal and Hypoxic influences upon DPG

ACCLIMATION CONDITIONS

Al moles DPG/g Hemoglobin

ROOM TEMPERATURE  COLD  COLD HYPOXIC
Figure 6

This is a graphical representation of red cell ATP/ADP ratios of room temperature, cold, and cold-hypoxic acclimated turtles. The respective means and standard deviations are given.
Thermal and Hypoxic influences upon ATP / ADP

![Graph showing ATP / ADP levels under different conditions: Room Temperature, Cold, Cold Hypoxic.](image)

ACCLIMATION CONDITIONS
Figure 7

This is a graphical representation of red cell ATP/AMP ratios of room temperature, cold, and cold-hypoxic acclimated turtles. The respective means and standard deviations are given.
Thermal and Hypoxic influences upon ATP / AMP
Table 1

This is a table of the respective mean hematocrit, hemoglobin and Hb/HCT values with standard deviations. The number of animals used was 12 for the room temperature group, 15 for the cold group, and 8 for the cold-hypoxic group.
<table>
<thead>
<tr>
<th></th>
<th>HEMOGLOBIN (gm%)</th>
<th>HEMATOCRIT (%)</th>
<th>Hb/HCT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ROOM</strong></td>
<td>5.60 ± 1.539</td>
<td>25.0 ± 9.60</td>
<td>2.24 ± 0.0796</td>
</tr>
<tr>
<td><strong>TEMPERATURE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>COLD</strong></td>
<td>9.47 ± 5.106</td>
<td>31.6 ± 5.02</td>
<td>0.305 ± 0.1600</td>
</tr>
<tr>
<td><strong>COLD</strong></td>
<td>8.69 ± 1.335</td>
<td>26.1 ± 7.47</td>
<td>0.330 ± 0.1375</td>
</tr>
<tr>
<td><strong>HYPOXIC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

In this study of selected organophosphates, levels of ATP, ADP, AMP, and 2,3-DPG were examined within the red blood cells of the fresh-water turtle, Pseudymys scripta elegans. Recent observations have clearly shown that these intermediates exert a strong controlling influence on hemoglobin function (Benesch and Benesch, 1967; Chanutin and Curnish, 1967; Benesch et al., 1968; Benesch and Benesch, 1969) as well as cellular metabolism (Cori et al., 1938; Mansour, 1963; Drummond et al., 1965; Hammermeister et al., 1965; Krebbs and Woodford, 1965; Atkinson, 1966). An examination of the influences of these phosphocompounds on hemoglobin function and cellular metabolism during these acclimation conditions should give insight into the adaptive response of this organism to hypothermia and/or hypoxia.

Hypothermic Influences: Although there is evidence for thermoregulation in reptiles (Templeton, 1970; Bartholomew, 1971), the body temperature of Pseudymys scripta elegans is passively determined by the ambient temperature (Lucey, 1974). Hypothermia shifts the oxygen-dissociation curve to the left (Benesch et al., 1969; Fry, 1970), resulting in an increase in hemoglobin's affinity for oxygen. The magnitude of this effect varies among genera of reptiles. Normal hemoglobin saturation values for reptiles (e.g. the Iguana, 63-72%; the Chelydra, never more than 85%) are much lower than those of mammals.
reptilian hemoglobins provides a less efficient system of oxygen delivery that is further reduced by a drop in the ambient temperature (Benesch et al., 1969; Fry and Hochachka, 1970). Consequently, there is less oxygen being delivered to the tissues for aerobic metabolism.

_Pseudymys scripta elegans_ adapts to low ambient temperatures through bradycardia, vasoconstriction in peripheral tissues, and an overall reduction in activity (Bartholomew, 1971). Through these adaptive responses the organism decreases its need for oxygen.

It would appear that cold-acclimation induces a discharge of high energy adenylate phosphates to AMP primarily at the expense of ADP as well as ATP pools. The result presented here for a poikilothermic vertebrate are similar to homeothermic vertebrate systems in which cold temperatures induce the cleavage of the terminal phosphate groups from both ADP and ATP (Prosser, 1973).

Although erythrocyte levels of ADP in the cold group (Figure 3) are reduced by 1/3 ($p < 0.05$) and those of AMP (Figure 4) are increased four-fold ($p < 0.001$), these phosphocompounds are less effective than ATP and 2,3-DPG in modulating hemoglobin function (Chanutin and Curnish, 1967; Gillen and Riggs, 1971).

A comparison of the cold-acclimated animals with the control group indicates a "sparing" of red cell levels of 2,3-DPG (Figure 5), while ATP (Figure 2) is significantly reduced by 1/5. It
appears that this key modulator (2,3 DPG) is maintained in spite of low environmental temperatures, thereby assuring a certain degree of effectiveness regarding the hemoglobin-oxygen interaction. The cell requires a 1/5 discharge of ATP but, possibly, maintains its ability to unload oxygen in the tissues.

Consideration of the hypothermic influences of low molecular weight intermediates (i.e. ATP, ADP, AMP) includes their relationships with the metabolic branchpoint enzymes of glycolysis (Atkinson, 1966). Studies concerning enzymes have revealed that certain enzymes exhibit increased activities at lower temperatures, such as, PFK of the Goldfish (Hochachka, and Somero, 1973). There is no available information concerning the concentrations of such enzymes, so predictions of relative enzymatic activities are speculative. However, the activities of many of the "mainline" catabolic reactions are enhanced during cold exposure, with this pattern of enhancement differing with the degree of change in temperature (Hochachka and Somero, 1973).

In general, there are four ways in which a poikilothermic organism can compensate for the reduction of enzyme activity that occurs when the ambient temperature is lowered (Van Hoft's rule). These methods are: 1) increasing the synthesis of a particular enzyme, 2) synthesizing an isozyme that is more active at the ambient temperature, 3) modulating enzymatic activities with low molecular weight metabolites and/or 4) regulating the proper positioning or enzymes within the cell (Prosser, 1973). It is the
third method which reflects the allosteric nature of the glycolytic branchpoint enzymes and their modulation by the adenylates (Passonneau and Lowry, 1962; Krebbs and Woodford, 1965; Uyeda, 1970).

An examination of the average concentrations of ATP, ADP, and AMP for the control and experimental groups allows certain predictions of the cell's state of energy metabolism.

Four potential effects of the high rise in AMP concentration in the cold group can be noted: 1) the resulting inhibition of FDPase activity will reduce the amount of gluconeogenesis occurring (Mendecinod and Vasarhely, 1963; Taketa and Pogell, 1963); 2) the inactive form of glycogen synthetase will be predominant and thus decrease the amount of glycogen formation from UDP-glucose (Atkinson, 1966); 3) the inhibiting effect of ATP on PFK activity will be overcome (Passonneau and Lowry, 1962; Mansour, 1963; Lardy and Parks, 1965); and 4) glycogen phosphorylase activity will be enhanced (Cori et al., 1938; Sutherland and Rall, 1958; Drummond et al., 1965).

Previous studies of cold acclimation have revealed patterns in the enzyme mechanisms of the poikilothermic animal. Cold acclimation of fish shifts metabolism of liver tissue to anaerobic pathways (Ekberg, 1958). Precht and other co-workers discovered a large variety of glycolytic, oxidative hexose monophosphate shunt and nonmetabolic enzymes which increase in activity during acclimation to cold (Precht, 1958; Prosser, 1962). This
The study supports the data of these findings and demonstrates that cold exposure might favor increased glycolytic activity in the erythrocyte of the fresh-water turtle. It appears that the energy metabolism of the erythrocyte tends towards catabolism during this condition, although any definite statement necessitates correlation with cellular adjustments of enzyme activity.

**Hypoxic Influence:** *Pseudymys scripta elegans* become hypoxic during diving (Belkin, 1962), relying principally upon anaerobic metabolism. The acidic end products of glycolysis accumulate in the blood in the form of lactate and CO₂ (Belkin, 1968; Clark and Miller, 1973) causing a progressive rise in acidosis (Robin, 1962; Robin, 1964).

With the development of the diving responses in *Pseudymys scripta*, bradycardia and vasoconstriction occur but the blood pressure remains stable (Bartholomew, 1971). There is also a marked reduction in the pulmonary stroke flow: prior to diving 60% of the cardiac output is to the lungs while during diving bradycardia, this value is diminished to 40% (Bartholomew, 1971). As a result of the intense vasoconstriction in hypoxic situations, oxidative metabolism is limited in most organs which must thus rely on anaerobic pathways (Belkin, 1962).

Significant changes are found upon comparison of the red cell organophosphate levels of cold-hypoxic turtles with those of room temperature animals (Figures 2-7). In the red cells of the cold hypoxic group, high energy phosphates discharge (Woods, 1972):
ATP levels rise 3.5-fold (Figure 4). The 1/3 reduction in 2,3-DPG (Figure 5) may indicate a reduced delivery of oxygen to the tissues enhancing the hypoxic condition in the tissues.

It can also be observed that the Hb/HCT ratios of the cold-hypoxic animals are 1/3 greater than those of the controls (Table 1). This increase in the concentration of hemoglobin is concomitant with a 2/3 reduction in the levels of ATP as well as 1/3 reduction in 2,3-DPG. This could be due to an intracellular volume change or an increase in Hb synthesis. Meints et al., (1975) found hypoxia-induced rises in hemoglobin synthesis in *Chrysemys picta* and Woods (1972) reported similar results in the eel. Such a trend suggests that the organism is able to load more oxygen due to the greater amount of hemoglobin present and unload the oxygen in the tissues because of the hypoxic, micro-environmental conditions which are developing. Mammalian adaptation to various hypoxic stresses employs reduced hemoglobin-oxygen unloading in the tissues via rises in red cell ATP and 2,3-DPG (Chanutin and Herman, 1969; Lenfant et al., 1970; Eaton 1974).

Research in other laboratories has shown that diminished oxygen availability can elicit an increased activity of anaerobic pathways in *Pseudomyis scripta elegans* as evidenced by a build-up of blood lactate, pCO₂, and glucose (Clark and Miller, 1973; Penny, 1974). Such changes can serve to offset, in part, the shift in hemoglobin-oxygen dissociation curve induced by decreased
ATP, and 2,3-DPG availability (Benesch et al., 1969; Chanutin and Herman, 1969). The studies of Belkin (1968) have clearly shown that glycolysis is the major source of energy during prolonged hypoxia for *Pseudomyys scripta elegans*. Woods (1972) has suggested that the presence of mitochondria and oxidative phosphorylation in nucleated red cells, account in part for differences in red cell phosphate distribution. It also seems likely that variations exist between mammals and the diving turtle in the effects of ambient oxygen levels on the regulation of the red cell adenylates and 2,3-DPG.

A comparison of cold-hypoxic animals with the controls demonstrates that the relative changes in the adenylates tend to negatively modulate gluconeogenesis as well as glyconeogenesis while favoring glycolysis. The 2/3 reduction of ATP with a concurrent 3.5-fold elevation in AMP concentrations enhances the activities of PFK (Passonneau and Lowry, 1962; Mansour, 1963; Lardy and Parks, 1965), glycogen phosphorylase (Cori et al., 1938; Sutherland and Rall, 1958; Drummond et al., 1965), and phosphorylase kinase (Krebbs et al., 1964). In addition, such alterations in the relative adenylate concentrations indicate inhibition of the activities of FDPase (Taketa and Pogell, 1963; Mendecino and Vasarhely, 1963) as well as the predominance of the inactive form of glycogen synthetase (Atkinson, 1966).

Since lowered oxygen availability results in a 2/3 reduction in erythrocyte ATP levels, it is likely that the operation of an oxidative phosphorylation and/or hexose monophosphate shunt...
contributes significantly to ATP levels of the red blood cell. Further elucidation of the involved enzymes is required to explain their pathways of operation within the erythrocyte of the turtle.
SUMMARY

Groups of female fresh-water turtles were maintained and acclimated to room temperature (22°C), cold (10°C), and cold-hypoxia (10°C) for three to five weeks. Animals were fed ad libitum twice weekly. At the appropriate times, turtles were killed by decapitation and exsanguinated into 10 mM EDTA--10 U/ml heparin--0.8% NaCl. Erythrocytes were isolated by centrifugation, hemolyzed in mM Tris, and boiled for 15 minutes. Content of Adenosine 5'-triphosphate was assayed fluorometrically while Adenosine 5'-diphosphate, Adenosine 5'-monophosphate, and 2,3-diphosphoglycerate were estimated spectrophotometrically. Hemoglobin estimates and hematocrits were also taken on each animal. The room temperature mean levels of ATP, ADP, AMP, and 2,3-DPG (expressed in micromoles/gm of hemoglobin) were $3.8 \times 10^{-4}$, $3 \times 10^{-3}$, $2 \times 10^{-3}$ and 2.3, respectively. The cold group experienced a 1/5 and 1/3 reduction in ATP and ADP, respectively, and a four-fold rise in AMP, while 2,3-DPG and Hb/HCT values remained constant. The cold-hypoxic group experienced a 2/3 reduction of ATP with a concomitant rise in Hb/HCT values, indicating an increase in the intracellular concentration of hemoglobin. There was also a 1/3 decline in ADP and 2,3-DPG and a 3.5-fold rise in AMP. The cold exposure may favor glycolysis.
with little or no modulation of hemoglobin function, while exposure of this poikilothermic organism to hypoxia results in increased intracellular hemoglobin concentrations as well as the possible enhancement of glycolytic activity (Belkin, 1968; Clark and Miller, 1973; Penney, 1974).
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The thesis submitted by Daniel J. Culkin has been read and approved by the following Committee:

Dr. Albert J. Rotermund, Jr.
Assistant Professor, Biology, Loyola

Dr. Edward Palincsar
Professor, Biology, Loyola

Dr. Kirt Vener
Assistant Professor, Biology, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 19, 1975

[Signature]
Director's Signature