

INTRODUCTION

The aim of the experiments in this thesis is to investigate the relatively unknown biochemical aspects of reptilian red blood cells, specifically in crocodiles. Work has been done on the overall metabolism of large reptiles, and examining their potential value as research models (Coulson et al, 1950; Coulson and Hernandez, 1971; Grigg, 1981). Still, Dessauer (1970) in his excellent review of information on the physiological aspects of reptilian blood chemistry acknowledges that only a bare beginning has been made in the study of reptilian blood.

Reptiles generally have lower metabolic rates than birds or mammals of comparable weight, but there is no such thing as a 'typical' reptile and, on a weight basis a very small reptile may have several times the metabolic rate of a large animal. In addition, the metabolic rate of any one species decreases as it grows, so you could vary a reaction rate 'in vivo' by varying the size of the animal. In turtles, crocodiles and fish the ratio of the weight of an adult to that of a newborn may exceed 20,000:1 versus a ratio of less than 20:1 in most mammals. Reptilian red cells have several interesting features; they have a relatively long lifespan and are nucleated, with a broader metabolic potential (Bishop, 1964) than mammalian red cells. Nucleic acid metabolism and protein synthesis occurs throughout its lifespan.

Serum morphology of red blood cells have been studied with a view towards speciation and inter-

relationships among different animals. Little data has been published on the metabolism of the red cell of reptiles. In his review of pathways of metabolism in nucleated and enucleated erythrocytes, Schweiger (1962) had only data on geese for his example of a nucleated erythrocyte. Hunter and Hunter (1957) in their comparative study of erythrocyte metabolism looked at four enzymes in humans, rabbits, birds, frogs, fishes and stingrays, but failed to include any species of reptilia. The presence of LDH and G-6-PD (Vessel and Brody, 1964; Bishop, 1964) has been demonstrated in certain reptiles, but most of the enzymes of the Embden-Meyerhoff pathway and the hexose-monophosphate shunt have not been demonstrated in reptiles, and no data has been published on enzymes in the red blood cells of crocodiles. Also, little biochemical data has been collected on healthy versus wild-caught crocodiles to define what 'normal' parameters would be. There is also a fair amount of controversy regarding levels of methaemoglobin in reptilian red blood cells which requires resolution.

The present study, with emphasis on crocodilian blood, used wild-caught and captive reptiles to collect data on basic haematological parameters (PCV, Hb, Hb electrophoresis etc.) and levels of glucose and some of its metabolites. The important enzymes of the Embden-Meyerhof pathway and the hexose monophosphate shunt were determined (and some partially characterised) in bloods from fresh and salt water wild-caught crocodiles from the Northern Territories and New Guinea.

Turtle, snake, lizard and chicken red blood cells were included for comparisons on some tests. Special attention was directed to methaemoglobin levels in 'healthy' reptiles and possible pathways of methaemoglobin reduction.

The present state of knowledge concerning red cells metabolism, with emphasis on nucleated red cells, is reviewed in the final chapter.

C H A P T E R I.

The Composition of Plasma and Red Blood
Cells of Captive and Wild Caught Reptiles

INTRODUCTION

Blood is the major transport medium in an organism for handling the diverse requirements of tissues and organs for nutrients, gas exchange and waste disposal. The mechanisms regulating blood volume and composition are fairly well known for mammals, but these mechanisms are likely to be different in reptiles, where normal physiological conditions (such as osmolarity, pH, Cl^- concentration, temperature, metabolic rate, etc.) would be incompatible with mammalian life. In addition, the knowledge that one seeks today is not that of an animal dissected from its environment, but a picture of the living healthy animal in its normal environment. To this end I analysed blood from "wild-caught" crocodiles from several different areas of Australia. I also collected blood from captive animals and compared a few of the parameters. Packed cell volume, haemoglobin concentration, whole-blood levels of glucose and lactate; and intracellular levels of ATP, DPG and NADH-MR were measured in three species of crocodylus.

The two terminal phosphate bonds of ATP serve as energy donors for several purposes. ATP is required and produced in glycolysis, it plays a role in cation transport, and ATP is utilized in the formation of glutamine and glutathione. The crocodilian red cell, like most mammalian red cells, is high in potassium and low in sodium (calculated from data of Caulson et al (1950) and this gradient against plasma concentrations could be maintained through the hydrolysis of ATP by ATPase.

Glutathione levels in three genera with enucleated red cells were compared with GSH levels in four genera with nucleated red cells. Glutathione is an important factor in countering oxidative damage in the red cell by provision of an alternative reactant for reactions with radicals or peroxides, and by restoring damaged proteins (haemoglobins, enzymes, etc.) by reducing disulfide bonds.

The comparative study of haemoglobins is often used to elucidate relationships between species. Haemoglobins may be separated by zone electrophoresis on paper, cellulose acetate, starch gel, agar gel, acrylamide gel or starch block. Each method has its own advantages and disadvantages. Cellulose acetate was chosen, as it was the medium with which I was routinely separating human haemoglobins. It gives good resolution and uses very little sample (less than 1 μ l). Within the order crocodilia there are only a few studies on haemoglobin electrophoresis. A single electrophoretic band in Caiman latirostris was reported by Dessauer et al (1957) using paper electrophoresis, but Reischl and Diefenbach (1976) using starch-gel as a support medium found a pattern of three major and one minor components. They also reported polymerization of the haemoglobins occurred with aging, but this was reversible with reduction and alkylation. Sullivan (1974) suggested that crocodilians as a family may really have a larger number of haemoglobin components, but no one has characterised, electrophoretically members of the

genus *Crocodylus*. Using cellulose acetate I studied the haemoglobins of two of the species of the genus *crocodylus*, *Crocodylus porosus* and *Crocodylus johnstoni*.

MATERIALS AND METHODS

Blood Collection and Handling

Chickens were decapitated at Ighams abbatoir and I collected their blood in tubes and stored it at 4°C until reaching the laboratory. Snake blood was collected at the University of New England courtesy of Dr. Agar and sent, in ice, to me.

Gordon Grigg of Sydney University collected blood from crocodiles, lizards and tortoises, both captive animals from their own collection and from Taronga Zoo, and the wild caught animals from the Northern Territory and New Guinea. Laurie Taplin provided blood from captive *C. porosus* and *C. johnstoni* at James Cook University, Queensland. Without the kindness and expertise of these people and their associates this thesis would probably be confined to studies on chicken blood. All blood collecting, whether in the field or in the laboratory, used part or all of the following protocol. The whole blood was collected in heparinized tubes and was well mixed, and the PCV determined. Then 0.5ml of blood was mixed with 0.5ml 5% TCA for ATP and DPG, determination 0.2ml of blood was mixed well with 0.6ml of 5% metaphosphoric acid for determination of glucose

and lactate. These two tubes were then stored frozen until analysis. For enzyme studies and other tests, 1 to 2mls of blood was added to cold ACD solution (.02M citric acid, .05M sodium citrate and .08M glucose) in a ratio of 1:5 and stored at 4°C. Any remaining blood was stored in the heparinized tubes for comparative analysis. Blood samples for the methaemoglobin reduction tests were collected within one hour of starting the tests.

Reagents used were purchased from Sigma Chemical Co., and stored according to label directions. Substrates and enzymes were checked for contamination before use in each assay. Distilled, deionized water was used in the preparation of all solutions. Reagents, with certain exceptions, were stored in the frozen state at -20°C and completely thawed and mixed before use. The Hb reagent for NADH-MR and β -mercapto-ethanol-EDTA stabilizing solution were stored at 4°C and made fresh fortnightly. NADH, NADPH and diluted enzymes were prepared fresh daily.

The 'modified' Drabkins reagent for haemoglobin determination was stored in a dark bottle at room temperature. All assay tubes were disposable plastic Camelec tubes and Oxford samplers were used with plastic disposable tips. Quartz cuvettes were used for measurements in the ultraviolet light range while either glass or quartz were used in the visible light range. All cuvettes had a critical volume of less than 1.0ml. Cuvettes were rinsed with distilled, deionized water,

then acetone, air dried, and stored dry. They were cleaned once a week with dichromic acid. Gilford's model 300N micro sampler spectrophotometer has a fixed slit width of 0.4mm; half-intensity band-width of 2.4nm at a wavelength of 340nm (NADH linked assays); 4.8nm at a wavelength of 412nm (GSH); 6.4nm at a wavelength of 450nm (glucose); 11nm at a wavelength of 540nm (haemoglobin); and 16nm at a wavelength of 630nm (met Hb). The Gilford model 2400 automatic recording spectrophotometer with a circulating water bath which was checked before each experiment, was used for all enzyme studies. The temperature in the cuvette compartment also was controlled. The wavelength and absorbance calibration on both machines were checked weekly and adjusted if necessary. Samples were centrifuged on a MSE refrigerated centrifuge usually at 2000 x g unless otherwise noted. The radiometer pH meter with a small combination electrode was calibrated daily with three different buffers to check electrode span. The pH meter was then standardized with a buffer that was within 1pH unit of the sample to be checked or adjusted.

Packed Cell Volume (PCV):

Microhaematocrit tubes were filled three quarters full with well aerated blood; the clean end flame sealed to a flat bottom and the tubes centrifuged in a Hawksley hematocrit centrifuge at 12,000 x g for 5 minutes. Samples were run in duplicate, read on a Hawksley reader, and the readings averaged to the

nearest tenth percent. Percent of trapped plasma was less than 1.5%. The blood was always well-aerated as the PCV of venous blood is approximately 1% greater than that of fully aerated blood which has lost CO₂ and taken up O₂.

Haemoglobin (Hb)

A modification of the cyanmethaemoglobin procedure (Matsubara and Shibata, 1969) was used. The potassium ferricyanide-cyanide reagent is prepared as follows: 200mg K₃Fe(CN)₆, 2.7ml of .5M NaCN, and 1.0ml of Triton X-100 is added to approximately 800ml of distilled deionized water. The pH is adjusted to 7.2 with approximately 10ml of 3% KH₂PO₄ and the volume brought to one litre. The solution is stored in a brown bottle with a Glenco adjustable 5ml reagent dispenser, and kept out of direct light. Whole blood, or hemolysates in the case of enzyme studies, is added to a measured amount of the reagent which oxidizes the haemoglobin to methaemoglobin, and then converts the methaemoglobin to cyanmethaemoglobin. The tube is mixed well and allowed to stand 5 minutes, then centrifuged at 2000 x g for 5 minutes. The optical density of the supernatant is read at 540nm (the maximum wavelength) in the Gilford 300N spectrophotometer and the concentration calculated

$$\begin{aligned} \text{Hb gm\%} &= \text{O.D.} / m_{\text{Hb}}^{\epsilon} \times \frac{\text{dil}}{10} \times \frac{\text{mMolecular Weight}}{65} \\ &= \text{O.D.} \times 36.1 \text{ for } 20\mu\text{l in } 5.00\text{ml} \\ &= \text{O.D.} \times 14.5 \text{ for } 50\mu\text{l in } 5.03\text{ml} \\ &\text{where } m_{\text{Hb}}^{\epsilon} = 45 \text{ and } \text{mMW} = 65 \end{aligned}$$

The colour is stable for at least 24 hours, the absorbance maximum of 540nm is relatively broad and flat, and the response is linear from .05 to .50 optical density units.

Glutathione (GSH)

Reduced glutathione (GSH) was determined according to Beutler et al (1963) with the following exceptions. Dilutions of chicken and reptilian filtrates were required because of the high level of GSH present in their red cells. One-tenth millilitre of whole blood (or water for a blank) is added to 1.9ml of distilled water and mixed well; then 1.5ml of precipitating solution (1.67% glacial metaphosphoric acid, 0.2% EDTA-2Na, and 30% sodium chloride w/v of water) is added to the hemolysate, mixed and allowed to stand for 5 minutes. The mixture is filtered through Whatman #1 (6cm) filter paper and 1ml of filtrate from human blood is added to 4 ml of 0.3M K_2HPO_4 , or 0.5ml of filtrate from reptilian blood is added to 4.5ml of K_2HPO_4 , or 0.25ml of filtrate from chicken blood is added to 4.75 of 0.3m K_2HPO_4 mixed, and the optical density read at 412nm(OD_1) in a Gilford 300N spectrophotometer. One half a millilitre concentration of DTNB in 100ml of 1% sodium citrate is added to each tube, and a second optical density (OD_2) is read, all against the water-blank filtrate. GSH is then

calculated with the following equations where
 $m\epsilon = 13.6$, PCV is packed cell volume as a percent,
 and Hb is the whole blood hemoglobin concentration in
 gm% and the molecular weight of GSH is 307.

$$\begin{aligned} \text{GSH } \mu\text{M/gHb} &= \frac{(\text{OD}_2 - \text{OD}_1)}{m\epsilon} \times \frac{2.5}{.1} \times \frac{5.5}{\text{ml.}} \times \frac{100}{\text{Hbgm\%}} \\ &= \frac{(\text{OD}_2 - \text{OD}_1)}{\text{Hb gm\%}} \times F_1 \quad \begin{array}{c} \text{filtrate} \\ \text{used} \end{array} \end{aligned}$$

where $F_1 = 1011$. for humans, 2022. for reptiles and
 4044. for chickens

$$\begin{aligned} \text{GSHmg/\%RBC} &= \frac{(\text{OD}_2 - \text{OD}_1)}{m\epsilon} \times \frac{2.5}{.1} \times \frac{5.5}{\text{ml.}} \times \frac{600}{\text{PCV\%}} \times \frac{307}{10} \\ &= \frac{(\text{OD}_2 - \text{OD}_1)}{\text{PCV\%}} \times F_2 \quad \begin{array}{c} \text{filtrate} \\ \text{used} \end{array} \end{aligned}$$

where $F_2 = 31040$ for humans, 62080 for reptiles and
 124160 for chickens.

The hemolysate should be precipitated within a
 few minutes since the GSH will be gradually oxidized,
 but GSH in the acid filtrate is stable for several
 hours. The yellow coloured anion formed by the
 disulfide DTNB and GSH, the main non-protein sulfhydryl
 in the red cell, is stable for about 10 minutes. The
 spectral band width at 412nm in the Gilford 300N is
 4.8nm (fixed slit width of .4mm) which is less than
 one-tenth the natural band width of the coloured
 thereby giving an accuracy of greater than 99%. The
 Alloxan "305" method as described by Patterson and
 Lazarow (1955) is able to measure glutathione with

equal molar amounts of cysteine, cysteinyl glycine and cystathionine producing absorption of less than 5% of the value for glutathione. A semi-micro scale (four times the amount in the micro scale) was used so the final volume in the cuvettes were 1.0ml. The acid filtrate of reptiles were diluted 1:2 with 5% MPA and the acid filtrate of chickens were diluted 1:4 before adding the Alloxan, to keep the optical densities within the range of the standards. Other than the extra dilutions, the procedure was followed exactly as given, and the amount of glutathione in the blood was determined by comparing the corrected optical densities for the sample extracts with the standard curve, and correcting for any dilutions.

Samples were done in triplicate and the average reported.

Glucose

Glucose was determined according to the method of Raabo and Terkeloson (1960) using a Sigma Kit # 510 kit but with the following modifications: The final concentration of O-dianisidine- HCl was increased to 4mg%, and the blood was deproteinized with cold 5% MPA, enabling measurement of glucose and lactate levels on the same deproteinized sample. These deproteinized mixtures were stored frozen and were thawed and centrifuged to obtain a clear supernatant just prior to analysis.

The principle of the test involves a two stage coupled enzyme reaction: first glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide, then peroxidase catalyzes quantitatively the transfer of an oxygen from the peroxide to ortho-dianisidine, a chromogenic oxygen acceptor. The colourless ortho-dianisidine when oxidized produces a brown colour with a maximum at 450nm, the intensity of which is a quantitative measure of the original glucose concentration. The response curve is linear between 0.09 to 1.0 absorbance unit.

The samples were thawed and centrifuged. Then 0.05ml of the protein-free supernatant was added to 2.0ml of combined colour reagent, and the tubes were mixed thoroughly. A standard glucose solution of 1mg/ml (5.55mM) and water (blank) were treated in the same way as the sample. All tubes were incubated at 37°C for 30 ($\pm$ 5) minutes. At the end of the incubation period the absorbance (A) of the test and standard were read at 450nm in the Gilford 240N using the blank as a reference. The mM concentration of glucose in the sample was calculated as follows:

$$\frac{A \text{ of sample}}{A \text{ of Standard}} \times 5.55 = \text{mM conc. of sample.}$$

Lactate

Lactate was determined according to the method of Marbach and Weil (1967).

Lactate is quantitatively determined by driving the following reaction to the right, against its normal

equilibrium



The glycine-hydrazine buffer is used to trap and complex pyruvate. Maintaining the pH at a high unphysiological value (9.0) and adding an excess of NAD both serve to shift the equilibrium in favour of pyruvate.

Bloods were deproteinized with cold 5% MPA (0.2ml of blood + 0.6ml cold 5% MPA) and either analyzed within 4 hours, or stored frozen. Semi-micro glass cuvettes were used in the Gilford 300N. Samples were thawed and centrifuged. Each cuvette contained the following: Glycine (.5M) - hydrazine (.4M) Buffer pH 9.0, 1.0ml; NAD 27mM, 0.1ml; protein-free filtrate, or water, or a standard lactate (4.27 mM in 5% MPA). The cuvettes were mixed. After standing at 25°C for 10 minutes the first absorbance was read at 340nm. Then 0.01ml of LDH 500u/ml was added to the cuvettes, the cuvettes mixed, left standing at 25°C and after 45 minutes the increase in absorbance was measured and the final concentration of lactate was calculated by the following formula -

$$\frac{A_2 - A_1}{6.22\text{m}} \times \frac{0.8\text{ml}}{0.2\text{ml}} \times \frac{1.16\text{ml}}{0.5\text{ml}} \times \frac{1000\mu\text{M}}{\text{mM}} = \mu\text{M lactate}$$

NADH-MR

NADH-methaemoglobin reductase (NADH-MR) was based on the method of Hegesh et al (1968). The reduction of

met-haemoglobin by NADH-MR normally proceeds very slowly, but a methaemoglobin-ferricyanide complex serves as an excellent substrate for measuring the activity of NADH-MR. The reaction is followed spectrophotometrically by measuring the reduction of the met-haemoglobin-ferricyanide complex at 575nm. I have assumed a molecular weight of 66,000, $m_{578}^{HbO_2} = 59.3$ and $m_{578}^{met.Hb} = 17.2$. I tried Hb substrates (hemoglobin solutions freed of met-haemoglobin reductase activity) prepared from human and from crocodile blood, and found the source of the haemoglobin did not affect the enzyme activity of either reptilian or human blood. As human blood (M.G.) was more abundant, it was used for the substrate in all assays. The red cells were washed, lysed, freed of NADH-MR with dry Whatman DE-11 DEAE-cellulose and the haemoglobin concentration adjusted to .17mM ($m_{540}^{Hb} = 46.0$). To 0.5ml of this substrate was added 0.5ml of 5mM EDTA, 0.5ml 50mM citrate pH4.7, 0.5ml of 1.1mM potassium ferricyanide, 0.02ml of the blood to be assayed, and 2.98ml of 0.2% Triton X-100. This reaction mixture was mixed well, and centrifuged at 3,000 x g at 4°C for 10 minutes. One ml of this solution was added to each of two semi-micro cuvettes. To one cuvette (blank) was added 0.01ml of water. To the other cuvette (sample) was added 0.01ml of 10mM NADH in 1% sodium bicarbonate. The cuvettes were mixed and the increase in optical density at 575nm was followed for 5 minutes. The blank rate per minute, if any, is subtracted from the sample

rate, and the enzyme activity is calculated from the following formula, where Δm_{578} is equal to $(m_{578HbO_2} - m_{578metHb}) = 42$, and Hb gm% is the haemoglobin concentration of the blood sample in gram percent.

$$\frac{\Delta OD / \text{min.}}{m_{578}} \times \frac{5.02}{.02} \times \frac{100}{Hb gm\%} = \text{Enzyme units}$$

ATP

The ATP determination was based on the procedure outlined in Sigma's technical bulletin # 366- UV which is based on the reactions as described by Adams (1963). In a coupled enzyme reaction, PGK catalyzes a reaction between ATP and 3-phosphoglycerate, then the 1,3-diphosphoglycerate formed is dephosphorylated with GAPD to glyceraldehyde-3-P. The last reaction reaction involves the oxidation of 1 mole of NADH for every mole of 1,3-diphosphoglycerate dephosphorylated. The decrease in optical density as NADH is oxidized to NAD^+ is measured at 340nm (m_{340} of NADH is 6.22), and is a measure of the amount of ATP originally present in the blood sample. One half ml of blood was mixed with 0.5ml of 12% TCA, mixed well and either frozen or let stand 10 minutes at 4°C. Frozen samples were then thawed prior to assay, and the mixture was centrifuged at 2000 x g for 5 minutes. One ml of a 0.018M 3-phosphoglyceric acid buffered solution, 1.2ml of water and 0.4ml of fresh 2mM NADH were mixed together and 1.0ml of the mixture added to a blank cuvette and to a sample cuvette. The protein-free supernatant (0.2ml) was added to each

cuvette, mixed by inversion, and the optical density (OD₁) at 340nm versus water recorded. Then 0.02ml of GAPD/PGK enzyme mixture (Sigma# 366.2) was added to the sample cuvette, the cuvette mixed by inversion and the optical density (OD₂) read at 10 minutes when the optical density has reached a minimum. The ATP concentration is calculated as follows -

$$\begin{aligned} \text{Haemoglobin ATP } (\mu\text{M/gm}) &= \frac{\Delta\text{OD}}{6.22} \times \frac{1.22\text{ml}}{0.20\text{ml}} \times \frac{1.00\text{ml}}{0.50\text{ml}} \times \frac{100}{\text{Hb(gm\%)}} \\ &= \frac{\Delta\text{OD}}{\text{Hb gm\%}} \times 196 \end{aligned}$$

Red blood cell ATP ($\mu\text{M/ml RBC}$) =

$$\begin{aligned} &= \frac{\Delta\text{OD}_{340\text{nm}}}{6.22} \times \frac{1.22\text{ml}}{0.20\text{ml}} \times \frac{1.0\text{ml}}{0.5\text{ml}} \times \frac{100}{\text{PCV\%}} \\ &= \frac{\Delta\text{OD}_{340\text{nm}}}{\text{PCV}} \times 196 \end{aligned}$$

This procedure is not specific for ATP. Any other nucleoside triphosphate (guanosine-5-triphosphate, inosine-5-triphosphate, and uridine-5-triphosphate) would give the same result. The lowest concentration of ATP which can be measured would be 50 n moles/ml blood (approximately a ΔOD of 0.025).

2,3 DPG:

DPG levels were determined on the same supernatant as ATP using Sigma technical bulletin #35-UV which is similar to the technique described by Lowry et al (1964), but instead of monitoring the NADH reaction

fluorometrically, it is followed spectrophotometrically and the amount of sample added to the system has been increased due to low levels in crocodiles. In brief 2,3 DPG is hydrolyzed by 2,3-DPG phosphatase to 3PGA and Pi with 2-phosphoglycolic acid acting as a stimulator. The 3-PGA formed reacts with ATP in the presence of PGK forming 1,3 DPG. In the third and last step GAPD catalyzes the reduction of 1, 3-DPG to G-3-P, oxidizing NADH to NAD. The decrease in the amount of NADH (ΔOD_{340nm}) is a direct measure of the concentration of 2,3-DPG that was present in the sample. One-half ml of blood was mixed with 0.5ml of 12% TCA mixed well and either frozen, or kept at 4°C if it was to be analyzed within 2 hours. Frozen samples were thawed and all mixtures were centrifuged at 2000 x g for 5 minutes.

To a sample cuvette is added 0.875ml of triethanolamine (TEA) buffer solution (Sigma #665.5), 0.125ml of 2mM NADH, 0.05ml of 0.2M ATP in TEA buffer and .10ml of supernatant. The cuvette is mixed by inversion then 10 μ l of GAPD/PGK enzyme suspension is added (800U/ml GAPD and 450U/ml 3.PGK) and 10 μ l of the enzyme PGM (2400U/ml) and the cuvette mixed by inversion again. After standing for 5 minutes, which allows any endogenous 2-PGA to be converted to 3-PGA by PGM, the initial OD_{340nm} vs. water is read. A blank cuvette with TEA buffer replacing the ATP is also prepared. Phosphoglycolic acid (50 μ l of a 0.02 μ M aqueous solution) is added to both cuvettes. After the optical density

reaches a minimum (about 30 minutes) the second optical density is read and the amount of 2,3-DPG present originally is calculated -

Haemoglobin 2,3-DPG ($\mu\text{M}/\text{gm Hb}$)

$$= \frac{\Delta\text{OD}}{6.22} \times \frac{1.22}{0.1} \times \frac{1.0}{0.5} \times \frac{100}{\text{Hb}(\text{gm}\%)} = \frac{\Delta\text{OD}}{\text{Hb gm}\%} \times 392$$

Red Cell 2,3-DPG ($\mu\text{M}/\text{ml RBC}$)

$$= \frac{\Delta\text{OD}}{6.22} \times \frac{1.22}{0.1} \times \frac{1.0}{0.5} \times \frac{100}{\text{PCV}\%} = \frac{\Delta\text{OD}}{\text{PCV}\%} \times 392$$

The lower limit of the test is $0.6\mu\text{M}/\text{ml}$ (ΔOD of 0.08) to $4.2\mu\text{M}/\text{ml}$. The upper limit of the test $4.2\mu\text{M}/\text{ml}$ (ΔOD of 0.55) is limited by the amount of NADH present in the reaction system and ultimately the linear response of the spectrophotometer.

Haemoglobin Electrophoresis

Samples were prepared according to the technique of Ascott (1970). One-half ml of blood and 0.25ml of normal saline were mixed by inversion and gently layered over a hemolyzing sucrose solution (30% w/v sucrose and 0.5% Triton X-100 made up in 0.01M Tris·pH9.0). The tubes were centrifuged at $3000 \times g$ for 8 minutes then some hemolysate was carefully removed from the lower third of that layer being careful not to disturb the bottom button of cells, or the upper layer of white cells and plasma. To the hemolysate is added a microdrop of 1M potassium cyanide and the sample is stored frozen until used.

Where quantities of blood were minimal, microhematocrit tubes were one third filled with a blood-saline mixture, then one third filled with haemolysing-solution. The clean (blood-saline) end was fired sealed and the tubes centrifuged in a Hawksley microhematocrit centrifuge (12,000 x g for 5 minutes). The tubes were carefully removed, laid flat and the section containing the haemoglobin haemolysate scored with a triangular file. The lower score was broken first, then the upper score was broken. The amount of solution recovered was generally greater than 10 μ l, quite adequate to load the Gelman semi-micro applicator at least once if not more. Electrophoresis was carried out in a Gelman sepratek chamber using their TRIS-EDTA-Glycine Buffer pH 9.2 with 1.5% w/v sucrose added to reduce the heat produced by the electrophoretic procedure.

Sepraphore III strips were pre-soaked in 0.25% Na_2CO_3 for 2 or 3 hours then in haemoglobin buffer for 10 minutes prior to electrophoresis. Eight samples were applied to the strip simultaneously and 425v was applied to the strip for 15 minutes. After electrophoresis the samples were scanned wet for any haemoglobin bands. The strips were then stained for 10 minutes in 0.5% Ponceau S in 7.5% TCA, rinsed in 5% acetic acid to remove background stain and cleaned using absolute methanol, 13% acetic acid in methanol, and drying in an 80°C oven. The strips were then scanned again in a Gelman automatic computing densitometer.

Stability of Enzymes and Metabolites

Table 1 lists the results of stability tests for enzymes and metabolites in the blood of C. porosus to simulate the effects of transportation times from the field to the laboratory. The data in Table 1 compares favourably with the stability data for human red blood cell enzymes and metabolites (Beutler, 1975). The most labile constituents were GSH, and the ATP precipitates. All tests were carried out within the appropriate time, usually within 14 days, so any loss of activity should have been less than 10%.

RESULTS

Table 2 lists the means $\pm$ 1 SD of blood parameters of captive and wild caught Crocodylus porosus, Crocodylus johnstoni and Crocodylus novaeguineae from four different locations.

The 80 wild caught C. porosus ranged from 100gm and 17cm (snout to vent) up to 46kg and 115cm, the median weight was 6.0kg and the median length was 60cm. The crocodile has the low Hb concentration typical of animals with a nucleated red cell.

Glutathione levels (Table 3) are higher than in mammalian red cells but lower than levels found in snakes, lizards or chickens; animals with nucleated cells, but higher metabolic rates than crocodiles.

From the histograms in Tables 4 and 5 we see that with the exception of lactate and DPG the parameters are normally distributed. Lactate values ranged from .6mM/L

Table 1: Stability of red blood cell enzymes and metabolic intermediates in blood stored at 4°C with and without ACD.

	<u>Heparin Only</u>	<u>ACD</u>
PCV	21+	21+
Hb	21+	21+
GSH	4	21+
Glucose ppt	21+	21+
Lactate ppt	21+	21+
ATP ppt	4	14
DPG ppt	21+	21+
* GPI	21+	21+
* LDH	21+	21+
* G-6-PD	21+	21+
* 6-PGD	21+	21+
* NADPH-MR	21+	21+

ATP & DPG glucose and lactate were immediately deprotenized and the whole precipitates were stored frozen until required when they were thawed and centrifuged and the supernatant then removed for analysis.

Blood and supernatants were tested at 0,4,7,14 and 21 days.

Numbers indicate days of storage with < 10% loss of activity.

* Determined according to the methods of Beutler (1975)

Table 2 : Some blood parameters from wild-caught (w) and captive (c) crocodiles from three different areas. (Means $\pm$ 1 SD)

	n	Type	PCV %	MCHC gm%	Hb gm% blood	GSH μ M/gHb	NADH-MR E. U.	ATP ^L μ M/gHb	DPG μ M/gHb	GLUC mM/L	LACT. mM/L
<u>Northern Territory</u>											
<u>C. porosus</u>	80w		24.92 $\pm$ 2.76	31.55 $\pm$ 3.63	7.84 $\pm$ 1.09	-	4.50 $\pm$.92	2.25 $\pm$.57	.85 $\pm$.46	6.50 $\pm$ 2.07	5.42 $\pm$ 3.01
<u>Sydney Zoo</u>											
<u>C. porosus</u>	4c		21.75 $\pm$ 3.86	37.67 $\pm$ 2.24	8.14 $\pm$ 1.11	9.77 $\pm$ 1.56		3.15 $\pm$.65	1.53 $\pm$.67	4.04 $\pm$.89	7.77 $\pm$ 7.22
<u>New Guinea</u>											
<u>C. porosus</u>	6w		-		5.68 $\pm$ 1.46	11.2 1.42		2.08 $\pm$.78	1.46 $\pm$.36	-	21.6 $\pm$ 3.14
<u>C. novaeguinea</u>	14w		-		6.81 $\pm$.99	7.80 $\pm$ 3.07		2.11 $\pm$.36	1.05 $\pm$.18	-	11.21 $\pm$ 6.89
<u>Townsville (starved 3 weeks)</u>											
<u>C. porosus</u>	1c		17.3 -	26.9 -	4.66 -	9.11 -		-	-	-	-
<u>C. johnstoni</u>	8c		19.94 $\pm$ 4.02	21.37 $\pm$ 2.20	4.24 $\pm$.88	8.80 $\pm$ 1.65		3.32 $\pm$ 1.01	1.13 $\pm$.26	-	-

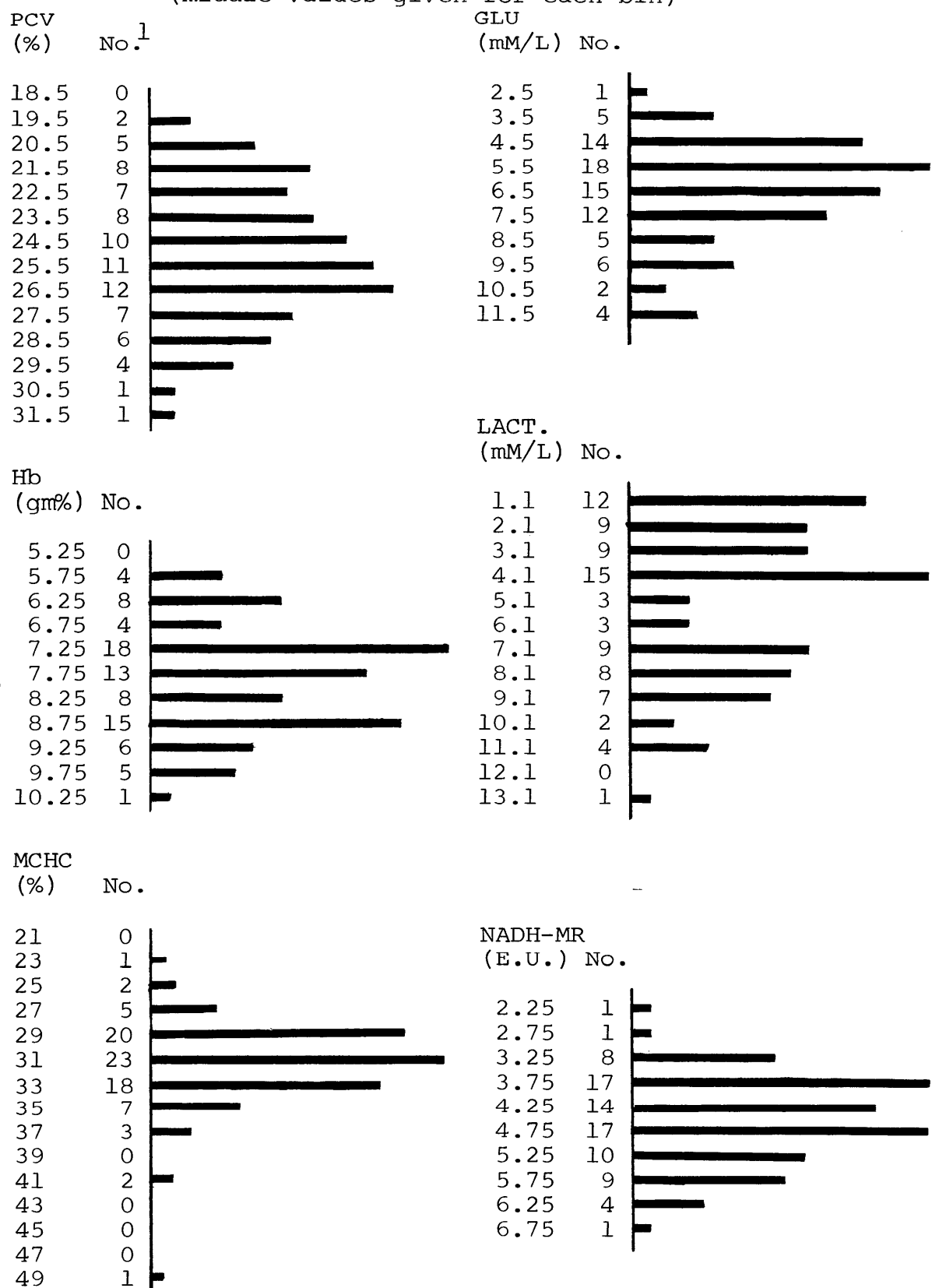
1. is not specific for adenosine triphosphate

Table 3 : Glutathione levels in nucleated and enucleate red cells. (mean $\pm$ S.D.)

<u>Species</u>	No.	GSH (μ M/gHb)	Red Cell
Human Newborn	6	8.94 $\pm$.73	enucleated
Human Adult	6	7.62 $\pm$.96	enucleated
Sheep Newborn	8	5.55 $\pm$.87	enucleated
Sheep Adult	6	5.68 $\pm$ 1.34	enucleated
Kangaroo Red	6	7.00 $\pm$ 1.59	enucleated
<u>Crocodylus porosus</u>	10	10.41 $\pm$ 1.55	nucleated
Lizard	6	22.98 $\pm$ 3.82	nucleated
Black Snake	6	22.18 $\pm$ 2.11	nucleated
Chickens	14	34.31 $\pm$ 5.93	nucleated

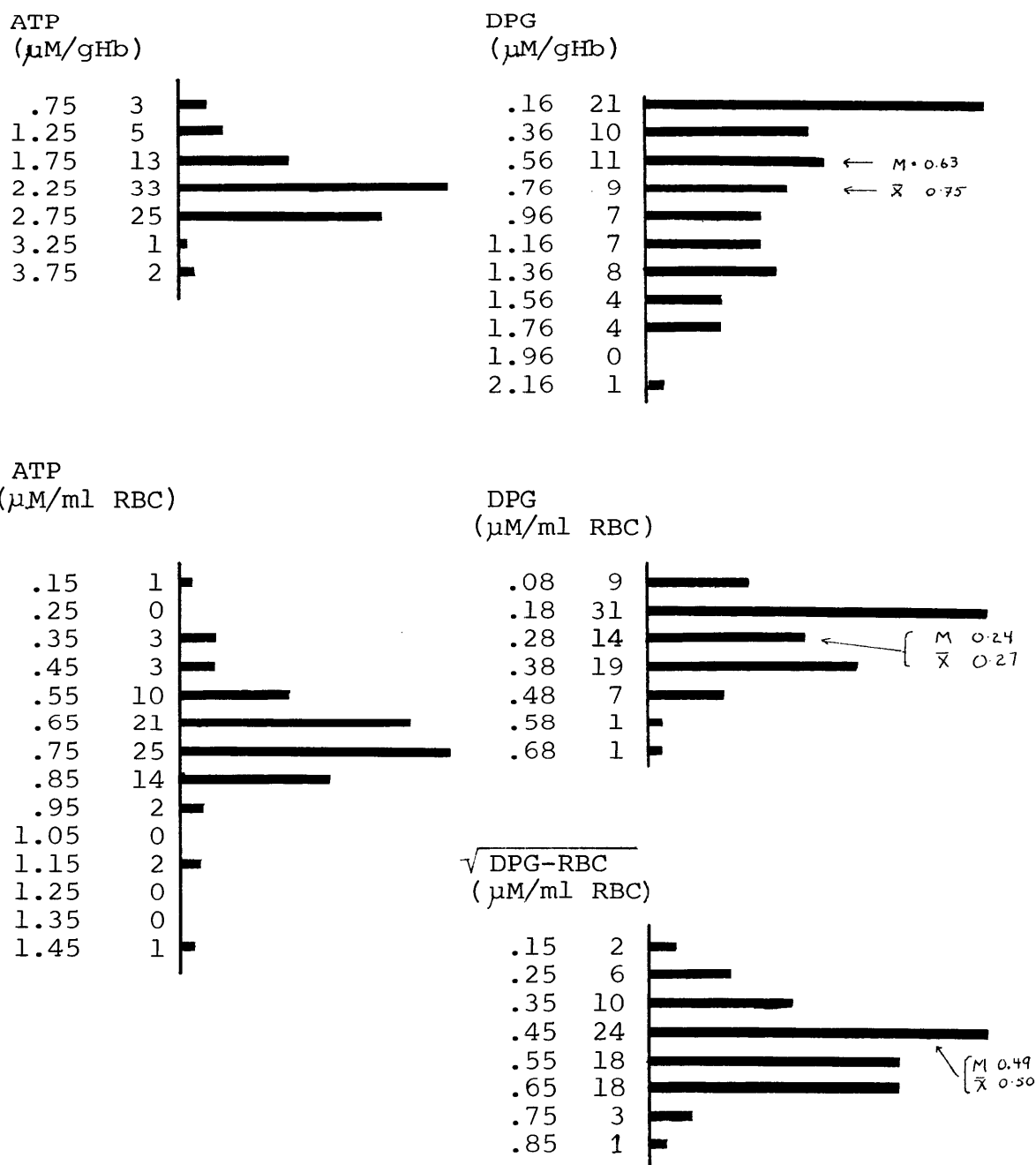
Table 4: Histograms of some of the blood parameters of 80 wild-caught Crocodylus porosus.

(middle values given for each bin)



1. number of values in each bin

Table 5 : Histograms of RBC metabolic intermediates
in 80 wild-caught Crocodylus porosus.



M denotes median value

$\bar{x}$ denotes mean value

to 11.5mM/L with a median value of 4.2mM/L. Lactate is produced as a response to excitement (anaerobic glycolysis), or after submersion when the animal surfaces and the vascular beds of the muscles dilate and the lactate produced during submersion diffuses into the blood, (Dessauer, 1970; Coulson and Hernandez, 1964; Grigg, 1981). This highlights the problem of reporting "normal levels" of lactate.

The lack of a normal distribution with DPG could be experimental. The values are at the low end of sensitivity of the test where slight variations in materials, techniques and instrumentation become increasingly important.

By taking the square root (a mild transformation) of DPG levels calculated as $\mu\text{M}/\text{mlRBC}$, the data takes on a normal distribution, with a mean of $.496 \pm .138$ and a median value of .490.

Note on Table 5 how the median, M, now falls in the bin with the greatest number. If we now do a correlation between the square root of DPG-RBC (S-D-R) and PCV, Hb or MCHC, Table 6, we find a good negative correlation between DPG and Hb. There is a similar negative correlation in the human RBC where DPG acts as a modifier of haemoglobin oxygen affinity.

Table 6: Correlation between blood parameters in 80 wild-caught Crocodylus porosus.

			r	p <
PCV	v	Hb	.641	.001
S-D-R	v	PCV	-.097	N.S.
S-D-R	v	MCHC	-.311	.01
S-D-R	v	Hb	-.330	.01

The three species of crocodiles all have low values of red cell organic phosphates. This supports the results of other workers (Rapoport and Guest, 1941, A. mississippiensis; Bartlett, 1978, C. moreletii and C. niloticus) and is similar to the low values we found for six Black Snakes $1.35 \pm .67 \mu\text{M/gHb}$ ATP and $.134 \pm .019 \mu\text{M/gHb}$, 2,3-DPG (Agar et al 1977).

The only other significant correlation with the rest of the data was the expected positive correlation ($p < .001$) between PCV and Hb, more red blood cells carry more Hb.

Cellulose acetate haemoglobin electrophoresis on 68 samples of C. porosus and C. johnstoni revealed one band which migrates faster than Hb A at pH 9.2 (Fig.1). No minor bands similar to human HbA₂ (1-2%) were apparent. The pattern remained as a single fast moving band at pH 8.5 and 7.5. Haemoglobin electrophoresis was performed on 56 wild caught C. porosus from the Northern Territory, 8 C. johnstoni from Townsville and 4 captive C. porosus.

Both species of crocodile (fresh and saltwater) had the same haemoglobin electrophoretic pattern. In contrast, electrophoresis of 6 samples of chicken haemoglobin showed the presence of two major haemoglobins, both of which migrate slower than human or crocodile haemoglobin. The faster of the two bands is approximately 25% and the slower band is about 75% of the total haemoglobin concentration. The pattern of two chicken haemoglobins agree with those of Johnson and Dunlap (1955), Saha et al (1957) and Kabat and Attardi (1967).

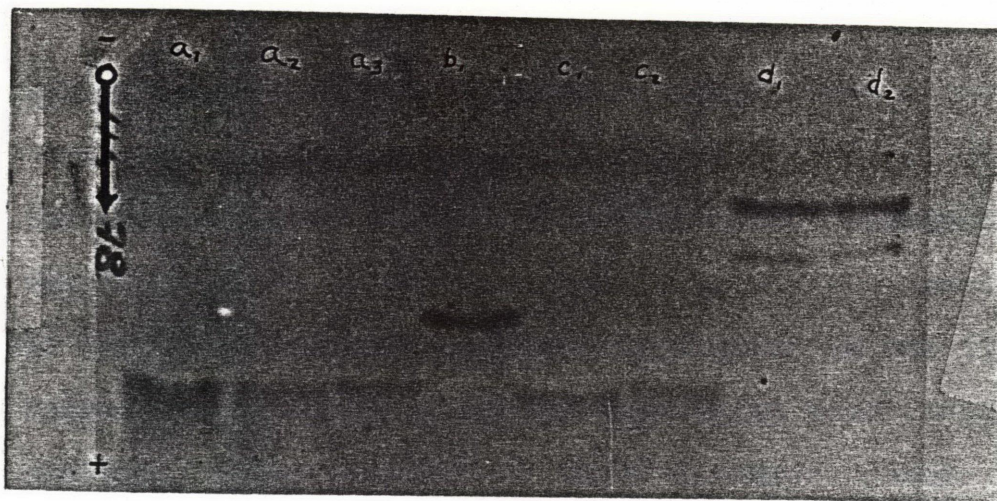


Figure 1: Cellulose acetate electrophoresis of C. porosus (a), normal human (b), C. johnstoni (c) and chicken haemoglobins (d) pH 0.2, 450v, 35 min.

DISCUSSION

The composition of crocodylian blood is typical of that of vertebrates in general. The nucleated red cell in a healthy wild caught animal makes up about 25% of the blood volume. Crocodylian red cells are large oval shaped red cells approximately $16 \times 9 \mu$ with an ellipsoid nucleus $5.5 \times 4 \mu$ centrally located (Saint Girons, 1970). The mean corpuscular haemoglobin concentration (MCHC) I found in the 80 wild-caught crocodiles of $31.6 \pm 2.5 \text{ gm/dL}$ is close to that of normal humans as determined using micro haematocrit PCV's (Dacie and Lewis, 1975). The concentration of haemoglobin, expressed as gm/100ml erythrocytes, is similar in all vertebrates (Wintrobe, 1934). The concentration of haemoglobin in the blood is directly proportional to the packed cell volume ($p < .001$) and the amount and complexity of the haemoglobin molecule(s) would relate to the availability of oxygen in the environment and the

activity of the organism and in a small part to the chance of non-lethal mutations in the globin molecule. The electrophoretic pattern, of one major component, was the same in all animals tested in the two species of the genus *Crocodylus*. This is similar to the findings of Dessauer et al (1957) who, using paper electrophoresis, found one band in three different genera of *Crocodylus* (*Alligator*, *Caiman* and *Crocodylus*) each genus specific.

Blood glucose levels are normally distributed (Table 4) and are similar to human levels. Lactate levels however, cover quite a range and reflect responses to excitement during capture, prior anaerobic condition such as diving, etc. ATP level, as $\mu\text{M}/\text{ml}$ RBC, is normally distributed and again similar to values found in human red blood cells, and probably serves many of the same functions; energy cofactors in several steps of glycolysis cation transport and in the formation of glutamine and glutathione.

Glutathione as calculated as $\mu\text{M}/\text{gHb}$ is present in quantities intermediate between enucleated cells (humans, sheep, kangaroos) and chicken, snakes and lizards. Glutathione is an important factor in maintaining the integrity of the red cell. It reacts with peroxides and radicals to form GSSG which is then reduced back to GSH by glutathione reductase with NADPH as a cofactor. The second way GSH counters

oxidative damage is by restoring damaged proteins or structures by reducing disulfide bonds.

Other systems mammals use for maintaining the integrity of their oxygen carrier, haemoglobin, appear to function in crocodiles as well. Methaemoglobin reductase is present in amounts similar to human levels. Scott and Griffith in 1959 showed that the enzyme which catalyzes the reduction of methaemoglobin by NADH was absent in Alaskan Eskimos who had hereditary methaemoglobinemia, that is a decrease in the ability of the red cell to reduce the iron in haemoglobin from the ferric state to the ferrous state. In humans NADH methaemoglobin reductase is the most important pathway for the reduction of methaemoglobin.

Red cell organic phosphates are present in very low levels. DPG is also an essential cofactor for monophosphoglyceromutase which catalyzes the conversion of 3-phosphoglyceric acid to 2-phosphoglyceric acid and it is also present as an intermediate in the 2,3-DPG bypath in the Embden-Meyerhof pathway, although this shunt does not appear to be significant in normal glycolysis (Yunis and Yasmineh, 1969).

DPG in low quantities would not serve as a major modifier of haemoglobin oxygenation as it does in humans.

We (Grigg and Gruca, 1979) have suggested that lowering red cell organic phosphates could be an adaptive strategy to reduce the fixed acid, Bohr effect,

as crocodiles are subject to large changes in their plasma pH after diving or anaerobic glycolysis. At low levels of organic phosphate it is known that the fixed-acid Bohr effect is much reduced. In whole crocodile blood Grigg and Cairncross (1980) found that the P_{50} is more accurately predicted by P_{CO_2} than by pH. Kay (1977) divided mammals into high and low red cell organic phosphate groups (RCOP). He found that animals in the high RCOP group were animals (man, rats, pigs and dogs) whose behaviour suggests that their tissues required sustained oxygen delivery at high rates. Low DPG animals (goats, cats, sheep and cows) were typically sprinters, darters and pouncers who extended energy in short bursts. Crocodiles would fall into this latter category.

Therefore the composition of blood of crocodiles is typical of that of vertebrates in general, but their red cells are nucleated and have a longer life span. Their red cells have also developed a strategy different from humans to modify the oxygenation of their haemoglobin.

CHAPTER II.

RED CELL METABOLISM - GLYCOLYTIC ENZYMES

INTRODUCTION

Although a number of papers have been published of metabolic studies in reptiles, most of these are concerned with oxygen consumption or whole body metabolism or respiration, (Coulson et al, 1950; Coulson and Hernandez, 1971; Grigg, 1981). Blood chemistry studies usually reflect attempts to follow population dynamics, or species variation or genetic affinities. Little is actually known about the metabolic behaviour of their red blood cells.

This section examines the activities of metabolically important enzymes in crocodiles and partially characterizes the relationship between some of the enzymes and their substrates. I have also compared the activities of enzymes in chickens and crocodiles, as a hypothesis of a common pseudosuchian origin for birds and crocodiles, independent of dinosaurs, has been proposed by Whetstone and Martin (1979). Information on red cell glycolysis in these two very different species could possibly aid in understanding how reptiles, with a very low metabolic rate, have survived and prospered even though they were surrounded by "metabolically more powerful" oxygen consuming mammals (Coulson, 1979).

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Company, except the phthalate esters which were purchased from Eastman-Kodak Company. The place and methods of blood collection were described in Chapter 1.

Because of the small amount of blood normally available to determine glycolytic enzymes, red blood cells were separated from white cells and plasma using a method Danon and Marikovsky (1964) developed to determine the density distribution of red cell populations. Dr. David Danon has separated practically every enzyme in the glycolytic pathways of the red blood cell using phthalates and found no change in activity (personal communication). Brok et al (1966) also used the phthalate method of separation when looking at the enzyme activities in human red blood cells of different age groups and did not find any interference due to the phthalates with his assays.

Briefly, methyl phthalate (SG 1.189) and di-n-butyl phthalate (SG 1.0416) were used to prepare a stock solution with a specific gravity of 1.078, and the SG of the mixture was checked with a hydrometer. The heparinized blood samples were allowed to settle at room temperature and most of the plasma layer was carefully removed. Glass macro-haematocrit tubes with a volume of approximately 200 μ l were used. Stock phthalate was drawn 5mm into a macro-haematocrit tube, the remaining blood was well mixed, drawn 5 cm into

the tube and the clean (phthalate) end was flame-sealed. The macro-capillaries are then centrifuged for 15 minutes at 12,000xg in a Hawksley haematocrit centrifuge fitted with a macrorotor. The tubes were carefully removed from the centrifuge and scored below the phthalate layer and at the very bottom. The upper score is broken first and then the bottom score is broken. The contents of the tube are then delivered into an appropriate amount (1 to 20 dilution) of Beutlers haemolyzing solution G (Beutler, 1975), mixed well, frozen in liquid nitrogen and thawed in 37°C water twice, and finally centrifuged at 4°C for 10 minutes at 2,000xg to give a clear haemolysate. The haemoglobin concentration is measured by adding 0.05ml of the haemolysate to 2.45ml of the ferricyanide-cyanide reagent used to determine whole blood haemoglobin. The haemolysates are kept at 4°C and analysed within 3 hours. Chicken blood was collected as described in Chapter 1, and separated and haemolyzed in the same manner. With the exception of catalase, all of the enzyme reactions listed below are linked to the reduction (an increase in optical density) or the oxidation (a decrease in optical density) of one of the coenzymes, NAD or NADP, as followed at 340nm. The following enzymes were analysed according to the methods of Beutler (1975).

HK: catalyses the reaction of glucose and ATP to G-6-P and ADP, and the G-6-P formed is oxidized by G-6PD to 6-phosphogluconolactone with the reduction of NADP^+ to NADPH.

PFK: catalyzes the phosphorylation of F-6-P by ATP to F-1,6-DP which is converted to DHAP through ALD and TPI reactions. The DHAP is then reduced by α -GDH, oxidizing NADH to NAD^+ .

ALD: cleaves F-1,6-DP into 1 molecule of DHAP, and GAP which is converted to DHAP by the addition of TPI. The DHAP formed is reduced by α -GDH to α -GP oxidizing NADH to NAD^+ .

PK: catalyzes the phosphorylation of ADP to ATP by PEP. LDH then reduces the pyruvate formed to lactate, with the oxidation of NADH to NAD^+ .

LDH: catalyzes the reduction of pyruvate to lactate by the oxidation of the coenzyme NADH.

G-6-PD: oxidizes G-6-P to 6-PGA with NADP^+ being reduced.

6-PGD: catalyzes the oxidation of 6-PGA to ribulose-5-phosphate and CO_2 . NADP^+ is reduced to NADPH during the reaction.

GR: reduces GSSG to GSH with the oxidation of NADPH to NADP^+ .

Catalase: catalyzes the breakdown of H_2O_2 to water and oxygen. The rate of decomposition of H_2O_2 is followed at its absorption maximum of 230nm.

The activity of Na^+, K^+ -ATPase was determined, in 8 crocodiles from whom larger quantities of blood could be drawn, according to the method of Brewer et al (1968) but using 0.1ml of haemolysate and a buffer pH of 8.0. Red cells were separated and washed, and frozen and thawed three times. Aliquots of haemolysate were then incubated

at 44°C in buffer with Na⁺, K⁺ Mg⁺⁺ and ATP with and without ouabain. A blank was prepared by precipitating a reaction mixture at 0 hours. After two hours the proteins were precipitated with 10% TCA, the tubes centrifuged and the supernatant analyzed for phosphorus ("P"). The erythrocytic ATPase activity is expressed as $\mu\text{M}^{\text{P}}/\text{gmHb}\cdot\text{hr}$ at 44°C. The results obtained in the absence or presence of 10⁻³M ouabain were taken as the total or the ouabain-insensitive enzyme activity, the difference representing the ouabain-sensitive Na⁺, K⁺ - ATPase.

Rough in vitro studies of five of the enzymes in the crude haemolysate were made altering the final concentrations of the substrate without changing the final volume in the cuvette. Tris buffers of different pH but with the same molarity (.1M final concentration) were used to test the effects of pH on the reaction in the cuvette.

The in vitro effects of sodium and potassium on PK activity was studied individually and in combination by varying the concentrations of the individual ion in the cuvette between 0 and 100mM final concentration, or by inversely varying the concentration of individual ions in combination to maintain a final concentration of 100mM in the cuvette.

Cellulose acetate electrophoresis of G-6-PD and 6-PGD was carried out in the same Gelman system used for haemoglobin electrophoresis using the same haemoglobin-buffer. Red cells were washed once and the cells haemolyzed by adding 25 μl of cells to 175 μl of Beutlers Solution "G" that has a final concentration of 1 μM NADP and .2% TRITON X-100 added. The cellulose acetate strips

(5.7cm x 12.7cm) were soaked for 15 minutes in 100mls of buffer with 5mg of NADP^+ added. The chamber was loaded, the samples (8) applied and a 4ma current applied for 30 minutes. The strips were removed and stained immediately according to the method of Sparkes et al (1969), but with 6-PGA and Mg added to detect 6-PGD activity. The stain, made up just prior to use and stored in a dark bottle, consisted of 2.3ml of buffered Mg^{++} (.13M TRIS-HCl pH8.0 and .013M Mg Cl_2), 0.1ml 8mM NADP^+ , 0.1ml 26mM G-6-P and 0.1ml of 20mM 6-PGA. The stain was poured onto a Perspex plate and a dry cellulose acetate strip was laid onto the stain. The stain soaked strip was then placed onto another perspex plate and the sample strip was laid gently over it and the air bubbles were removed by rolling a glass rod over the surface of the the strips ensuring complete contact. The strips were then incubated in a dark, moist chamber at 37°C for 10 minutes. The strips were washed in deionized water for a a minute and scanned directly in a Gelman densitometer at 440nm for the formazan colour, as they faded with time. All bands migrated toward the anode (+) and appeared in order from the origin as 6-PGD, Hb and G6PD.

RESULTS

Table 7 lists the enzymes activities of C. porosus red cell enzymes at two different temperatures, both within the range of normal preference temperatures for this genus (Cott, 1961) and near the temperature of maximal rate of glycogenolysis (28°C) obtainable in the alligator (Stevenson et al, 1957). Among all the enzymes,

Table 7 : Enzyme activities¹ in the red cells of C. porosus and chickens at two different temperatures. (mean $\pm$ 1 S.D. No. of animals in parenthesis)

Enzymes	<u>C. porosus</u> (12)		α^2	<u>Chickens</u> (6)		α^2
	20°C	30°C		20°C	30°C	
Hexokinase	.58 $\pm$.19	.78 $\pm$.33	1.34	.98 $\pm$.12	1.72 $\pm$.22	1.76
Phospho-fructokinase	1.97 $\pm$.53	3.68 $\pm$.86	1.87	3.55 $\pm$ 1.04	10.20 $\pm$ 2.60	2.87
Aldolase	3.45 $\pm$.64	7.22 $\pm$ 1.50	2.09	.93 $\pm$.17	1.70 $\pm$.23	1.83
Pyruvate kinase	12.31 $\pm$ 4.00	15.65 $\pm$ 2.91	1.27	24.86 $\pm$ 2.64	36.21 $\pm$ 5.01	1.46
Lactate dehydrogenase	7.96 $\pm$ 1.74	13.93 $\pm$ 3.20	1.75	51.93 $\pm$ 4.90	77.09 $\pm$ 3.69	1.48
Glucose-6-phosphate dehydrogenase	11.44 $\pm$ 3.09	19.14 $\pm$ 2.31	1.67	11.84 $\pm$ 2.13	16.63 $\pm$ 3.34	1.40
6-phosphogluconate dehydrogenase	.50 $\pm$.16	1.08 $\pm$.42	2.16	1.16 $\pm$.07	1.96 $\pm$.17	1.68
Glutathione reductase	7.68 $\pm$ 1.08	12.05 $\pm$ 1.56	1.56	1.53 $\pm$.30	1.70 $\pm$.25	1.09
Catalase	1.91 x 10 ⁴ $\pm$.187					
ATPase ³ . Total	56.8 $\pm$ 10.3			* 273 $\pm$ 36		
Insen.	55.8 $\pm$ 1.0			146 $\pm$ 26		
Sensitive	1.0 $\pm$.8			127 $\pm$ 13		

1. Enzyme activities are expressed as uM/min gHb
 2. Activation coefficient as Enzyme activity at 30°C/enzyme activity at 20°C
 3. n = 8 animals for this enzyme. Activity measured at 44°C as μ M^{*} liberated /gHb hr.
 * From Gupta et al 1973

HK, PFK and 6-PGD are present in the lowest levels in C. porosus. This is consistent with data for fish (Bachand and Leray, 1975) and snakes (Agar et al 1977) and supports the idea that they are rate-limiting steps in nucleated erythrocyte glycolysis as Chapman et al (1962) suggested they are in humans. The activity of PFK is about 30% of that found in humans. ATPase activity is about twice the activity of human ATPase; and within experimental error, crocodile ATPase is insensitive to ouabin inhibition.

PFK levels in the chicken are similar to that found in humans. PK levels in crocodiles are higher than in humans, but have about half the activity of chicken erythrocytes. Jacobasch et al (1974) found species with low 2,3-DPG have particularly high PK activities. Lactate dehydrogenase is present in intermediate quantities, but as reptilian red cells have a broader metabolic potential (Bishop, 1964; Dessauer, 1970) so lactate is not necessarily the last step of glycolysis as it is in mammalian red cells. The level of 6-PGD is quite low, and the ratio G-6-PD/6-PGD is quite high in both C. porosus and the chicken. Snakes (Agar et al 1977) fish (Bachand and Leray, 1975) and marsupials (Agar et al, 1976) have a similar high ratio of G-6-PD to 6-PGD. The level of GR in C. porosus is sufficiently abundant for the reduction of glutathione, but the low level of GR in chicken red cells would suggest it serves as some type of regulator for glutathione reduction.

A rough study of HK in vitro in relation to glucose concentration (Fig.2) showed a maximum rate of reaction at 2×10^{-4} with an apparent K_m of $5 \times 10^{-5}M$. The reaction at a glucose concentration of $2 \times 10^{-3}M$ had only 86% of the maximum activity. In Figure 3 we see a strong effect of pH on the apparent reaction rate, an increase in hydrogen ion concentration decreases the activity of the enzyme. Human HK has a pH optimum of 8.1-8.3 with the activity declining gently on the acid side (Jacobasch et al, 1974). G-6-PD is a part of the reaction mixture for the assay of HK, but as can be seen in Figure 7, G-6-PD activity is independent of the pH of the reaction mixture between pH7 and pH8.

Figure 4 is a plot of the effect of pH on the rate of reaction of PK at two different levels of substrate in C. porosus. There is a strong positive effect of hydrogen ions on the rate of reaction of PK particularly at the lower concentration of PEP. In human red cells Rose and Warms (1966) have concluded that PK normally is operating far below saturation with respect to PEP.

Calculating from the data of Coulson et al (1950), alligator red cells contain an average of 134 mEq/L of potassium as compared with an average plasma level of 4 mEq/L, a characteristic electrolyte gradient shared with lizards (Dessauer, 1952), snakes (Nutton, 1958) and man. The effects of sodium and potassium on the rate of reaction of PK are shown in Figure 5. PK is activated by potassium and magnesium (Dixon, 1958). The intracellular potassium concentration plus the level added to the reaction mixture in vitro are enough to ensure complete activation.

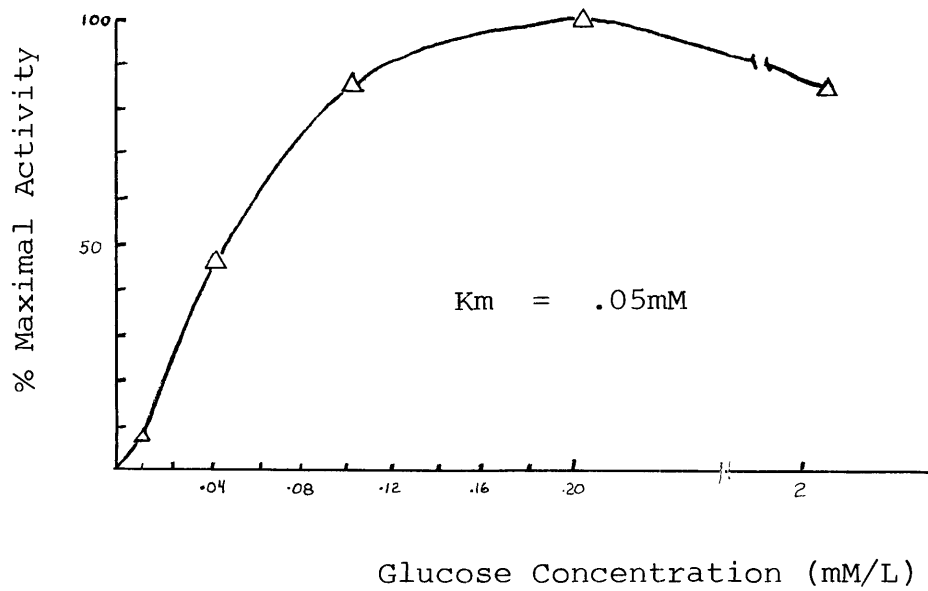


Figure 2: Rate of reaction of HK in relation to glucose concentration at 30°C in C. porosus.

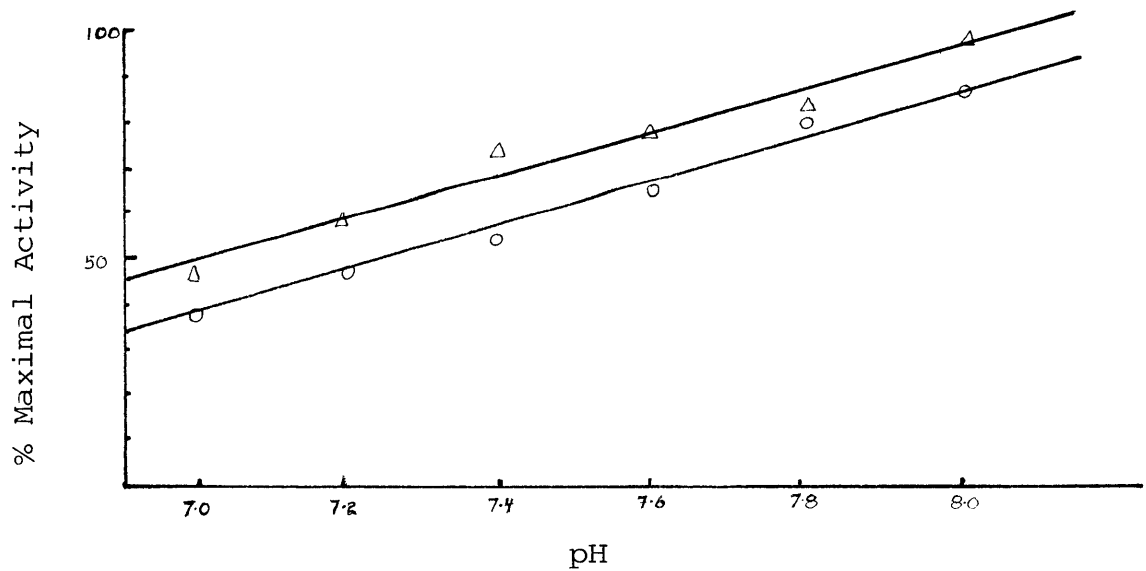


Figure 3: Rate of reaction of HK in relation to pH of reaction mixture using 10^{-4}M (Δ) and $2 \times 10^{-3}\text{M}$ ($\circ$) glucose at 30°C in C. porosus.

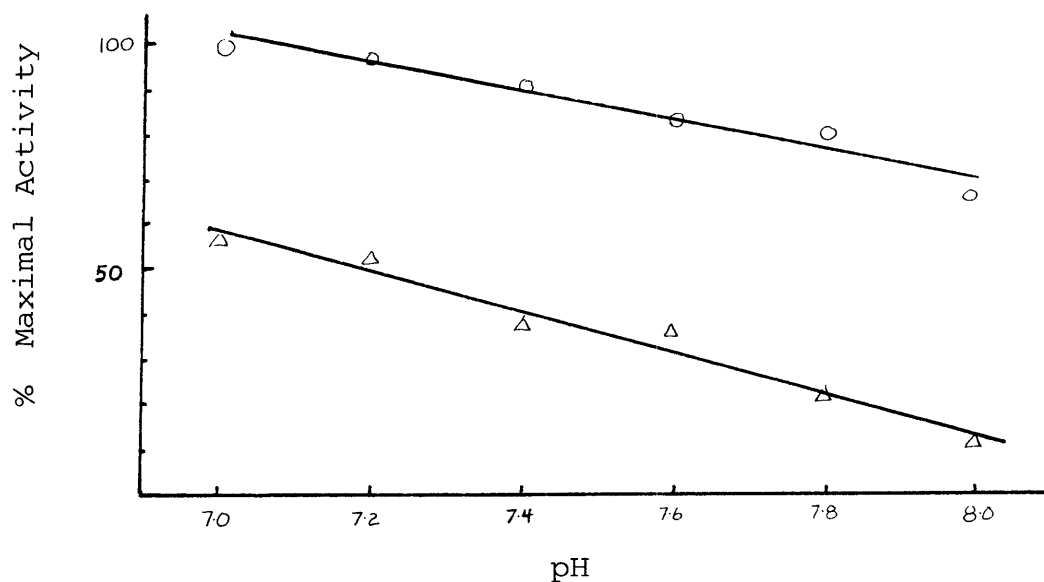


Figure 4: Rate of reaction of PK in relation to pH of the reaction mixture using $5 \times 10^{-4}M$ and $5 \times 10^{-3}M$ PEP at $30^{\circ}C$ in C. porosus

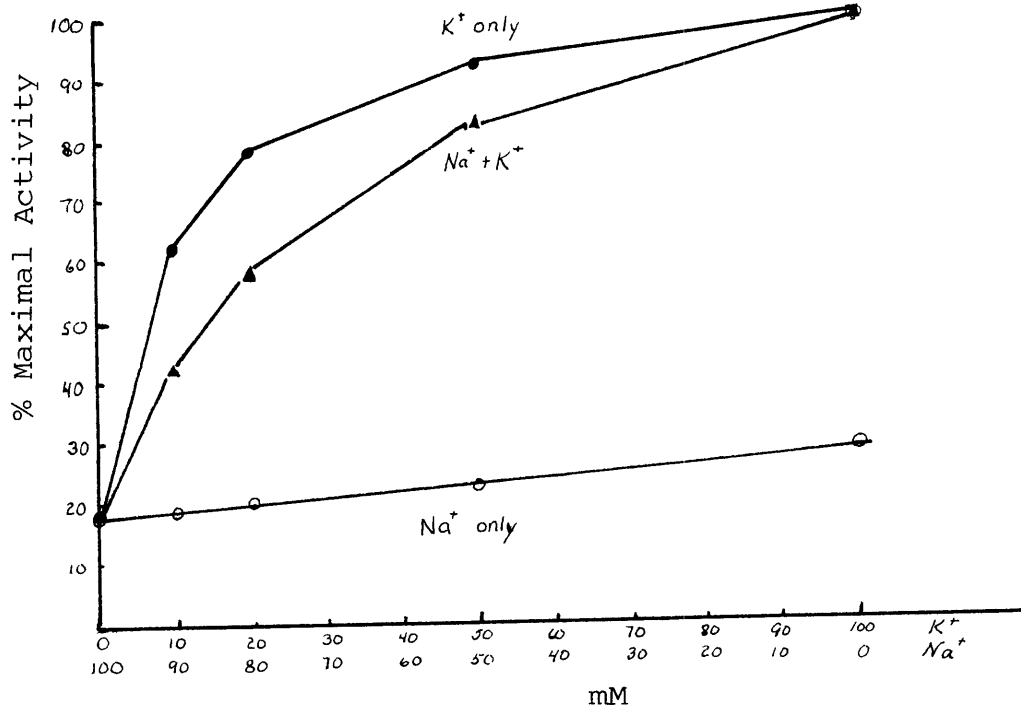


Figure 5: Rate of reaction of PK in relation to the amount of Na^{+} or K^{+} present in the reaction mixture, $1.5mM$ PEP, $30^{\circ}C$ in C. porosus

A rough in vitro study of G-6-PD in relation to the concentration of G-6-P (Figure 6) shows a maximum rate of reaction at 2-3mM with an apparent K_m of 0.06mM. As mentioned before there is no hydrogen ion effect over the range of pH7 to pH8 (Figure 7).

Cellulose acetate electrophoresis of red cell G-6-PD (Figure 8) shows a double banded polymorphic pattern. Both bands migrate slower than the normal single band of human G-6-PD. The concentration of 6-PGD in crocodilian red cells is too low to be detected by this staining method. McDermid et al (1977) also found a

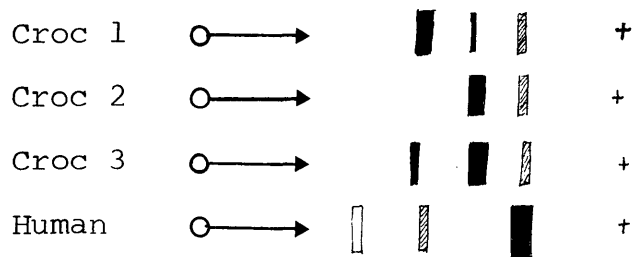


Figure 8: Diagramatic representation of cellulose acetate electrophoresis of Red Cell G-6-PD of three C. johnstoni and one human.

Run at pH 9.2 30min 475v 4ma

G-6-PD [thick black band] 6-PGD [thin white band] Hb [thin hatched band] and

origin and direction of travel O →

double banded G-6-PD electrophoretic pattern in the red blood cells of copperhead snakes. The rate of reaction of 6-PGD in relation to 6-PGA (Figure 9) has an apparent K_m of 0.025 with a maximal rate of reaction at 0.4mM 6-PGA. The rate of reaction of 6-PGD like G-6-PD is fairly independent of pH over the range of 7 to 8 (Figure 10).

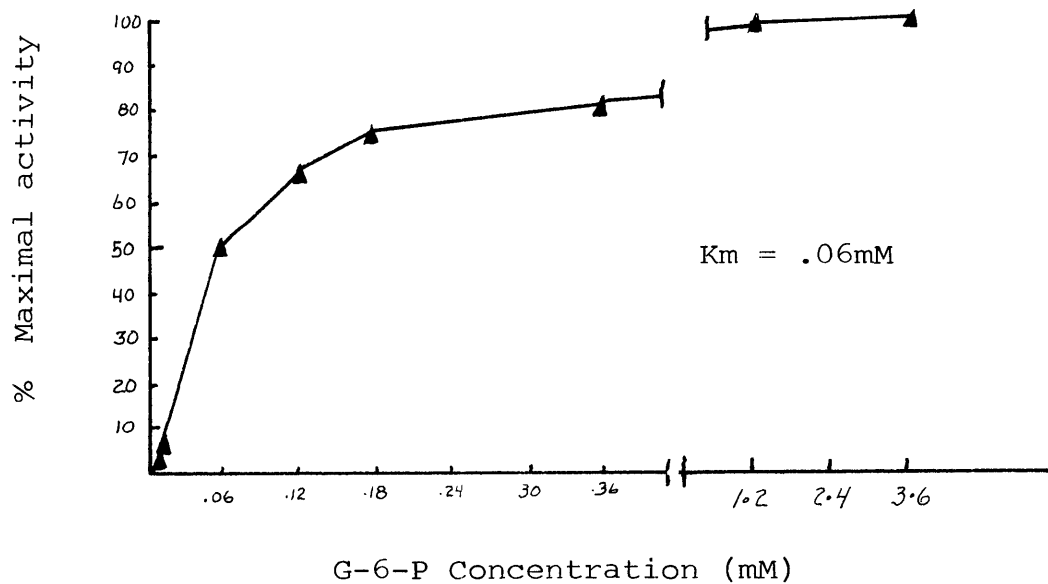


Figure 6: Rate of reaction of G6PD in relation to G-6-P concentration at 30°C in C. porosus at pH 8.0.

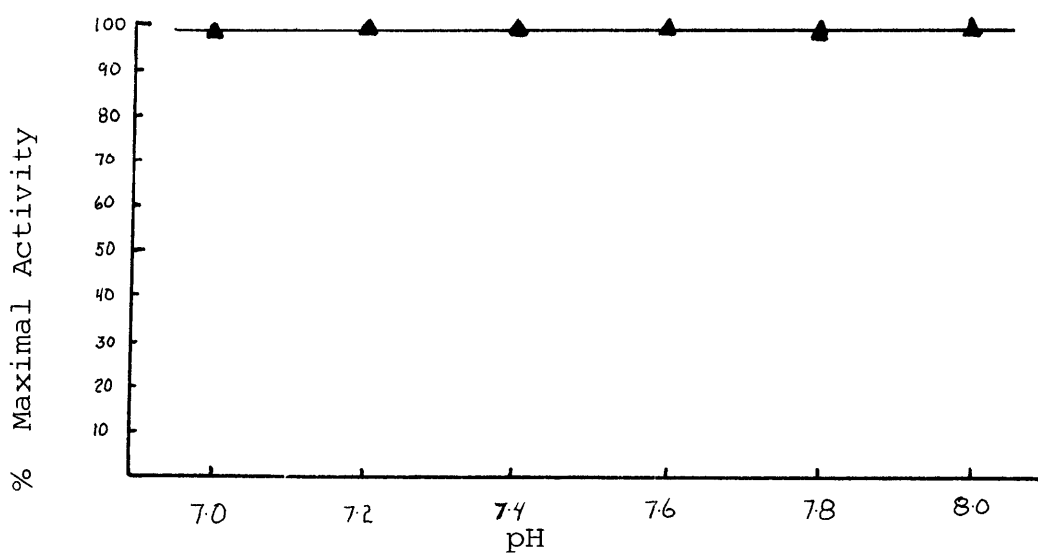


Figure 7: Rate of reaction of G6PD in relation to pH of the reaction mixture using $6 \times 10^{-4}M$ G-6-P at 30°C in C. porosus.

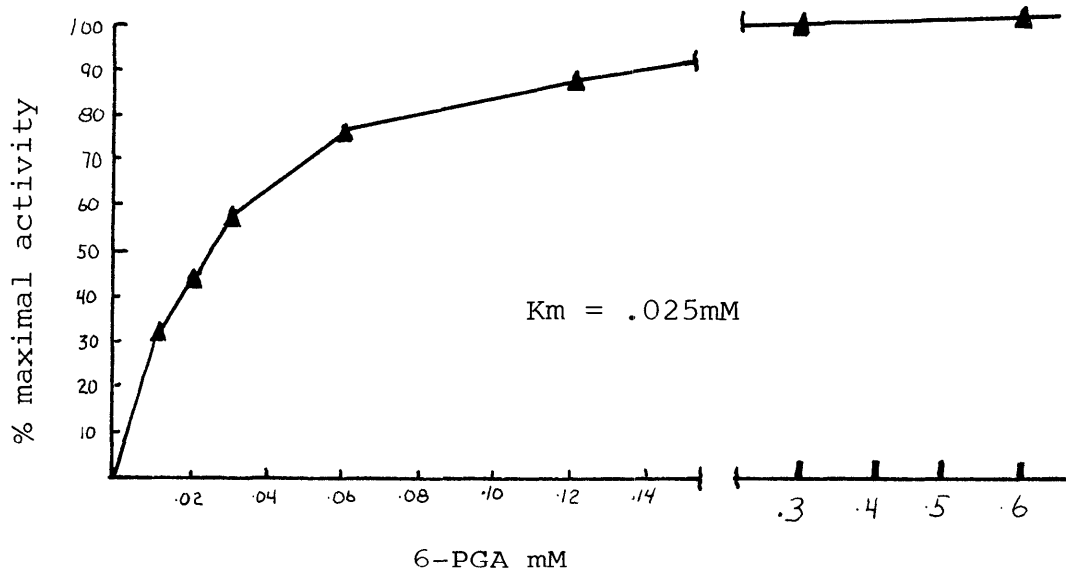


Figure 9: Rate of reaction of 6PGD in relation to 6-PGA concentration at 30°C in C. porosus at pH 8.0.

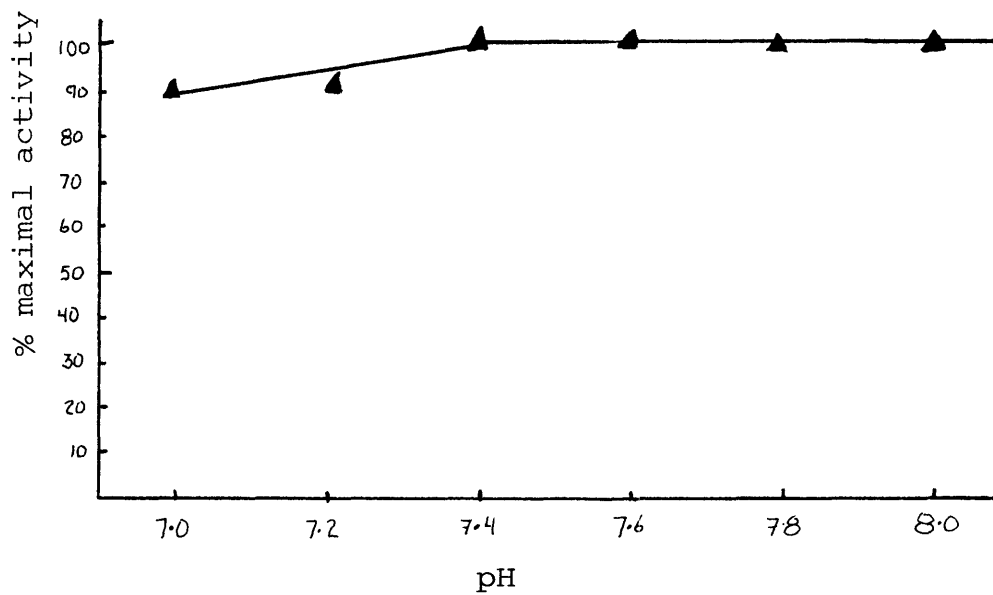


Figure 10: Rate of reaction of 6-PGD in relation to pH of the reaction mixture using 0.6M 6PGA at 30°C in C. porosus.

Similarly the rate of reaction of LDH is independent of pH over the "physiological range" of 7 to 8. A rough study of the rate of reaction of LDH in relation to Sodium Pyruvate concentration (Figure 11) shows an apparent K_m of 0.5mM with a maximal rate at 5mM and then a strong inhibition is exhibited at concentrations of Sodium pyruvate above 10mM. In human red cells which depend primarily on anaerobic glycolysis to meet their energy requirements pyruvate diffuses through the red cell membrane and is distributed through plasma and red cells at a normal concentration of 53.3 μ M (Beutler, 1975). Enig et al (1976) suggest that pyruvate inhibition is the mechanism whereby lactate dehydrogenase channels pyruvate into the Krebs cycle in aerobic metabolism in frog heart, whereas skeletal muscle LDH which is less inhibited effects swift conversion to pyruvate to lactate in this anaerobic tissue. Preliminary tests on plasma LDH in crocodiles suggests that it is present in high concentrations and has a much higher K_m for pyruvate than red cell LDH.

CONCLUSIONS:

Although these enzyme activities were measured in vitro with substrate saturation and a pH probably not found inside the red cell, one is still cautiously tempted to remark on the relationship between enzymes and reflect on the differences between reptilian, avian and mammalian red cells. Crocodiles and chickens, like most mammals, have red cells with high potassium and low sodium ionic

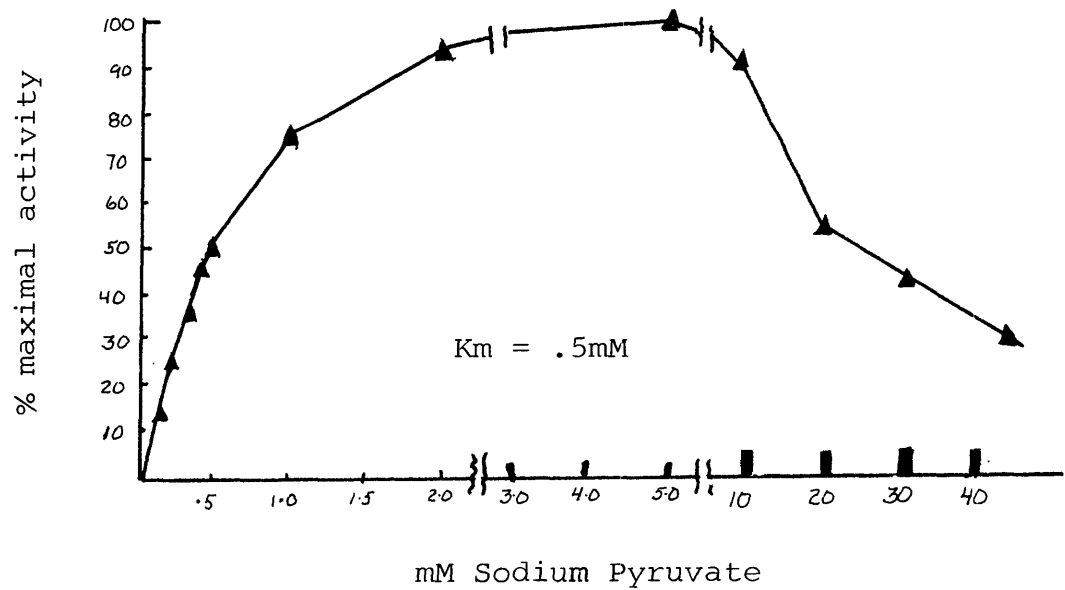


Figure 11: Rate of reaction of LDH in relation to Sodium Pyruvate concentration at 30°C in C. porosus at pH 8.0.

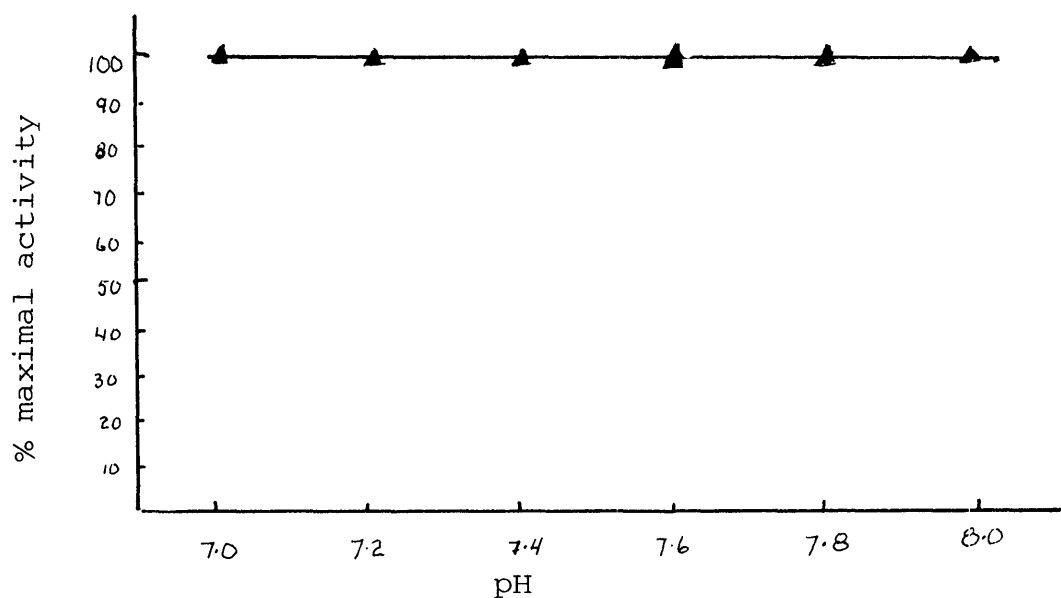


Figure 12: Rate of reaction of LDH in relation to pH of the reaction mixture using 10M Sodium pyruvate at 30°C in C. porosus.

composition versus a low potassium-high sodium environment in the plasma. This active transport of potassium across the cell membrane, balanced against the passive diffusion of sodium back down its electrochemical gradient, is driven by the energy derived from the dephosphorylation of ATP. This ATP is regenerated in humans via the anaerobic breakdown of glucose to lactate in the Embden-Meyerhof Pathway. The high G6PD/6PGD level in crocodiles and chickens seems typical of nucleated red cells (Agar et al, 1977) and could indicate the utilization of some other sugar, say inosine, ribose (from RNA degradation) or ribulose, which could enter the hexomonophosphate pathway of the erythrocyte after the 6PGD step. Pig red cells metabolize free ribose instead of glucose which enters the pentophosphate pathway at ribose-5-phosphate (Duhm, 1974). The low levels of 6-PGD would tend to direct the substrates on through the pathway toward the Emben-Meyerhof pathway, possibly at the point of GAP. The end product, pyruvate, (note low levels of LDH and high levels of PK, in both chickens and crocodiles) could then be channelled into the TCA cycle.

It is interesting to note than many sugars bind to human haemoglobin. Bunn and Higgins (1981) in their paper on reactions of monosaccharides with haemoglobin show that glucose is one of the least reactive of the aldo-hexose. They propose "the emergence of glucose as a primary metabolic fuel in mammalian red cells may in part be due to the high stability of its ring structure which limits potentially deleterious nonenzymatic glycosylation of proteins". This

binding of sugars occurs at the NH_2 -terminal amino of the β chain, the same area where 2,3-DPG and, to some extent ATP, binds. As we have described in a paper on the possible adaptive significance of low red cell organic phosphates in crocodiles (Grigg and Gruca, 1981) crocodilian haemoglobin has a low oxygen affinity, insensitivity of O_2 affinity to organic phosphates, high sensitivity to CO_2 and low levels of red cell organic phosphates. Human red cells using the DPG pathway consume 30% of the glucose metabolized and 37.5% of ATP produced by the Embden-Meyerhof pathway to maintain the high levels of DPG involved in modifying haemoglobin oxygen affinity (Kaneko, 1974). The lowering of red cell organic phosphates reduces the fixed-acid Bohr-effect in animals subject to large changes in plasma pH (Grigg and Gruca, 1979). As remarked on in Chapter I plasma lactate levels can reach high concentrations from diving, or flight, events that depend on anaerobic glycolysis. Coulson and Hernandez (1971) looked at the "alkaline tide" involved in feeding. Plasma pH varied from 7.3 to 7.8 as the plasma chloride content decreased (and passed into the stomach) and the plasma bicarbonate content increased to a similar magnitude (up to 60 MEq/L). Having a haemoglobin, whose O_2 affinity is insensitive to pH and to organic phosphates, yet responsive to CO_2 levels can be seen as a functional adaptation for a diving poikiotherm like the crocodile at such times when low plasma pH would otherwise lower O_2 affinity just when there is a premium on O_2 uptake at the lungs. A haemoglobin with a high sensitivity to CO_2 would

be less of a liability, as its rapid diffusion at the lungs would allow a left shift of the O_2 equilibrium curve, facilitating O_2 loading.

Adult chicken haemoglobin, on the other hand, is strongly modified by inositol 1,3,4,5,6 - pentophosphate (IPP) (Johnson and Tate, 1969), and in embryonic chickens, 2,3-DPG followed by ATP modify the oxygen affinity of haemoglobin (Isaacks et al, 1975). The IPP could be produced from G-6-P by a cyclase and phosphorylase. Notice the high levels of HK, PFK and PK in chicken red cells (Table 7). Chickens, then, like humans, fall into the group of vertebrates with high red cell organic phosphates who have haemoglobins with high oxygen affinities that are right shifted into the functional range by these organic phosphates. It appears then that factors that control concentration of enzymes of substrates may be less influenced by the metabolic rate of the animal, but rather to its physiology, the amount and rate of oxygen and substrates to be delivered to an enzyme-site and, whether the animal derives most of its energy from aerobic or anaerobic reactions. The normally sluggish crocodile when excited is able to convert all his muscle glycogen to lactic acid and ATP many times faster and more completely than an excited man (under circumstances short of mania), the compensation for a low metabolic rate being anaerobic glycolysis (Coulson, 1979).