RED BLOOD CELL PATHOPHYSIOLOGY: COMPARATIVE AND DIAGNOSTIC ASPECTS

Brandon W. Weber

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Dedicated to my family

and to

the glory of my Lord and Saviour

Jesus Christ.

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ABSTRACT

Captive black rhinoceroses in the United States are threatened by three major clinical disorders. Among these, haemolytic anaemia ranks as the number one cause of death. The object of this study therefore was to determine the metabolic lesion responsible for the haemolytic anaemia.

Since black rhinoceros red blood cells were shown to be susceptible to oxidative stress, the first stage of the work focused on the characterisation of the hexose monophosphate shunt in these cells. Results were compared to human red blood cells. To determine how these cells responded to oxidative stress and to stimulation of this pathway, rhinoceros red blood cells were incubated with the known shunt stimulants ascorbate and methylene blue. Red blood cells were incubated with ^{14}C-labelled glucose and then acid extracted. The rate of flux through the shunt was measured by counting ^{14}C trapped on a filter placed inside the incubation vessel and saturated with NaOH. Red cell extracts were used for lactate and reduced glutathione determinations. These experiments showed that black rhinoceros red blood cells were capable of increasing flux through the shunt in response to oxidative stress although the magnitude of this response was significantly lower than that observed in human red blood cells. To determine whether the cells were capable of recycling metabolites through the shunt in response to prolonged oxidative stress, the red cells were incubated with 2-^{14}C-labelled glucose. Recycling through the shunt was observed to function efficiently in these cells.

Since no particular metabolic lesion could be defined with respect to the functioning of the HMP, we shifted our attention to an unusual peak with cytidine-like absorbance properties which dominated the rhinoceros HPLC profile. A range of cytidine nucleotides and other nucleotides were analysed by reverse phase HPLC in an attempt

to match the elution position of the unknown peak. This search yielded a surprising candidate, the amino acid tyrosine. Co-elution of this peak with standard tyrosine strengthened this identification. Diode array analysis of the HPLC peak yielded an identical wavelength maximum to standard tyrosine. Amino acid analysis of rhinoceros red blood cell extracts showed that rhinoceros red blood cells did indeed have elevated levels of tyrosine relative to human red blood cells. These levels were in the range of 20 to 50 times that measured in human red blood cells. The peak was then fractionated, concentrated by freeze drying and analysed by mass spectroscopy, which showed that it had the molecular weight expected for the amino acid tyrosine. Red blood cells of wild black rhinoceroses were found to contain 0.78 ± 0.11mM (n=8) tyrosine. The finding of such a high concentration of a free amino acid in red blood cells appears to be unprecedented.

In an attempt to elucidate the function of tyrosine in rhinoceros red blood cells we drew on the analogy of taurine which is present at very high concentrations in heart epithelial cells and neutrophils. Taurine protects these cells against the respiratory burst oxidants during periods of acute inflammation.

Exposure of rhinoceros red blood cells to hydrogen peroxide resulted in the accumulation of dityrosine, a highly fluorescent tyrosine dimer. The formation of dityrosine has previously been shown to occur between protein tyrosyl residues. In our system we describe the crosslinking of free tyrosine in response to hydrogen peroxide in rhinoceros red blood cells. The formation of dityrosine was followed by fluorimetry. Human red blood cells showed no significant production of dityrosine under the same conditions. The accumulation of dityrosine in rhinoceros red blood cells was found to be reciprocally related to GSH concentration. A series of cell-free

experiments showed that dityrosine only accumulated in the absence of sufficient GSH and that its production was catalysed by haemoglobin.

This study therefore describes the identification of tyrosine present in rhinoceros red blood cells at levels approaching 1mM. Haemoglobin catalyses the production of dityrosine in response to hydrogen peroxide in a manner that is inversely proportional to the GSH concentration.

It is our hypothesis that this tyrosine response to hydrogen peroxide may form a backup antioxidant mechanism for the glutathione peroxidase / reductase system in these cells which are relatively deficient in catalase, a major enzyme for the protection from hydrogen peroxide in other mammals.

Analysis of red blood cell extracts of 30 captive black rhinoceroses was found to have significantly lower tyrosine levels (0.37 ± 0.14mM) than the wild rhinoceroses (0.78 ± 0.11mM) analysed. These low levels of tyrosine combined with an iron overload syndrome recently described by D. Paglia at UCLA, suggests that black rhinoceroses in captivity may face a higher level of oxidative challenge. The unusual mechanisms in the rhinoceros red cell described here for handling oxidative stress, in which catalase has been replaced by reliance on the glutathione peroxidase system together with a tyrosine buffer, seem to be inadequate under the environmental and dietary circumstances of the captive state. These findings however may give new insight into therapeutic or preventative measures based on dietary modification which will address iron absorption and anti-oxidants.

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ABBREVIATIONS

μCi	micro Curie
λ_{em}	emision wavelength
λ_{ex}	excitation wavelength
μl	microliter
$^{\bullet}NO_2$	nitrogen dioxide
$^{\bullet}OH$	hydroxyl radical
1,3-DPG	1,3-diphosphoglyceric acid
2,3-DPG	2,3-diphosphoglyceric acid
2-PGA	2-phosphoglyceric acid
3-PGA	3-phosphoglyceric acid
A	alanine
A_{260nm}	absorbance at 260 nanometers
A_{280nm}	absorbance at 280 nanometers
ADA	adenosine deaminase
ADP	adenosine-5'-diphosphate
AK	adenylate kinase
AMPDA	adenosine-5'-monophosphate deaminase
APH	acetylphenylhydrazine
ATP	adenosine-5'-triphosphate
BSO	L-buthionine-(*SR*)-sulfoximine
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
cps	counts per second

CTP	cytidine-5'-triphosphate
DCFP	dialysed cell free preparation
DHA	dehydroascorbic acid
DHAP	dihydroxyacetone-P
DPGM	diphosphoglyceromutase
DPGP	diphosphoglycerophosphatase
dpm	disintegrations per minute
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
E	glutamate
EMP	Emden Myerhof pathway
G	glycine
G-6-PD	glucose-6-phosphate dehydrogenase
GAP	D-glyceraldelhyde-3-P
GAPD	glyceraldehyde phosphate dehydrogenase
GP	glutathione peroxidase
GPI	glucose phosphate isomerase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione dimer
H_2O_2	hydrogen peroxide

HBSS	Hank's balanced salt solution
h.c	haematocrit
HI	heat-inactivated
HMP	hexose monophosphate pathway
HOCl	hypochlorous acid
HPLC	high pressure liquid chromatography
Hx	hexokinase
I	isoleucine
keV	kiloelectron volts
L	leucine
LDH	lactate dehydrogenase
min	minutes
ml	milliliter
mM	millimolar
MPGM	monophosphoglyceromutase
N	asparagine
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
nm	nanometers
nmole	nanomole
NO_2Cl	nitryl chloride
NP	nucleotide phosphorylase
P5N	pyrimidine-5-nucleotidase
PBS	phosphate buffered saline
PCA	perchloric acid

PEP	phosphoenolpyruvate
PFK	phosphofructokinase
PGK	phosphoglycerate kinase
PK	pyruvate kinase
Q	glutamine
R-5-P	ribulose-5-phosphate
rbc	red blood cells
rpm	revolutions per minute
SOD	superoxide dismutase
TPI	triose phosphate isomerase
TROLOX	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate
UDPG	uridine diphophsoglucose
UV	ultraviolet
V	valine
Y	tyrosine

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1. Introduction

1.1 Background

Poaching and habitat encroachment have drastically decreased the global population of free ranging black rhinoceroses (*Diceros bicornis*) from over 60 000 in 1970 to approximately 2 600 presently in Africa. As if this situation was not dismal enough, since 1986 it has become apparent that captive black rhinoceroses in the United States have fallen prey to yet another threat. This threat comes in the form of three major clinical disorders.

These are: - haemolytic anaemia (1-3)

- mucocutaneous ulcerations (4,5)
- leucoencephalopathy (6,7)

Among these haemolytic anaemia ranks as leading cause of death among captive black rhinoceroses, being responsible for approximately a 75% mortality rate among 36 black rhinoceroses experiencing a combined total of 44 documented haemolytic episodes (2). This condition has only been documented in black rhinoceroses born in captivity, newly captured from the wild and in wild-caught animals following long-term captivity. No other species of rhinoceros seems to be plagued by this disorder. Earlier studies have eliminated haemoglobinopathies, auto-immune phenomena and exposure to specific toxins as causes of the haemolytic anaemia (8,9).

Encephalomalacia is a disorder which describes the degeneration of the brain tissue which takes the form of central nervous system tissue necrosis. The first sign of this disorder is often acute lethargy and depression. The animals usually enter a comatose state and dies

within 48 hours after the initial onset of signs. Miller *et al.* describes the fatal progression of the disorder in three black rhinoceroses from different zoos in the United States over a 9-year period (6).

Leptospirosis (infection by *Leptospira interrogans*) is a disease commonly associated with moist environments and is transmitted by rodents in their urine. The clinical signs of leptospirosis may include haemolytic anaemia. Leptospiral infections have been described in wild and captive rhinoceroses and therefore its possible link with the haemolytic problem in black rhinoceroses has been investigated (10-13). In a study by Jessup *et al.* which included serum samples from 63 free-ranging and 29 captive black rhinoceroses, the incidence of antibodies to leptospira, which indicated infection at one time or another, was found to vary in rhinoceroses captured in different geographical and ecological areas (13). Although leptospirosis has not been shown to be the cause of all cases of haemolytic anaemia, it has been associated with at least nine of 21 known fatalities. It would therefore seem that although it may not be the sole cause of the haemolytic anaemia, it may have acted as an agent which exploited a particular defect in the red blood cells of these rhinoceroses.

The aim of this project therefore is to characterise the metabolism of healthy, wild rhinoceros red blood cells and compare it to that of affected captive rhinoceroses so that the metabolic basis of the haemolytic anaemia may be elucidated. This may enable appropriate therapies to be devised which would be used to treat or prevent the crisis in black rhinoceroses in captivity.

1.2 Red blood cell metabolism

The red blood cell is a relatively simple cell which has been extensively studied and this has lead to the fairly complete understanding we now have of its metabolic processes. The primary function of the erythrocyte is to supply the body's tissues with O_2 and assist in ridding it of CO_2.

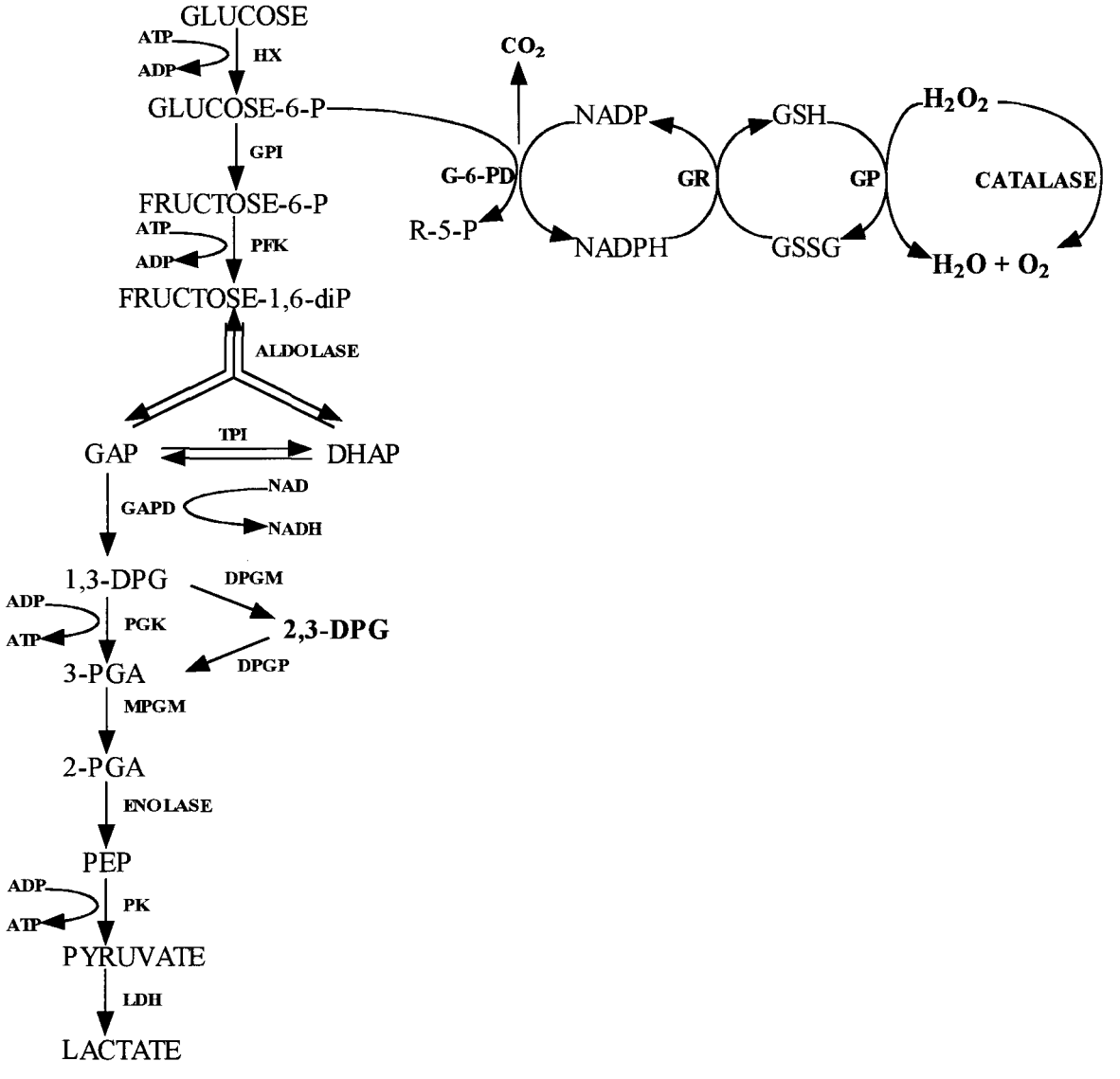

Figure 1. Diagrammatic representation of the Emden Meyerhof pathway (glycolysis) and the Hexose Monophosphate pathway.

The most abundant protein of the red cell, haemoglobin, was the first protein to have its molecular mass accurately determined, to be characterised by ultracentrifugation and to be associated with a physiological function (14). For it to fulfil its role in O_2 transport and delivery, the integrity of the red cell itself is required and this is dependent on two major metabolic pathways operative in the erythrocyte. These are the Emden Myerhof pathway (glycolysis) and the Hexose Monophosphate Shunt pathway (HMP) (fig.1).

Glycolysis has a threefold function:

- production of 2,3-DPG which modulates the oxygen affinity of haemoglobin,
- production of ATP which is basically the cell's energy currency, and
- the maintenance of NADH which forms part of the reducing power of the cell.

At rest the glycolytic pathway metabolises more than 95% of the glucose in the red cell and the remainder is diverted through the HMP.

It is the function of the HMP pathway to generate enough NADPH so that GSH levels can be maintained. Failure of this system would result in gradual oxidation and denaturation of cellular lipids and proteins.

Although some comparative work has been done on other species, overfamiliarity with the metabolism of the human red blood cell may have lead researchers to assume that all mammalian red cells behave similarly. This notion is proven wrong by the comparative aspect of this project which highlights remarkably interesting differences between the red blood cells of different mammalian species.

1.3 Unique metabolic characteristics of rhinoceros red blood cells

Initial studies by Paglia *et al.* indicated that black rhinoceros red blood cells possessed unique metabolic characteristics compared to other mammalian species. These background studies have established the following:

(i) Rhinoceros red blood cells contain very low levels of ATP (2-5% human levels) (15,16). Since ATP is the cell's 'energy currency' it was initially thought that this was the cause of the haemolytic anaemia. ATP supplies the energy for a number of crucial processes in the red cell:

- the Na^{+}-K^{+} ATPase pump
- other membrane pumps e.g. Ca^{2+}, Mg^{2+}
- synthesis of GSH and PRPP
- phosphorylation of membrane proteins

However, since normal individuals of all species of rhinoceros were found to exhibit this feature, it was eliminated as a direct cause for the haemolytic anaemia, although this does not exclude it from playing a contributory role in the metabolic process.

(ii) Enzyme studies revealed that black rhinoceros red blood cells have markedly altered enzyme levels relative to man and other species of rhinoceros (fig. 2). One must be careful, however, not to confuse the in vitro Vmax with intracellular activity as regulatory aspects differ.

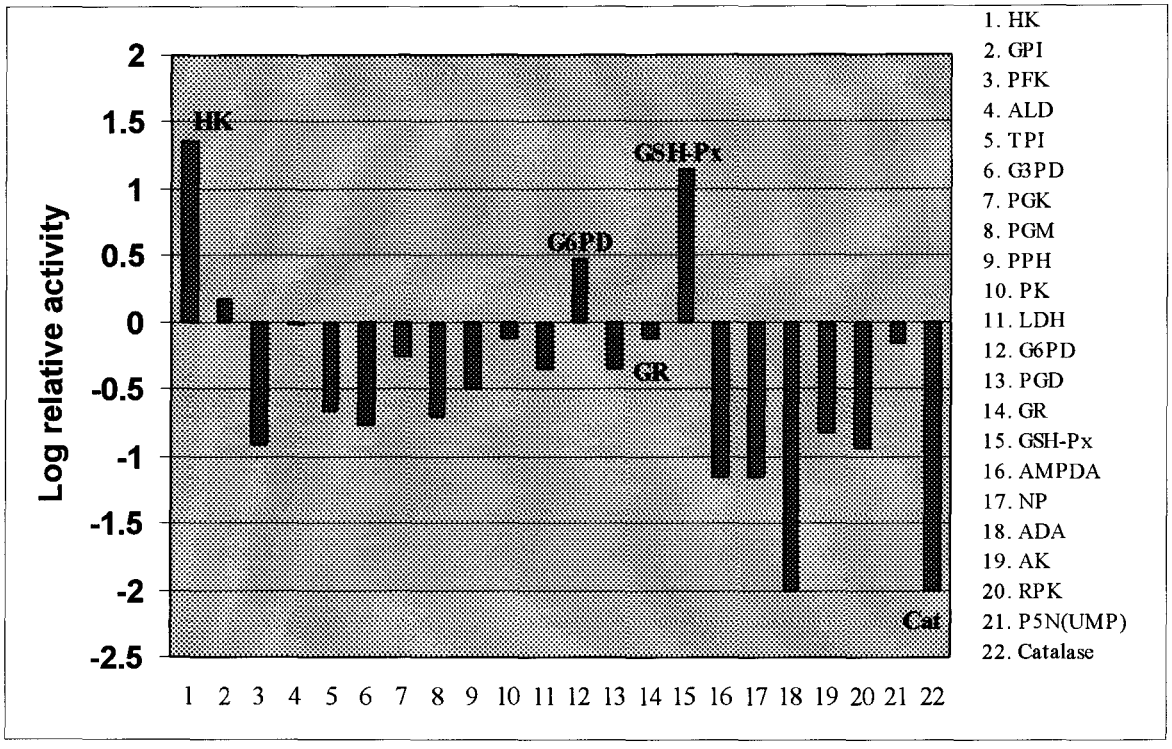

Figure 2. Black rhinoceros red blood cell enzyme activities relative to man. Levels are based on the findings of Paglia *et al.* (15).

The most striking difference is the relative deficiency in catalase activity (17,18). The dog and the duck are two animals which share this erythrocyte trait with the black rhinoceros (19,20). Most other mammalian cells possess this enzyme in the greatest abundance to neutralise hydrogen peroxide, a toxic oxidant generated under many normal and pathological conditions. This apparent hypocatalasemia may contribute to the mucocutaneous ulcerations suffered by black rhinoceroses in captivity since the human deficiency of this enzyme (Takahara's disease) is characterised by gangrenous oropharyngeal and gingival ulcers (21-23). Other species such as African white (*Ceratotherium simum*), Asian greater one-horned (*Rhinoceros unicornis*) and Sumatran

(*Dicerorhinus sumatrensis*) rhinoceroses display red cell catalase activity that is intermediate between the activities observed in black rhinoceroses and primates (17). The fact that other species of rhinoceros are not afflicted with the susceptibility to haemolytic anaemia and ulcerative disease as are black rhinoceroses suggests that the relative acatalasaemia and therefore impaired oxidant defences contribute to the pathogenesis of these disorders in the captive black rhinoceros.

In humans acatalasaemia, or Takahara's disease as it is also known, is associated with oral gangrene and ulceration. Rare cases of this disorder are primarily found in Japan and Switzerland. The Japanese form of the disease is more severe than the normally asymptomatic Swiss form. Japanese patients have between 2 and 4 percent of normal catalase activity whereas Swiss patients generally have ~ 15 percent (24-26). A deficiency of catalase predisposes the patient to infection with peroxide-generating bacteria. This may be the cause of the oral gangrene and ulceration which may lead to extensive tissue damage, especially affecting the gums. Aside from the symptoms mentioned above patients with acatalasaemia have no other serious effects. Acatalasaemia predisposes haemolysis.

It must also be noted that glutathione peroxidase activity is increased several-fold relative to human levels which would suggest a greater dependency on GSH recycling during episodes of oxidative stress.

(3) All rhinoceroses contain large amounts of non-adenine compounds not normally found in other mammalian red blood cells, the nature and function of which are still unknown.

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This study will shed light on one of the major unidentified UV absorbing species observed in rhinoceros red blood cells.

The observation that black rhinoceros red blood cells are particularly susceptible to oxidative stress (27) initially focused our attention on the HMP since it is the function of this pathway to protect the red blood cell against reactive oxygen intermediates. The optimal functioning of this pathway is therefore critical to the survival of the red blood cell under conditions of oxidative stress.

The antioxidant machinery of the red blood cell consists of catalase and the glutathione peroxidase / reductase system. Both rely on the production of NADPH as an essential co-factor for the enzymatic reduction of hydrogen peroxide.

1.4 Glucose 6-Phosphate Dehydrogenase deficiency

Glucose 6-phosphate dehydrogenase (G-6-PD) catalyses the first reaction of the hexose monophosphate pathway or the oxidative "shunt" as it is also known. It produces NADPH which is an important co-factor for reducing enzymes in cells. NADPH is the co-factor for glutathione reductase which serves to maintain reduced glutathione (GSH) levels in the cell and is therefore essential in protecting the cell against oxidative damage. GSH reduces H_2O_2 to H_2O stoichiometrically via glutathione peroxidase.

Much controversy has surrounded the role played by catalase in the detoxification of H_2O_2 in human erythrocytes. The observation that red blood cells deficient in glucose-6-phosphate and NADPH are susceptible to lysis by peroxides while acatalasaemic red blood cells are not (28) has lent support to the notion that the glutathione peroxidase/reductase

system is the principle means of peroxide detoxification in erythrocytes (29). This lead to the assumption that catalase was only important above a certain threshold level of H_2O_2 concentration (29). The mechanism by which catalase remains active in the presence of its own substrate, hydrogen peroxide has for a long time been poorly understood. It has been reported that catalase contains tightly bound NADPH, four molecules per tetrameric enzyme molecule (30). The function of this NAPDH is to prevent and reverse the accumulation of compound II, an inactive form of the enzyme (31,32). In a system where normal and acatalasaemic erythrocytes could be lysed, reconstituted with catalase and resealed, it has been shown that catalase and not GSH concentration decreased oxidant sensitivity of the erythrocytes (33). Therefore it was concluded that catalase was at least as important as GSH in the protection of erythrocytes against H_2O_2. However work done by Gaetani *et al.* has proved that catalase is responsible for more than half of the peroxide removal when cells are incubated with H_2O_2 concentrations comparable to that which causes haemolysis in G-6-PD deficient red blood cells (28). In a paper which clears up the controversy of the mechanism of H_2O_2 detoxification to a considerable degree, Gaetani *et al.* shows that increased dependence on the glutathione peroxidase / reductase system only occures after 98% of the catalase had been inactivated. Therefore these two systems function in a co-ordinated way in which the glutathione dependant system is the rate-limiting step (34). Catalase and glutathione therefore share the load of removing H_2O_2 in human erythrocytes.

It was found that catalase activity was severely impaired in G-6-PD deficient erythrocytes in the presence of H_2O_2 compared to normal cells (35). The addition of NADPH was found to significantly decrease catalase inactivation whereas the addition of NADP had no

effect. Despite the addition of NADPH to acatalasaemic mouse erythrocytes, the addition of catalase was required to ameliorate the effect of oxidant sensitivity. It was therefore postulated that decreased catalase activity is a major contributor to the oxidant susceptibility of G-6-PD deficient red blood cells (35).

A deficiency of G-6-PD results in an inability to maintain adequate NADPH levels which eventually results in haemolysis in one of two main ways. Chronic haemolysis is a feature of class I G-6-PD deficient variants. In these red cells NADPH production is inadequate and this results in the failure to maintain sulfhydryl groups of key proteins in the reduced state. Inter- and intramolecular disulphides are formed which lead to protein aggregation and a decrease in red cell deformability. The cell surface of the erythrocyte may be modified in such a way that it is recognised by macrophages as abnormal and this then leads to removal of these cells by the spleen and therefore extravascular haemolysis.

In class II and III variants episodic haemolysis occurs as a result of the exposure to an exogenous substance. These haemolytic agents often stimulate the HMP indicating that increased NADPH is needed in their presence. Lack of sufficient NADPH in G-6-PD deficient red cells leads to low GSH levels which is always associated with a haemolytic event. The inability to deal effectively with oxidative stress leads to the formation of Heinz bodies within the red blood cells which also mediate haemolysis (36). Heinz bodies are insoluble aggregates of denatured proteins which form inside erythrocytes.

Therefore the inability of these cells to generate sufficient NADPH during an oxidative challenge to deal with hydrogen peroxide and oxygen radicals leads to acute intravascular haemolysis.

The following syndromes are associated with G-6-PD deficiency: drug-induced haemolysis, infection-induced haemolysis, neonatal jaundice (NNJ), chronic nonspherocytic haemolytic anaemia and favism (37).

Drug-induced haemolysis occurs when affected cells are exposed to certain drugs such as antimalarials (e.g. primaquine), sulfonamides (e.g. sulfacetamide), sulfones (e.g. dapsone), antipyretics/analgesics (e.g. acetanilid) and other drugs such as naphthalene and methylene blue (37).

Bacterial, viral and rickettsial infections have been associated with haemolysis but particularly important are infectious hepatitis (38), pneumonia (39) and typhoid fever (40). The severity of the haemolysis is influenced by various factors such as concurrent administration of oxidant drugs, hepatic function, level of haemoglobin and age.

Favism refers to the occurrence of acute haemolysis after the ingestion of broad beans (*Vicia faba*). Favism is prevalent in the Mediterranean countries, Middle East, Far East and North Africa (41). These are areas where the consumption of fava beans is customary. This type of haemolysis is similar to that observed in primaquine-induced haemolytic anaemia which implicates oxidative stress as the precipitating factor. Prevention comes simply by avoidance of fava beans and treatment is blood transfusion in severe cases (37).

Since black rhinoceros red blood cells showed sensitivity to oxidative stress and also the observation of Heinz body production when incubated with ascorbic acid, it was initially assumed that these cells were G-6-PD deficient. Subsequent enzyme assays showed that

these enzyme levels were in fact increased relative to humans therefore ruled out this as a cause of the haemolytic anaemia.

1.5 Stimulation of the HMP

1.5.1 Ascorbic acid: pro- or antioxidant ?

Ascorbic acid has been ascribed many physiological roles since its discovery about six decades ago by Szent-Gyorgyi and Waugh and King (42,43) yet its exact function has not been completely elucidated. Its role in preventing and treating scurvy is well documented. Ascorbic acid is an effective reducing agent and has long been known to play a role in free radical metabolism in tissues. This fact and its ability to stimulate the HMP (44) brings it into the scope of relevance with respect to the present problem. Its interaction with GSH has been the subject of much controversy with this being the focus of much research especially since the connection was made between reactive oxygen intermediates and certain diseases. Interest in cellular antioxidants has therefore heightened.

The oxidised form of ascorbic acid, dehydroascorbic acid (DHA), is taken up by cells more readily than is ascorbic acid itself (45-47). Once in the cytosol the DHA is reduced back to ascorbic acid by GSH in a process which has recently been found to be nonenzymatic (48). The existence of a DHA reductase has been dismissed (49). The redox coupling between GSH/GSSG and ascorbic acid/ DHA is nonenzymatic:

$$2GSH + DHA \longrightarrow GSSG + \text{ascorbic acid}$$

Ascorbic acid may be described as a double edged sword since it acts as both an anti- and pro-oxidant under various conditions. Its role as an antioxidant will first be addressed.

Meister and colleagues have shown that ascorbic acid 'spares' GSH and vice versa (50,51). Their experimental system was newborn rats which do not synthesize ascorbate. The rats were made GSH deficient by the administration of BSO, an irreversible inhibitor of γ-glutamylcysteine synthetase. Cellular damage that results in the liver, kidney, lung and brain was prevented by the supplementation of ascorbic acid. The administration of ascorbate to the animals resulted in an increase in the mitochondrial GSH levels in the tissues of rats given BSO plus ascorbate compared to those only given BSO (50). Thus ascorbate 'spares' GSH. The 'sparing' of ascorbate by GSH refers to the reduction of DHA to ascorbic acid (by GSH) which then shares the load of reacting with free radicals.

Johnson *et al.* have shown that ascorbic acid (500mg/d) significantly elevates red blood cell glutathione levels in the blood of healthy adults and therefore improves the antioxidant protection capacity of blood (52).

Doba *et al.* have reported that ascorbic acid is an effective inhibitor of peroxidations in the aqueous phase but not so in the lipid phase (53,54). It is therefore a very good synergist with phenolic antioxidants such as α-tocopherol or its water-soluble analogue TROLOX. Ascorbate reduces oxidised α-tocopherol thereby enabling it to carry on its antioxidant effect in the cell membrane. It has been proposed that ascorbate acts as a chain breaking antioxidant by reducing peroxyl radicals in the aqueous phase before it enters liposome and causes lipid oxidation. Vitamin E on the other hand acts as an antioxidant in the membrane, preventing lipid peroxidation by oxidants. Investigation of the vitamin E levels of wild versus captive rhinoceroses by Dierenfeld *et al.* showed that these levels were significantly higher in free ranging animals than those in captivity (55). Vitamin E supplementation was then indicated. In a later study by Ghebremeskel *et al.* vitamin E

levels in supplemented captive rhinoceroses were found to be comparable to those of rhinoceroses in the wild (56).

Ascorbate, at ten times the concentration normally found in human erythrocytes i.e 1mM, is effective at reducing nitroxide radicals in the erythrocyte membrane. NAD, NADP and unknown heat-sensitive components enhance this ability. Other antioxidants such as CAT, glutathione peroxidase, NADH, NADPH, SOD, vitamin E and CO-gassed haemoglobin (HbCO) play a negligible role in the reduction of membrane nitroxides (57).

Ascorbic acid enters the red blood cell as DHA. DHA is converted to ascorbate by GSH and this ascorbate works in conjunction with any left over GSH to reduce intracellular free radicals. This is accomplished by the reduction of DHA by GSH to form ascorbic acid which in turn reduces nitroxides along with a thiol-DHA complex (58). Low concentrations of ascorbate in erythrocytes is enough to have a significant effect since the reaction is linked to the HMP enzymatic machinery.

Work done by Winterbourne and co-workers have shown that ascorbate protects G-6-PD deficient erythrocytes from methaemoglobin formation and Heinz body production when incubated with acetylphenylhydrazine (APH) (59). APH is one of many redox drugs that cause Heinz body formation and haemolysis in G-6-PD deficient red blood cells. Reaction of the drug with oxyhaemoglobin results in its oxidation to form the drug free radical, hydrogen peroxide and methaemoglobin. Further reaction produces denatured haemoglobin derivatives which precipitate and form insoluble inclusions within the red cells, termed Heinz bodies. In G-6-PD deficient cells where the HMP is impaired and GSH production is reduced, these reactions proceed with subsequent haemolysis.

Circulating free iron is extremely hazardous since it has the ability to generate toxic amounts of free radicals via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Iron is therefore sequestered by two types of iron-binding proteins, transferrin and ferritin. Transferrin is a high affinity, low capacity protein which binds two atoms of iron per molecule of transferrin. Ferritin is a lower affinity protein with a high capacity which binds a maximum of 4500 atoms of iron per molecule of ferritin. Iron is bound within ferritin as Fe^{3+} which is harmless. Ascorbic acid, in the reduced state and when at high serum concentration, penetrates the ferritin shell and converts the Fe^{3+} to catalytic Fe^{2+}. These reactive Fe^{2+} atoms leak out of the protein shell, react with ascorbate and generate free radicals (60). In the presence of transition metal irons, ascorbic acid behaves as a pro-oxidant.

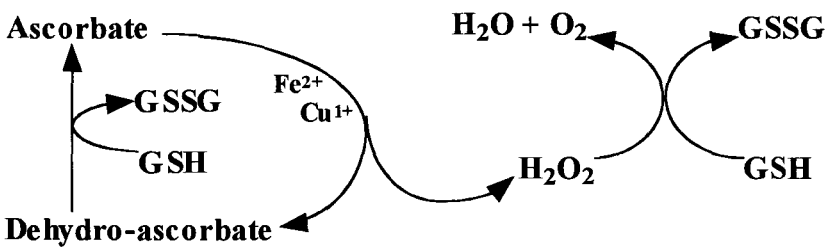

Figure 3. The mechanism of action of excess ascorbate in the red blood cell.

Udomratn and co-workers have reported that ascorbate causes haemolysis in human red blood cells which are G-6-PD deficient when administered in large doses (61). The damage is in the form of haemoglobin oxidation as evidenced by the production of Heinz bodies within these erythrocytes. A specific case reported by the same group involved a patient who having received large doses of ascorbic acid intravenously on two successive

days, developed intravascular haemolysis and died (62). Mengel and Greene have reported the increased in vitro lytic sensitivity of normal erythrocytes to hydrogen peroxide when incubated with 5g of ascorbic acid (63).

Another case of ascorbate inducing haemolysis in a patient with G-6-PD deficiency is reported by Rees and colleagues (64). After consulting with a nutritionist the patient was found to have low red cell and serum GSH. It was not known at this time that the patient was G-6-PD deficient. He was prescribed very high dosages of ascorbic acid and eventually presented with what proved to be oxidative haemolysis. After appropriate enzyme assays were performed G-6-PD deficiency was confirmed.

In summary then, in patients with G-6-PD deficiency, low doses of ascorbate have been found to protect red blood cells against haemolysis whereas high doses have been shown to precipitate episodes of haemolysis. Studies in rats with G-6-PD deficiency confirm this (61). This may be explained by the fact that at low concentrations ascorbic acid 'spares' GSH as discussed earlier and helps to increase the GSH levels in G-6-PD deficient red cells, thereby increasing the antioxidant status and protecting the cell against oxidative damage. At high concentrations however the ascorbic acid manages to penetrate the ferritin shell and release catalytic Fe^{2+} atoms. The reaction of this catalytic Fe^{2+} with hydrogen peroxide results in the generation of the OH radical, a potent oxidant.

1.5.2 Methylene Blue

Methylene blue, also a known stimulator of the HMP, is an organic dye which acts as a redox compound. Its oxidised form reacts directly with NADPH to form NADP which is a regulator of HMP activity (65). The stimulatory action of methylene blue was first demonstrated by Harrop and Barron (1928) (66).

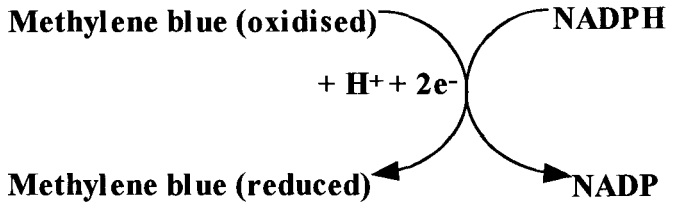

Figure 4. Stimulation of the HMP by methylene blue.

The electrophilic properties of methylene blue enable it to convert NADPH to NADP while the dye is reduced to a colourless leuco-compound which, in contact with air, can rapidly be oxidised to the blue form. This mode of action was first suggested by Gibson in 1948 (67). Later work done by Metz and co-workers (68) showed that high concentrations (~ 0.1mM) of methylene blue are associated with the production of peroxide. This then implies that at high concentrations methylene blue stimulates the shunt via the glutathione peroxidase / reductase reaction.

The stimulatory action of methylene blue on the HMP is considerable since at a concentration of 0.16mM the pathway is stimulated approximately forty times so that it becomes the major route for consumption of glucose, bypassing phosphofructokinase (69).

1.6 Types of Heamolytic Anaemia

Haemolytic anaemias are separated into two broad categories: (1) haemolytic anaemias due to abnormalities of the red cell itself and (2) haemolytic anaemias due to abnormalities of the environment of the red blood cell.

The first category is divided into congenital abnormalities and acquired abnormalities. Congenital abnormalities refer to disorders of the membrane, disorders of metabolism and haemoglobinopathies (70). Disorders of the membrane such as hereditary spherocytosis and hereditary elliptocytosis have been excluded as possible causes of the haemolytic anaemia problem in the black rhinoceros. Haemoglobin structural studies have not shown any abnormalities either (8). Black rhinoceros red blood cells were found to have markedly different enzyme activity profiles as already mentioned. The relative deficiency in catalase activity in particular suggests that these animals must have a novel way of dealing with oxidative challenges. The red cells were shown not to be deficient in G-6-PD and any other enzyme which may more typically lead to haemolysis. Therefore the known disorders of metabolism cannot be given as a cause of the haemolytic anaemia experienced by the black rhinoceros.

Chaplin *et al.* performed haematologic and immunohaematologic studies on the red blood cells of 6 black rhinoceroses: 3 healthy nonrelated rhinoceroses, 1 rhinoceros with iron deficiency anaemia and 2 rhinoceroses with intravascular haemolysis (8). Results indicated that osmotic fragility, erythrocyte membrane protein composition, haemoglobin electrophoresis and haemoglobin stability did not distinguish between healthy and affected rhinoceroses. Immunological data also provided no significant link with the haemolytic crisis in the two haemolysing rhinoceroses.

Since the possibilities of immunologic and red cell membrane abnormalities as well as structural changes of the haemoglobin protein have been eliminated as direct causes of the haemolytic syndrome, this project is aimed at identifying a metabolic disorder as a possible explanation for the haemolytic anaemia syndrome plaguing black rhinoceroses in captivity.

2. Materials and methods

2.1 Preparation of blood samples

Blood samples were collected in sterile Vac-u-test heparin-lithium tubes. The plasma and buffy coat were removed after centrifugation at 4500rpm for 5 min in a Sigma 302K centrifuge. The red cells were washed twice in isotonic saline.

2.2 CO_2 Assay

2.2.1 Preparation

The assay was performed using 35 x 10mm petri dishes (Corning). Using a heated needle, a hole was pierced into the lid and then sealed with Beckman vacuum grease. 25mm glass microfibre filters (Whatman, England) were placed into position on top of the lids using vacuum grease. The rims of the petri dishes were also greased to make the dishes airtight.

2.2.2 Labelling conditions

The labelling medium was prepared using 0.1uCi/ml of either D[1-^{14}C] glucose, specific activity 54mCi/mmol , (Amersham Lifescience, England) or D[2-^{14}C] glucose, specific activity 55mCi/mmol, (American Radiolabelled Chemicals, St.Louis, USA). An amount of 1.5ml of medium containing all the label was pre-incubated at room temperature in a 35 x 10mm petri dish, with a filter saturated with 150µl of 2M NaOH attached to the lid, for 30min to equilibrate the labelled medium. After incubation the labelled medium was added to the total amount of Hanks balanced salt solution (HBSS) (GibcoBRL) required for the assay.

2.2.3 Experimental Procedure

The method used was a modification of the method by Dancis et.al (71). 0.5ml of the labelled medium and the required amount of HMP stimulant was pippeted into 5ml polypropylene tubes (Elkay). Red blood cells were resuspended in HBBS (GibcoBRL), counted using a Coulter counter (Coulter electronics inc., Hiahleah, Florida) and the suspension was adjusted to $\pm 10^6$ cells per microlitre which was then pippeted into 5ml tubes (Elkay) which contained 0.5ml of the labelled medium and the required amount of HMP stimulant. The contents of the tubes were inverted, mixed and dispensed into the petri dishes. The filters were saturated with 150µl of 2M NaOH (Saarchem). The lids were secured on the dishes and incubated for 4 hours at 37^0C in a Memmert dry incubator while shaking on a GFL 3015 shaker. After 4 hours 150µl of 1.2M PCA (Merck) was injected through the hole in the lids to lyse and precipitate the red blood cell proteins. The dishes were then placed on ice for 30min after which the filters were placed into radioactivity counting vials filled with 7ml of Ultima Gold XR scintillation fluid (Packard instrument company, Meridan, USA) and 500µl of distilled water. Radioactivity was measured in the filters after 24 hours in a Beckman liquid scintillation spectrometer model LS3801 using a window set at 425-675keV. The supernatant in the petri dishes was transferred to 5ml polypropylene tubes and centrifuged at 4500rpm for 5min in a Sigma 302K centrifuge to remove cellular debris. The supernatant was split between two tubes and only half was neutralised with 2.5M K_2CO_3 (BDH).

All CO_2 assays were done in duplicate and two blanks, without red blood cells, were run with each assay. The neutralised samples were used for lactate determinations and the non-neutralised samples for reduced glutathione assays.

Calculation of CO_2 concentration

$$X * 0.0252 = \text{nmoles } CO_2$$

0.0252 is a constant which takes into account the specific activity of labelled glucose expressed as dpm/nmole.

X is the counts of $^{14}CO_2$ given off and trapped on the filter (in dpm).

2.3 Lactate assay

The lactate content of the neutralised PCA extracts were determined by the method of Beutler et.al (72). 1ml of lactate buffer pH 9.2 was added to 200ul of 1% NAD solution (Boehringer Mannheim, Germany), 500µl of PCA extract and 1.3ml of distilled water in a 10x10x45mm Greiner plastic cuvette. The initial absorbance at 340nm was read on a Beckman DU-62 spectrophotometer against a blank which did not contain PCA extract. The absorbance of 100, 200 and 400mM of lactate standard (Boehringer Mannheim, Germany) was also read. 10µl of lactate dehydrogenase (100mg/10ml , Boehringer Mannheim , Germany) was added to each of the cuvettes, mixed and incubated at room temperature for 30min. The absorbance at 340nm was read. The concentration of lactate (mmoles/10^6 rbc) in the sample was then calculated by the following formula:

$$\frac{(OD_2 - OD_1)}{OD_2 std - OD_1 std} \times \frac{\text{Total vol.}}{3} \times \frac{100}{500}$$

2.4 Glutathione Assay

The concentration of reduced glutathione present in the non-neutralised PCA extracts was determined by a modification of the method by Beutler et.al (73).

2ml of 1M K_2HPO_4 was added to 0.5ml of PCA extract and mixed in 5ml polypropylene tubes. This was incubated on ice for 10min to allow precipitation of the resulting $KClO_4$. The tubes were centrifuged at 4500rpm for 10min in a Sigma 302K centrifuge to sediment the precipitate. 2ml of the supernatants was dispensed into 10x10x45mm Greiner plastic cuvettes and 250ul of 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) (*) (Koch-Light Laboratories, England) was added. The solution turned a yellow colour and the absorbance was read at 412nm on a Beckman DU-62 spectrometer. The absorbance was read against the sample which served as a blank in the CO_2 assay. As a control for the experiment, a standard 2mM solution of GSH was also assayed.

The concentration of GSH (mmoles/litre) in the samples was calculated by the following formula:

$$\frac{OD}{13.6} \times \frac{\text{dilution factor}}{1}$$

*DTNB solution: 20mg of DTNB per 100ml of 1% sodium citrate solution.

2.5 Preparation of blood samples for HPLC analysis

Blood was collected in Vac-u-test heparin-lithium tubes and centrifuged at 4500rpm for 5 min at room temperature in a Sigma 302K centrifuge. The plasma and buffy coat were removed and the packed cells washed twice with isotonic saline. Washed erythrocytes were resuspended in one volume of saline.

2.5.1 Acid extraction of red blood cells

The samples were deproteinated with two volumes of ice cold 1.2M PCA (Merck) added to the resuspended erythrocytes while mixing vigorously on a Vortex-genie2 vortex mixer (Scientific Industries Inc., New York, USA). The proteins were precipitated by

centrifugation at 4500rpm for 10 min. The supernatant was neutralised with 2.5M K_2CO_3 (BDH) and filtered through Millex-GV 0.22μm filters (Millipore, Bedford, USA).

2.5.2 Alkaline extraction of red blood cells

Packed red blood cells were resuspended in an equal volume of ice cold 0.16M KCl (BDH) solution containing 5mM glucose (BDH). Two volumes of ice cold 0.5M KOH (Kanto Chemicals) was added while mixing vigorously on a Vortex-Genie 2 vortex mixer (Scientific Industries). 600μl of ice cold water was added and the solution quickly transferred to a pre-cooled Centrifree filter unit (Millipore) and centrifuged at 1600g for 10min. The clear ultrafiltrate was collected and neutralised with 1M KH_2PO_4 (BDH).

2.6 Preparation of fibroblasts for HPLC analysis

Fibroblast cell lines were grown to confluency in $25cm^2$ or $75cm^2$ Greiner tissue culture flasks (Labortechnik, Germany) and maintained on Dulbecco's Modified Eagle Medium (DMEM) (GibcoBRL) and 10% foetal calf serum (FCS) (Delta). The adhering fibroblasts were washed three times in the flasks with saline. 500ul or 1ml of ice cold 1.2M PCA was added to $25cm^2$ or $75cm^2$ culture flasks respectively. The flasks were swirled to ensure that the PCA covered the entire layer of fibroblasts and then incubated on ice for 10min. The liquid content of the flasks was then pippeted into 1.5ml eppendorf tubes and neutralised with 2.5M K_2CO_3 (BDH).

2.7 HPLC analysis

PCA extracts of red blood cells and fibroblasts were analysed by anion exchange or reverse phase HPLC. The HPLC system consisted of a Beckman 126 programmable solvent module and a 168 diode array detector.

The anion exchange method used was essentially that of Simmonds et.al (74). A 250 x 3mm Hypersil 5 NH_2 (5μm) column (Phenomenex, Torrance, California, USA) was used. Buffer A consisted of 5mM KH_2PO_4 pH2.8 (BDH Laboratory Supplies Poole, England). Buffer B consisted of 0.5M KH_2PO_4, 1.0M KCl (E. Merck, Darmstadt, West Germany) at pH 3.5. Starting conditions were 1 % buffer B, 99 % buffer A. This changed after 1 min to 99 % buffer B over 15 min. These conditions were maintained for 1 min then returned to initial conditions over 7 min. The total run time was 30min.

The column used for reverse phase separations was a 250 x 4.6mm Ultrasphere C18 (5um) column (Beckman Instruments Inc., San Ramon,California, USA). Buffer A consisted of 10mM KH_2PO_4 pH 5.6 (BDH Laboratory supplies, England). Buffer B consisted of 50% methanol in water. Initial conditions were 1% buffer B, 99% buffer A and changed after 5min to 20% buffer B over 3min. These conditions were maintained for 15min then returned to initial conditions over 3min. The total running time was 31min. Extracts (100μl) were injected onto the column and the UV absorbance was monitored at 260 and 280 nm.

Compounds were identified by retention time, 280/260 ratio and co-elution with standards. All HPLC buffers were made with distilled deionised water.

Assays were performed which involved incubating red cells with label to follow the uptake of the label into the various nucleotides. The red cells were extracted as described previously and nucleotide analysis performed by anion-exchange HPLC linked to a radio-chromatography detector (Flo-One Beta , series A-100 , Radiomatic).

In this way an absorbance nucleotide profile as well as a labelled nucleotide profile was produced.

2.8 Source of blood samples

Human blood samples were donated by healthy laboratory personnel. Rhino blood samples were obtained opportunistically from various game parks in Natal (Umfolozi, Hluhlwe, Augrabies), Gauteng (Kruger National Park) and Zimbabwe. Horse blood was obtained from the Blue Cross Veternary hospital. Bovine blood was collected at the Maitland abattoir. Rabbit blood was obtained from experimental animals in the department of Tissue Immunology, UCT Medical School. All donor animals were apparently healthy.

2.9 Freezing of red blood cells

The blood was centrifuged at 3500rpm for 5min and the plasma and buffy coat were aspirated. The red blood cells were then washed once with isotonic saline. Packed cells were diluted 1:1 with the freezing medium and allowed to incubate at room temperature for at least 20min. This red blood cell mixture was then frozen drop-wise in liquid nitrogen and stored until needed. When needed the red cells were rapidly thawed in saline, heated to ~40^{0}C and washed three times with saline.

Freezing medium:

Sucrose (BDH) 77g

D-Glucose (BDH) 29g

NaCl (B&M Scientific) 1.48g

Dissolve in distilled water and make up to 500ml.

2.10 Uricase assay

The test for uric acid as in the case of the urate riboside in bovine red blood cells was performed by incubating 2μg (1μl of 2mg/ml stock) of uricase (Boehringer Mannheim) with 50μl of PCA extract at 37^{0}C for 15min. The characteristic peak shift on subsequent HPLC analysis confirmed the identity of the peak as being uric acid or a urate nucleotide.

2.11 HOCl experiments

Red blood cell pellets were thawed and washed three times with isotonic saline. Cells were then resuspended in Hanks balanced salt solution (HBSS, GibcoBRL) containing 2.5mM HOCl (prepared by titrating NaOCl with HCl) to a haematocrit of 12.5% and incubated at 37^{0}C. Aliquots of red blood cell suspension were taken and acid extracted at various timepoints. Samples were analysed by reverse phase HPLC. Standard tyrosine solutions and rhinoceros red blood cell extracts were also incubated with HOCl and analysed by reverse phase HPLC.

2.12 Amino acid analysis

Amino acid analysis was performed on a Beckman System 6300 High Performance Analyser using a 10cm cation exchange column and lithium based buffers according to a standard method developed by the manufacturers.

2.13 Dityrosine formation

Rhinoceros red blood cells were pre-incubated with Hanks balanced salt solution (GibcoBRL) at 37^{0}C for 1hour. Cells were then washed three times with isotonic saline and resuspended in HBSS (GibcoBRL) containing 2mM H_2O_2 (Fluka) (or as indicated in the legend to the figures). 2.5ml aliquots were then taken at the times indicated, acid

extracted, neutralised and analysed by reverse phase HPLC. Time 0 aliquots were always taken just before resuspension in H_2O_2-containing HBSS.

Red blood cell free lysates were prepared by resuspending packed red blood cells in an equal volume of distilled water and freeze thawing six times. Lysates were then centrifuged at 10 000rpm for 25min in a Sigma 302K centrifuge to remove cellular debris. Supernatants were dialysed at 4^0C for 24hours against a solution of PBS. A basic enzyme preparation remained which was reconstituted as indicated in the results section.

Heat inactivated red blood cell lysates were prepared incubating the lysates in boiling water for 5min. The lysates were centrifuged and the supernatants (heat inactivated lysates) used in subsequent experiments.

0.5mM tyrosine (Sigma) and 2mM H_2O_2 (Fluka) were incubated with either human or rhinoceros heat inactivated or non-heat inactivated red blood cell lysates at 37^0C for various times before analysis for dityrosine production by fluorescence spectroscopy. Additions were 0.5mM GSH (Sigma), 0.1mM ferrous sulfate (BDH) or 10μM hemin (bovine from Sigma).

Standard dityrosine was produced by a modification of the method by Malencik et.al (75). 800μl of 7mM tyrosine (Sigma) was heated until dissolved and cooled to 30-40^0C. 200μl of PBS and 0.5μCi of L-[U-^{14}C]tyrosine (Amersham) was added and incubated at 37^0C for 5min. 25U/ml of horse radish peroxidase (Boehringer Mannheim) was added, the solution mixed and 2.5mM H_2O_2 (Fluka) was added. The solution was incubated at 37^0C

for 30min then centrifuged in a benchtop ultrafuge (LASEC) for 1min before being analysed on reverse phase HPLC.

2.14 CDNB-mediated GSH depletion

After pre-incubation with HBSS for 1hour at 37^{0}C, red blood cells were washed three times with saline and resuspended in HBSS containing 1mM 1-chloro-2,4-dinitrobenzene (Sigma). This suspension was incubated at 37^{0}C for 30min then the cells were washed three times with saline. Cells were then incubated in HBSS containing 2mM H_2O_2 (Fluka) and acid extracted at various times. Red blood cell extracts were analysed on reverse phase HPLC.

2.15 Haem-catalysed dityrosine production

A stock solution of 10mM hemin (bovine from Sigma) was prepared in 20mM NaOH (Saarchem). Dilutions of 0.1, 1 and 10μM in PBS were incubated with 0.5mM tyrosine (Sigma) and 2mM H_2O_2 (Fluka) at 37^{0}C and acid hydrolysed with PCA (Merck) at various timepoints. Samples were neutralised with 2.5M K_2CO_3 (BDH) and dityrosine was assayed for by fluorimetry.

2.16 Haemoglobin catalysed dityrosine production

A stock solution of 1mM haemoglobin (Sigma) was prepared in distilled water. Dilutions of 1 and 10μM in PBS were incubated with 0.5mM tyrosine (Sigma) and 2mM H_2O_2 (Fluka) at 37^{0}C and acid hydrolysed with PCA (Merck) at various timepoints. Samples were neutralised with 2.5M K_2CO_3 (BDH) and dityrosine was assayed for by fluorimetry.

2.17 Mass Spectroscopy

Tyrosine and dityrosine fractions were collected from reverse phase HPLC, freeze dried in a Freezemobile 6 freezedrier (Virtis) and analysed by Matrix Assisted Laser Desorption mass spectroscopy with time-of-flight detection (MALDI-Tof) on a Perseptive Biosystems DE-PRO MALDI mass spectrometer.

2.18 Fluorimetry

Red blood cell extracts were diluted 1:4 with distilled water and analysed on a SPEX FluoroMax spectrofluorometer. Excitation and emission scans were obtained by using wavelength windows of 250-350nm and 350-450nm respectively. Tyrosine has an λ_{ex} and λ_{em} maximum of 280nm and 300-305nm respectively. Dityrosine is intensely fluorescent and has an λ_{ex} and λ_{em} maximum of 315nm and 409nm respectively.

3. Results

3.1 Comparative red blood cell nucleotide composition

3.1.1 Rhinoceros versus human red blood cells

Rhinoceros red blood cells, as already mentioned, have an unusual nucleotide composition with ATP levels being as low as 2-5% of human levels and the presence of large amounts of uridine and "cytidine-like" compounds. The identity and function of the "cytidine-like" compound will be discussed later.

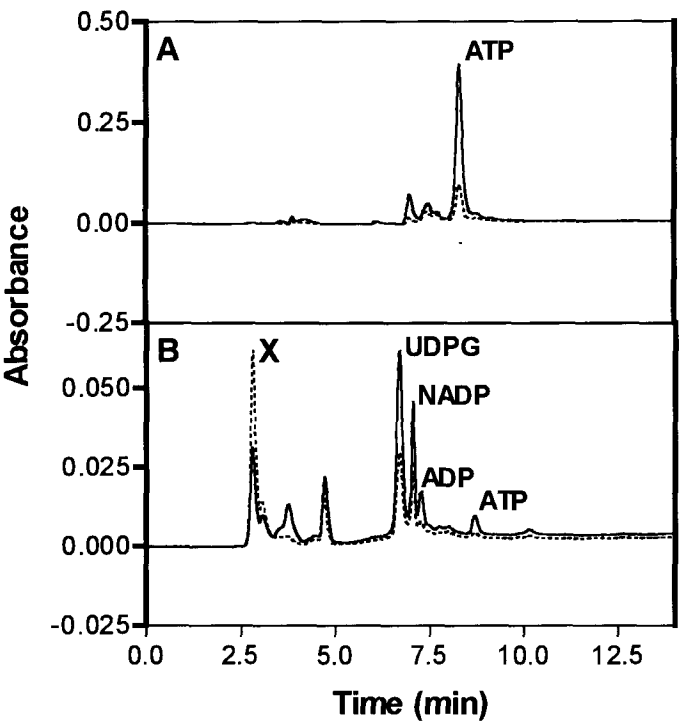

Figure 5. Anion exchange HPLC of nucleotide extracts of human (A) and rhinoceros (B) red blood cells. —— A_{260nm}, ····· A_{280nm}.

It is interesting to note that while rhinoceros red blood cells have extraordinarily low ATP levels, rhinoceros fibroblasts have ATP levels which are similar to those in fibroblasts from

other orders of mammals. High Pressure Liquid Chromatography (HPLC) has proven to be an indispensable tool for studying nucleotide composition.

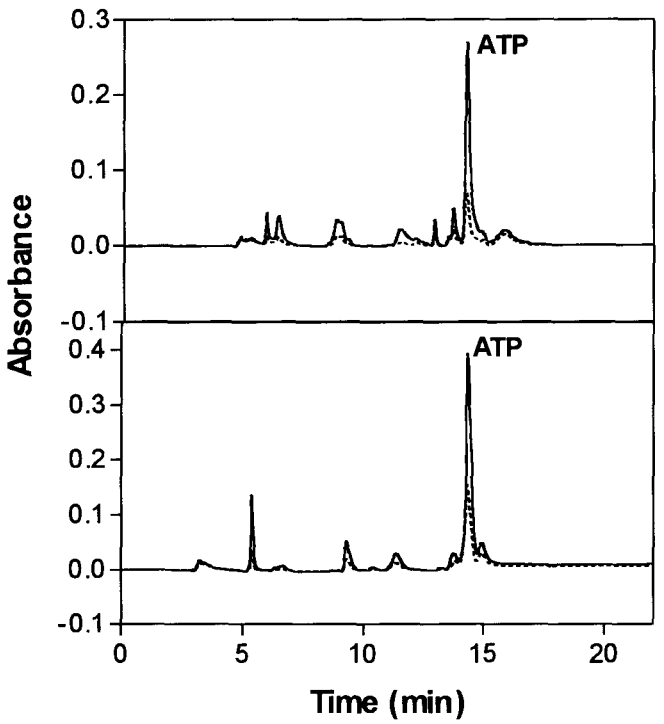

Figure 6. Anion exchange HPLC nucleotide profiles of PCA extracts of rhinoceros (A) and human (B) fibroblast cultures.

Note the absence of any cytidine-like compounds in the human red blood cell profile. . Rhinoceros fibroblast ATP levels approach that seen in human fibroblasts unlike the case with the red blood cells. These profiles (fig.5) are representative of >30 rhinoceros red blood cell nucleotide profiles and ten human red blood cell nucleotide profiles.

These striking differences in red blood cell nucleotide composition between two mammalian species, rhinoceros and human, has lead to a broader comparative study. It

includes four other mammalian species and these are the horse, rabbit and cow. Each of the species studied exhibited characteristic features not seen in the other red blood cells.

3.1.2 Horse red blood cells

The red blood cells of horses were found to contain a significant amount of cytidine triphosphate (CTP)(fig.7). None of the other species studied has shown this feature. Out of ten horse blood samples analysed six showed the presence of a CTP peak. Cytidine nucleotides are the only nucleotides in the red blood cell that are involved in the activation and transfer of alcohols therefore the presence of CTP in horses may indicate a greater demand for fatty acid synthesis relative to other mammals. It is also interesting to note that although horses belong to the same order of mammals as rhinoceroses, their nucleotide profiles are significantly different with respect to the ATP content. The ATP content of horse red cells were in the range of approximately 6-10% of human red cells whereas that of rhinoceroses, as mentioned earlier, were consistently between 2 and 5% of human values.

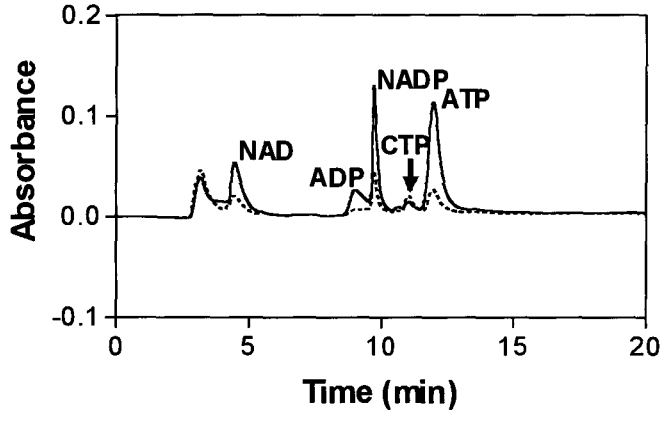

Figure 7. Anion exchange HPLC of horse red blood cell nucleotides.

3.1.3 Rabbit red blood cells

Rabbit red blood cells were found to have the highest nucleotide content of all other species studied (fig.8). These cells contained similar levels of ATP compared to that observed in human red blood cells. The following figure shows the high nucleotide content of rabbit red blood cells. This profile (fig.8) is representative of ten rabbit red blood cell nucleotide profiles. Note Y-axis scale.

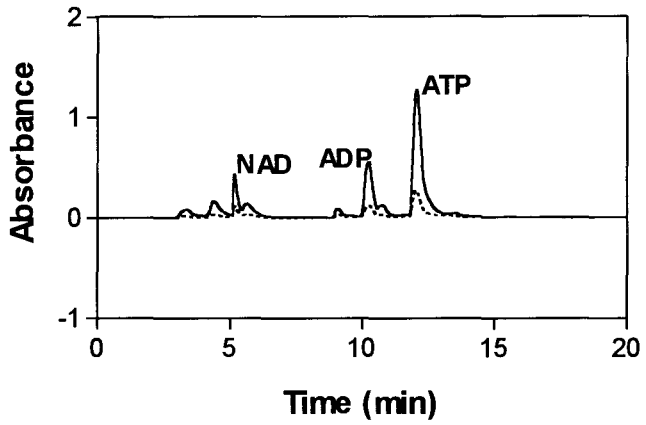

Figure 8. Anion exchange HPLC nucleotide profile of rabbit red blood cells.

3.1.4 Bovine red blood cells

Bovine red blood cells exhibited a peak similar to the "cytidine-like" peak seen in rhinoceros red blood cells with respect to the $A_{280/260nm}$ ratio (fig.9).

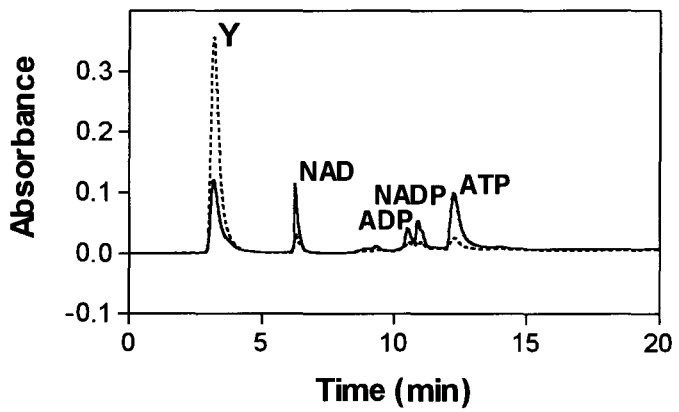

Figure 9. Anion exchange HPLC nucleotide profile of bovine red blood cells.

This compound (peak labelled Y) dominated the bovine red blood cell nucleotide profile and dwarfed the ATP peak so that it would appear similar to the ATP-deficient profile of rhinoceros red blood cells. Bovine red blood cells do however have up to 10% of the ATP levels seen in human red blood cells (note Y-axis scale). The identification of the dominant peak in this profile will be dealt with later.

3.1.4.1 Characterisation and identification of "bovine peak"

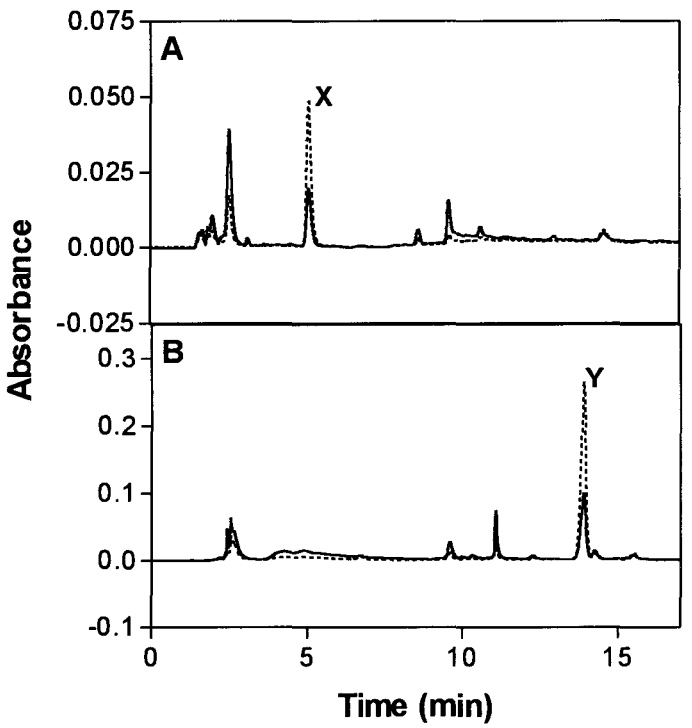

Figure 10. Reverse phase HPLC analysis shows the difference between the "cytidine-like" peak (X) of rhinoceros red blood cells and the "bovine peak" (Y) of bovine red blood cells.

Bovine, rhino and horse red blood cells were found to contain a cytidine-like peak near the origin of anion exchange HPLC nucleotide profiles. This peak present in bovine red blood

cells will be referred to as the "bovine peak". Reverse phase HPLC demonstrated that the cytidine-like peak in rhinoceros red blood cells and "bovine peak" are two distinct species despite the similarity displayed on anion exchange profiles (fig.10).

Several fractions containing the "bovine peak" were manually collected and freeze dried so that it could be concentrated. The peak eluted in distilled water which was favourable since there would be no salt to interfere with subsequent analysis. The concentrated sample of the peak was subjected to acid hydrolysis by incubation with concentrated perchloric acid at 100 ^{0}C for 1.5 hours. Acid hydrolysis would liberate the base from the compound which could then be identified. The acid hydrolysed sample was analysed by reverse phase HPLC and two peaks were observed, one accounted for residual unhydrolysed compound and the other was the base liberated by acid hydrolysis. The elution time and $A_{280/260nm\ ratio}$ of the base was similar to that of uric acid. Diode array analysis of standard uric acid and the base showed that the two compounds were identical. The peak was confirmed to be uric acid by adding uricase and observing the characteristic peak shift. Reaction, or rather non-reaction with alkaline phosphatase further aided identification of the peak as urate riboside.

3.1.5 Comparative nucleotide analysis

Table 1

Red blood cell nucleotide composition of five mammalian species.

	NAD	NADP	UDPG	ADP	ATP
Human	101.6 (43.9)	132.4 (52.0)	61.0 (49.4)	321.6 (88.7)	2314.0 (1524)
Rhino	10.6 (2.1)	15.3 (6.6)	60.5 (17.4)	16.76 (8.4)	3.3 (1.1)
Bovine	71.4 (42.6)	39.7 (20.3)	47.5 (22.3)	42.1 (23.0)	206.0 (112.2)
Horse	41.9 (24.3)	31.1 (11.3)	33.4 (29.0)	30.1 (16.8)	136.8 (77.5)
Rabbit	363.6 (157.4)	186.4 (47.2)	126.4 (88.0)	612.5 (211.7)	2406 (651.4)

Human: n=6, Rhino: n=6, Horse: n=10, Rabbit: n=10, Bovine: n=9.

Figures, based on anion exchange HPLC profiles, are given as μM with standard deviation given in parenthesis.

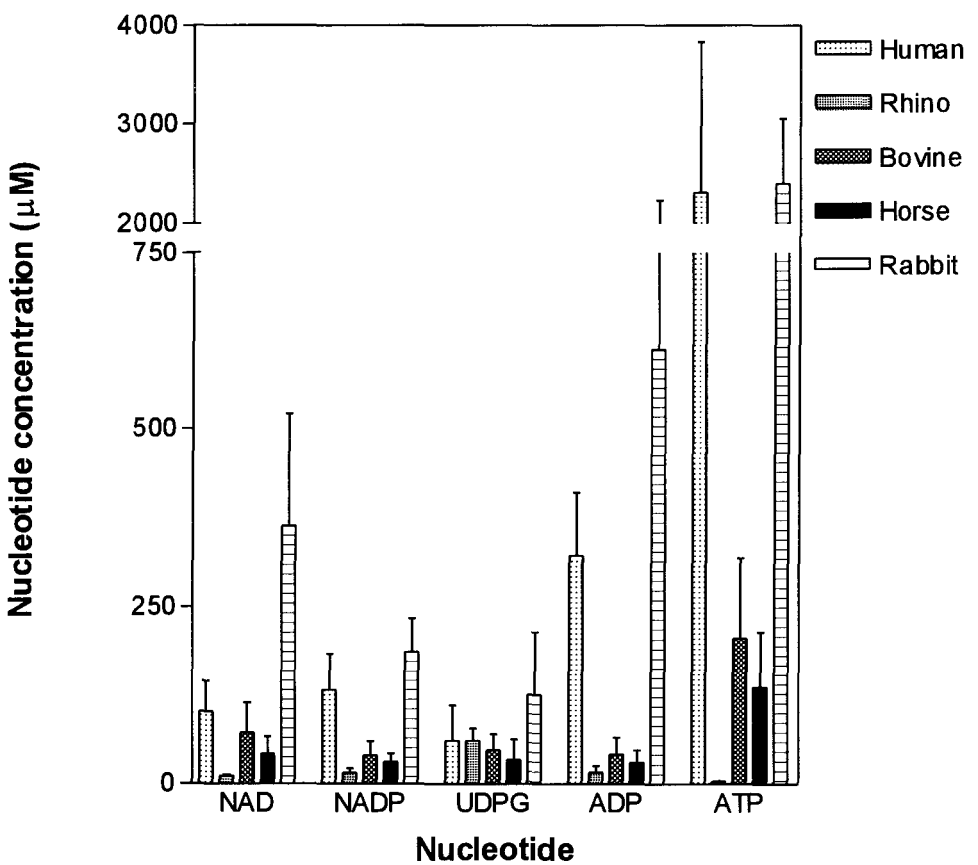

Figure 11. Relative red blood cell nucleotide content of five mammals.

A striking feature of the above figure is the relatively high ATP content of human red blood cells compared to other mammals. The rabbit is the only mammal represented here that has similar levels of ATP. The ATP content of horse and bovine red blood cells, although only one-seventeenth and one-tenth that of human red cell ATP respectively, is considerably higher than the rhinoceros red blood cell ATP. Rhinoceros red blood cells have the lowest nucleotide content in this study group, most notably being the precariously low ATP levels. To investigate whether rhinoceros red blood cells were able to maintain essential pathways with such low levels of ATP, a metabolic study was undertaken which focused primarily on the hexose monophosphate pathway shunt (HMP).

3.2 Characterisation of the rhinoceros Hexose Monophosphate Pathway

Black rhinoceros red blood cells are known to be particularly susceptible to oxidative stress (27). For this reason attention was focused on the characterisation of the hexose monophosphate shunt which has the function of protecting the red blood cell against oxidative stress. Ascorbate and methylene blue are reported to increase flux through the HMP (65-68) and were used as the HMP stimulants in this study. The first reaction of the HMP is the conversion of glucose-6-phosphate to ribose-5-phosphate which results in the liberation of one molecule of carbon dioxide. Red blood cells were incubated with 1-^{14}C-glucose and the rate of flux through the shunt was determined by measuring the labelled CO_2 liberated.

To determine the appropriate concentration of ascorbate to use, dose response experiments were performed on rhinoceros red blood cells (fig.12).

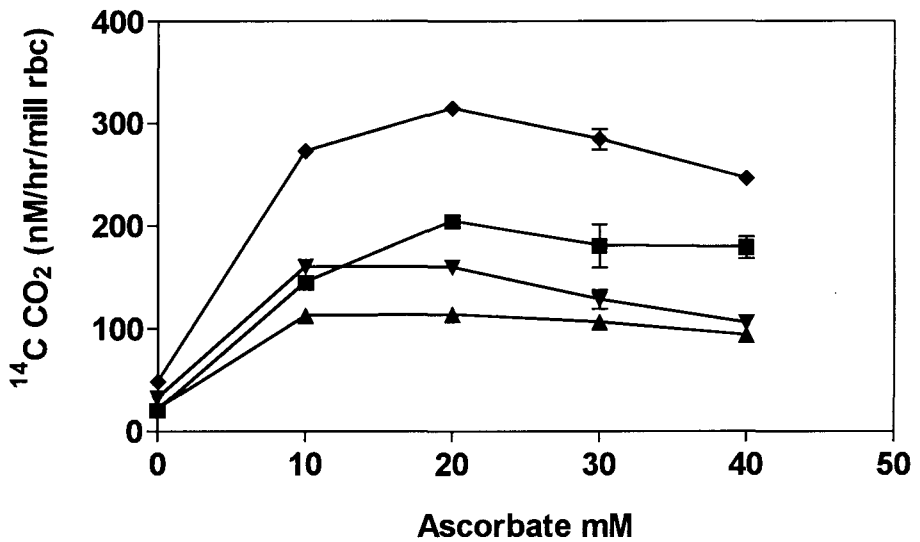

Figure 12. The effect of increasing ascorbate concentration on the hexose monophosphate pathway of rhinoceros red blood cells. Names of rhinoceroses: —■— Hlu 2; —▲— Magnum; —▼— Goliath; —◆— Natal 5.

All rhinoceros red blood cells exhibited a plateau response at 10mM beyond which there was no significant increase in shunt activity and therefore 10mM was taken to be the appropriate concentration to use in this study.

The concentration of methylene blue required to induce shunt stimulation was 6.7μM.

3.2.1 Stimulation of HMP shunt

As part of the evaluation of the ability of rhinoceros red blood cells to deal with oxidative stress the rate of flux through the HMP of black and white rhinoceros red blood cells were compared to that of red blood cells of healthy humans. Red blood cells were incubated with 1-^{14}C-glucose in the presence and absence of the HMP stimulants ascorbate and methylene blue. Labelled carbon dioxide measurements served as a measure of the rate of flux through the HMP, and lactate determinations measured flux via the glycolytic pathway (fig.13-14).

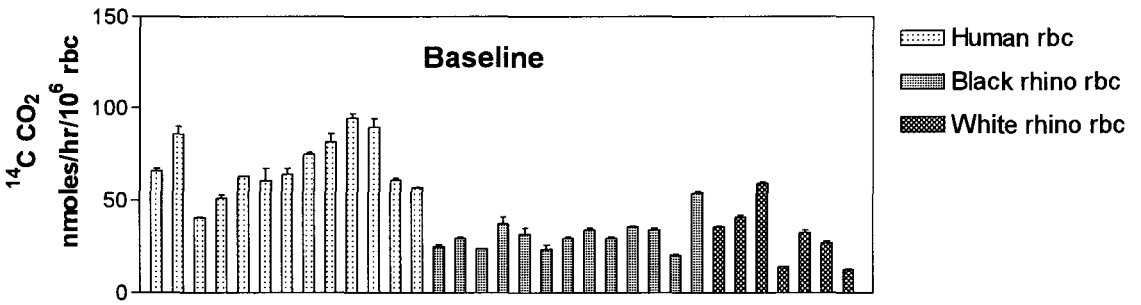

Figure 13. Basal rates of flux through the HMP of human and black and white rhinoceros red blood cells.

Basal metabolic rates through the HMP of rhinoceros red blood cells were approximately half of that observed in human red blood cells. Human rates ranged from 40.5 to

94.5nmoles ^{14}C CO_2/hr/10^6 rbc with a mean of 68.35, whereas black rhinoceros rates had a mean of 31.42 and white rhinoceroses had a mean of 31.64.

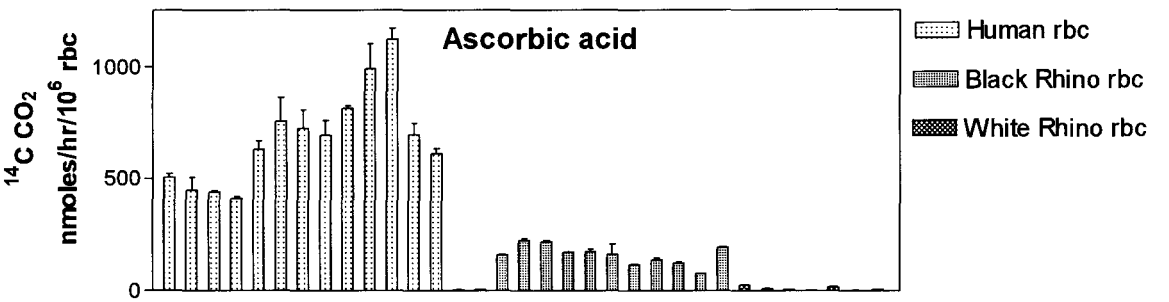

Figure 14. The effect of ascorbate on the flux through the HMP of human and black and white rhinoceros red blood cells. Red blood cells were incubated with 10mM ascorbate in HBSS at 37°C for 1hr.

When exposed to ascorbate the rate of flux through the human HMP was stimulated ten times above basal levels and ranged from 412 to 1122. Black rhinoceros red blood cell HMP activity increased five times above basal rates and ranged from 77 to 226.5, considerably lower than human red blood cells under these conditions. White rhinoceros red blood cells were stimulated poorly under these conditions and rates ranged from 2.5 to 25.5. Despite low basal rates of flux black rhinoceros red blood cells showed considerable increases in HMP metabolic rates when incubated with ascorbic acid.

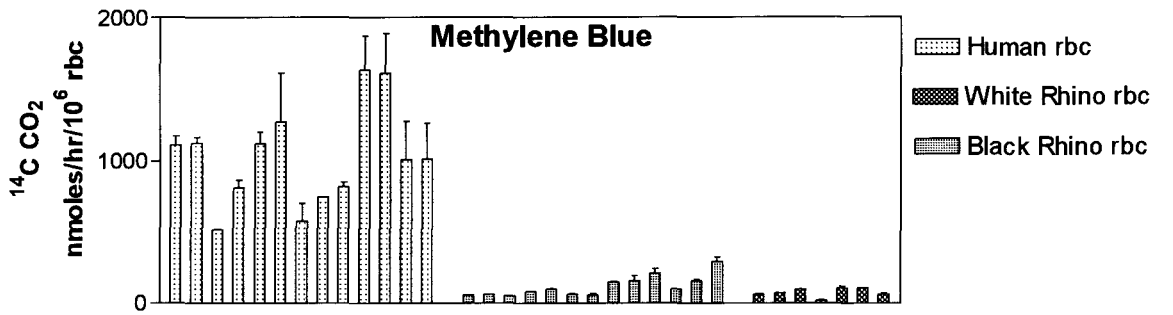

Figure 15. The response of the HMP of human and black and white rhinoceros red blood cells to the shunt stimulant methylene blue. Red blood cells were incubated with 6.7μM methylene blue in HBSS at 37°C for 1hr.

Human red blood cells showed an even greater response when incubated with methylene blue, being stimulated approximately fifteen times above basal levels with a mean of 1027. Interestingly black rhinoceros red blood cells only showed a modest increase of approximately three times above basal level with an average of 118.7 in the presence of methylene blue. White rhinoceros red blood cells were only stimulated approximately two times above basal levels. Although the HMP metabolic rate was significantly increased above basal levels when incubated with methylene blue the response was not proportional to that seen in human red blood cells.

In order to determine the effect of these shunt stimulants on the Emden Myerhof Pathway lactate assays were performed (fig.16).

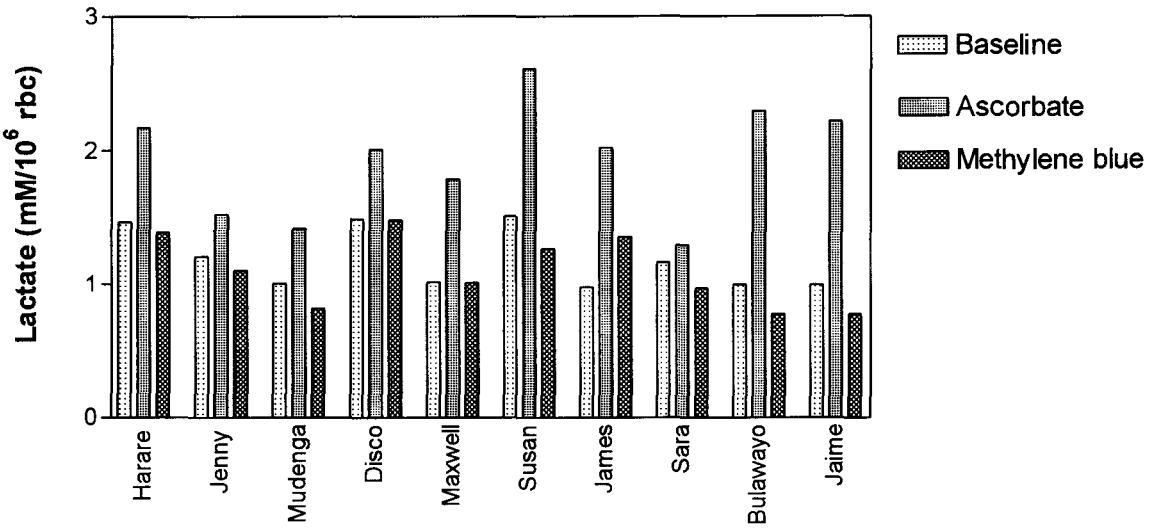

Figure 16. The effect of shunt stimulation on the Emden Myerhof pathway in the red blood cells of 11 individual black rhinoceroses.

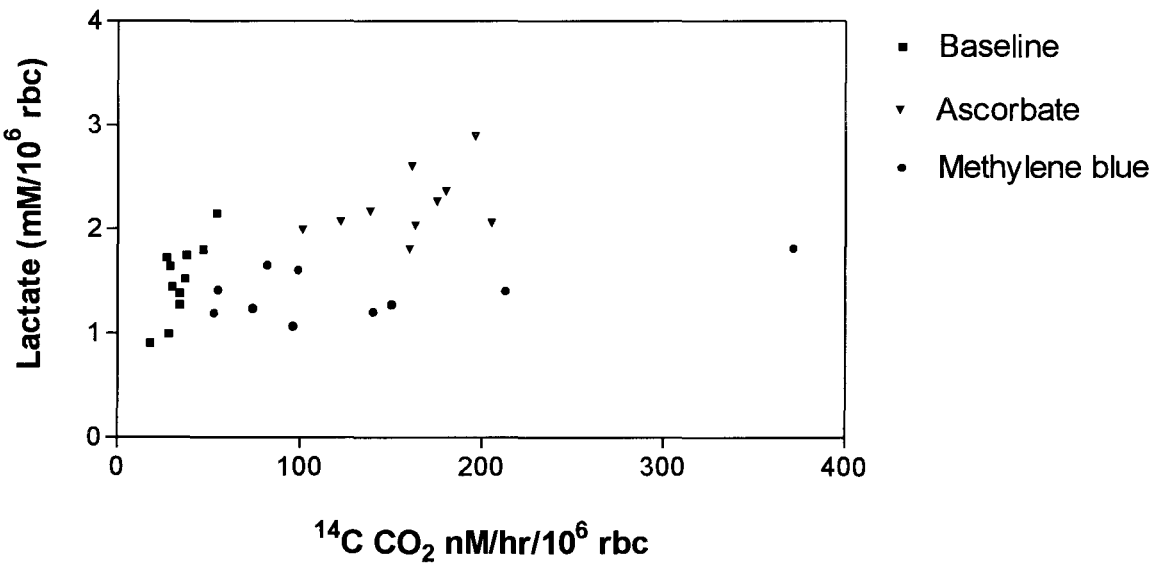

Figure 17. A graphic illustration of the interrelationship between the EMP and the HMP under basal conditions and when exposed to shunt stimulants.

Contrary to what was expected, lactate values in all samples except one (which was not determined) increased relative to basal levels when black rhinoceros red blood cells were incubated with ascorbate. Methylene blue did not have a significant effect on lactate values relative to basal levels (fig.16). Lactate values were plotted in relation to labelled CO_2 values in fig.17 which clearly illustrates the stimulant effect of the ascorbate and methylene blue on the HMP (increase in CO_2 values). Once again the increase in lactate produced in response to ascorbate is observed as ascorbate values clearly segregate from methylene blue-stimulated values which show no significant difference relative to basal values.

Human red blood cells were also assayed for lactate after being exposed to the shunt stimulants

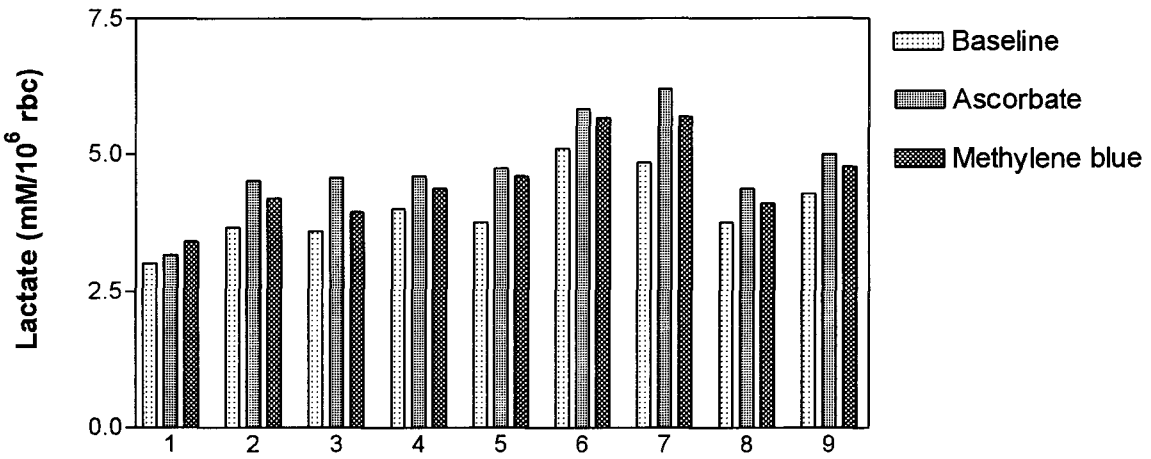

Figure 18. The effect of the shunt stimulants on lactate values in human red blood cells.

Once again, as seen with the rhinoceros red blood cells, when incubated with ascorbate, lactate values increase although not to the same extent as in human red blood cells. Although lactate levels were increased slightly above basal levels when incubated with methylene blue, these levels were lower than levels produced by ascorbate in all except one sample.

The effect of the shunt stimulants on human red blood cell GSH was also investigated (fig.19).

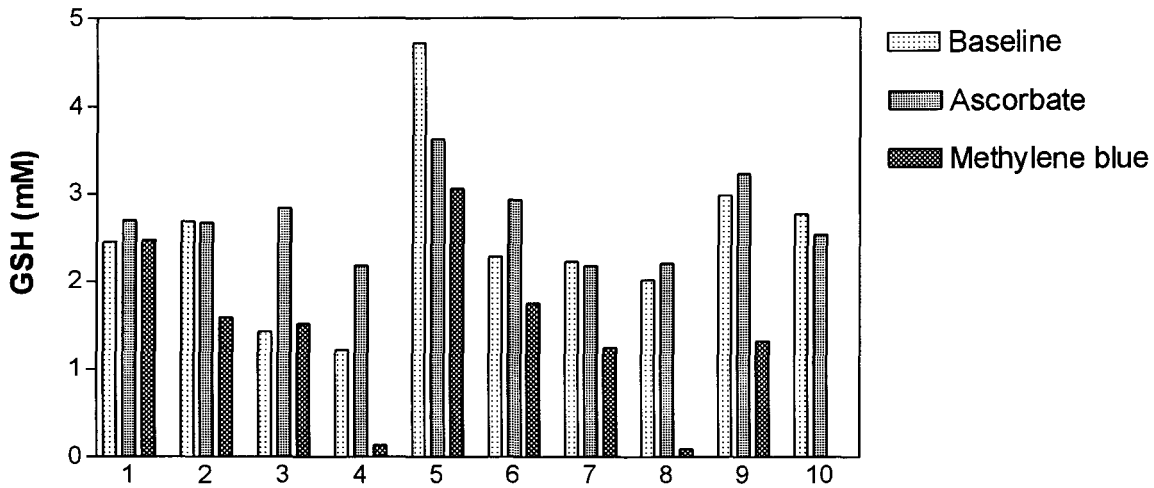

Figure 19. Human GSH values under basal conditions and when exposed to ascorbate and methylene blue.

In seven out of nine of the samples assayed the GSH in the red blood cells treated with methylene blue decreased significantly relative to basal levels. In six out of the ten samples ascorbate had the unexpected effect of apparently increasing the GSH and in the other four samples there was no effect on the basal GSH concentration.

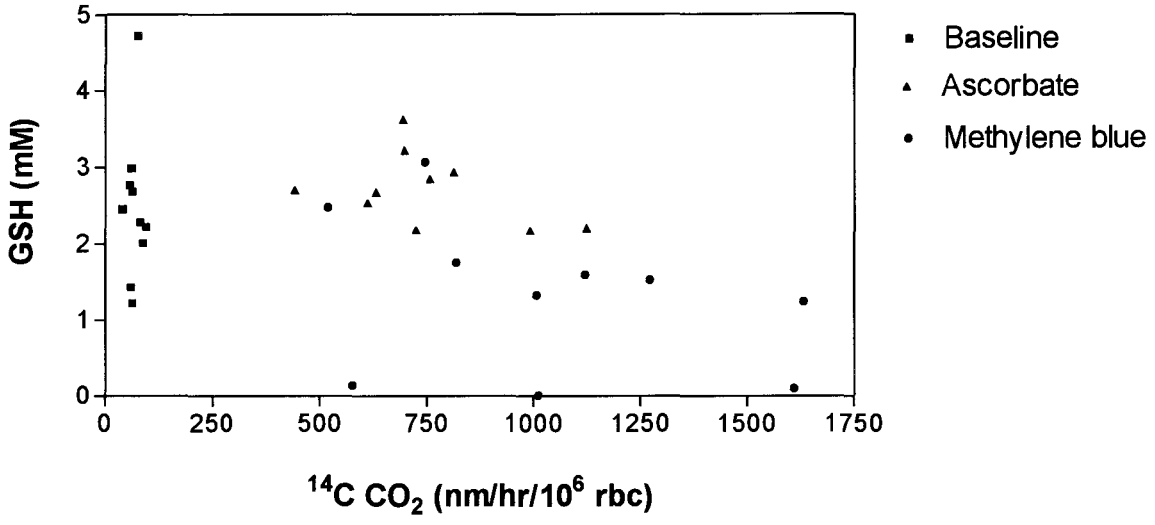

Figure 20. The effect of shunt stimulation on human red blood cell GSH levels.

The low shunt activity at basal conditions i.e. in the absence of ascorbate and methylene blue can clearly be seen. In the presence of ascorbate and methylene blue shunt activity increases significantly. The apparent maintenance of GSH levels by ascorbate is contrasted to the utilisation of GSH in the presence of methylene blue.

3.2.2 Recycling through the HMP

In order to deal with prolonged periods of oxidative stress, rhinoceros red blood cells would need to have the capacity to recycle intermediates through the hexose monophosphate pathway. The ability if these cells to do this was investigated by incubating the red blood cells with glucose labelled in the second carbon position (2-^{14}C glucose), in the presence of the shunt stimulants (fig.21).

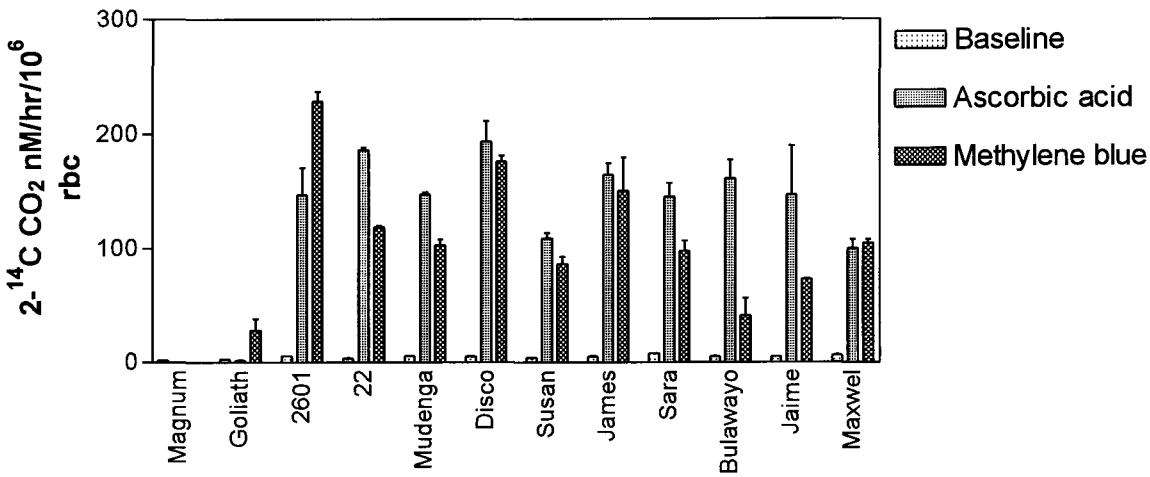

Figure 21. Rate of recycling through the HMP of black rhinoceros red blood cells when incubated with 2-^{14}C glucose.

At basal conditions a very low rate of recycling is observed through the HMP. In ten of the twelve samples studied both ascorbate and methylene blue produced a significant increase in the flux through the hexose monphosphate pathway. The greater response to methylene blue observed when the red cells were incubated with 1-14C labelled glucose is not seen in this case where ascorbate generally produces a greater overall effect. Two samples, Magnum and Goliath responded poorly to stimulation by ascorbate and methylene blue. That these samples were older than the rest may account for this lack of response.

3.2.3 The effect of sodium azide on the HMP

To investigate the effect of catalase inactivation on flux through the HMP rhinoceros red blood cells were incubated with sodium azide and then stimulated with ascorbate and methylene blue (fig.22). Sodium azide is a haem poison which inhibits catalase.

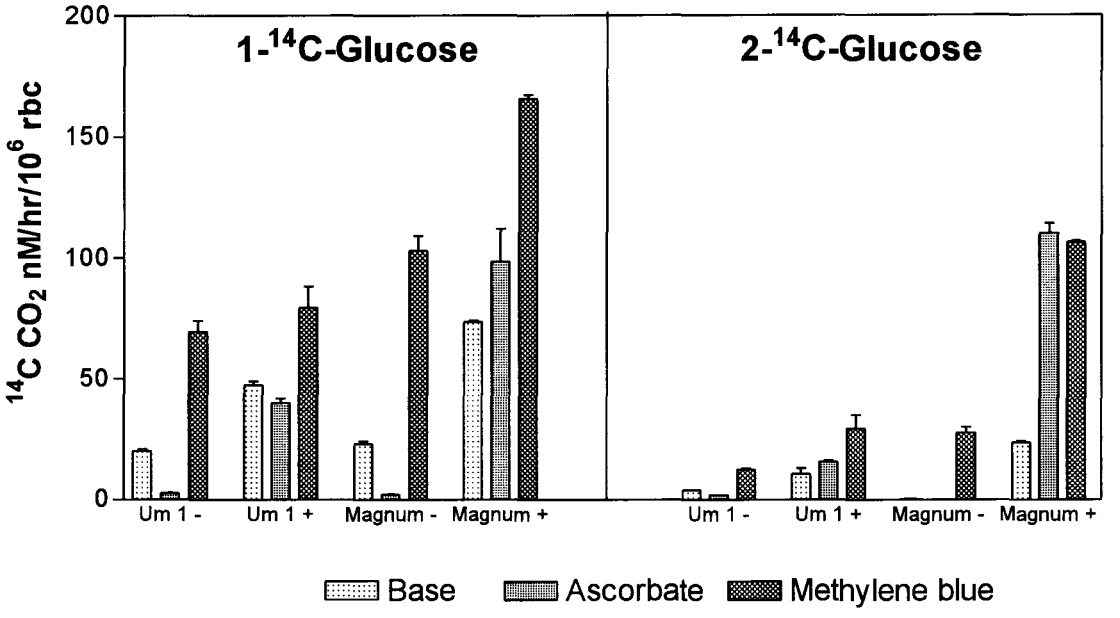

Figure 22. The effect of sodium azide on the rate of flux through the HMP when incubated with 1-^{14}C-glucose and 2-^{14}C-glucose.

It was expected that sodium azide would enhance the effect of the shunt stimulants since all the oxidative stress would have to be dealt with by glutathione peroxidase which in turn would increase flux through the HMP. When response to ascorbate is compared between red cells without and with sodium azide a significant increase in flux through the shunt is observed. The same response is seen with methylene blue but to a lesser extent. The basal response in the presence of sodium azide is also significantly greater than that in the absence of sodium azide.

The same pattern of response is seen when the red cells were incubated with C^{14}-2-glucose. This result also confirms that rhinoceros red blood cells are capable of recycling intermediates through the pentose phosphate pathway to deal with prolonged oxidative stress.

Taken together, these results give valuable insight into the metabolic capabilities of the rhinoceros red blood cell but does not disclose the lesion responsible for haemolytic anaemia in the black rhinoceros.

3.3 "Cytidine-like" compound

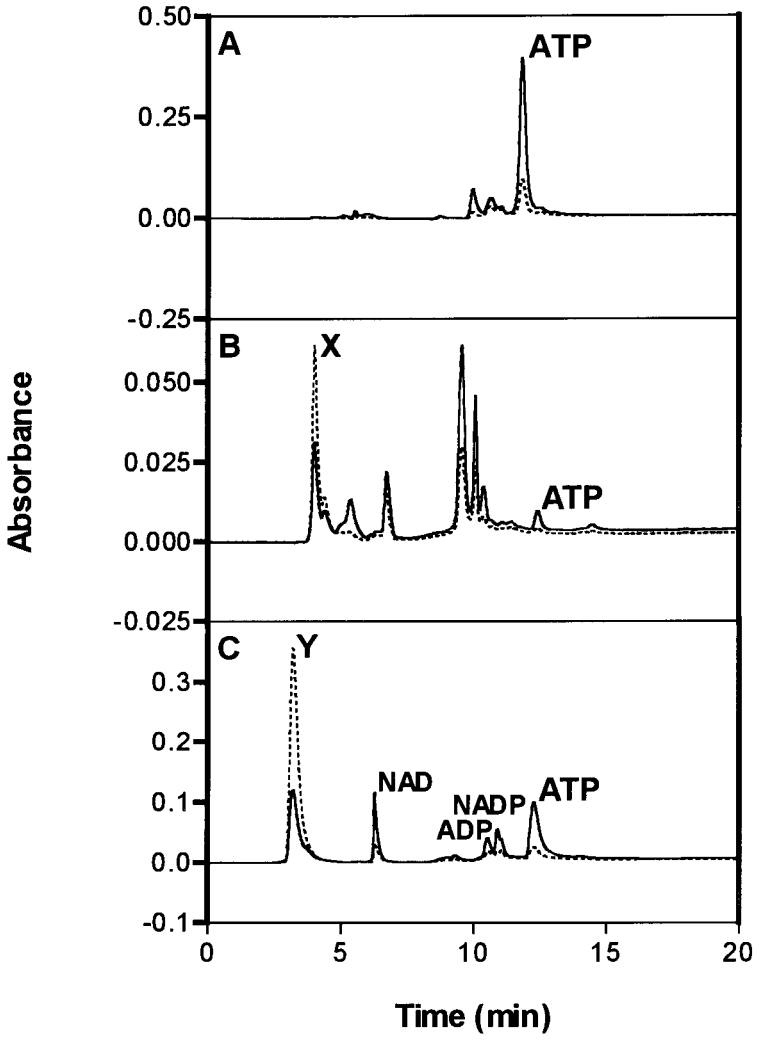

Figure 23 compares the nucleotide profiles of human (A), black rhinoceros (B) and bovine (C) red blood cells analysed by anion exchange HPLC.

Anion exchange analysis of rhinoceros (black and white) red blood cell extracts consistently revealed the presence of an early eluting compound with spectral characteristics resembling those of cytidine in terms of $A_{280/260nm}$ (peak "X") (fig.23). This compound, while the predominant feature of rhinoceros red blood cells, was not present in

human red blood cells. A similar peak was observed in the ultraviolet absorbance spectra of bovine red blood cells (fig.23).

3.3.1 Identification of "cytidine-like" compound

To identify the compound, rhinoceros red blood cell extracts were subjected to reverse phase HPLC analysis and compared to a range of standards with respect to elution position. This method of chromatographic separation revealed that the bovine and rhinoceros compounds eluted in different positions (fig.24). The compound present in bovine red blood cells was identified as urate riboside (fig.10, 3.5). In an attempt to identify the peak "X", a series of nucleotides known to absorb in the ultraviolet range were analysed on HPLC. The elution position of the "cytidine-like" compound in rhinoceros red blood cells coincided with that of the amino acid tyrosine and co-eluted with authentic tyrosine standards.

This was a highly unexpected finding therefore it was necessary to vigorously confirm the identity. The characteristic absorption profile given by diode array scan, elution position and ninhydrin positivity on amino acid analysis using a Beckman System 6300 High Performance Analyser (fig.25), fluorescence spectrometry, mass spectrometry (fig.25), as well as identical behaviour to tyrosine standards in metabolic conversion experiments to be described below, confirmed the identity of this species as tyrosine.

The concentration of the tyrosine in rhinoceros red blood cells was calculated relative to authentic tyrosine standards (see methods) to range from 0.5 to 1mM. Human red blood

cells contain approximately 20 μM tyrosine, therefore rhinoceros red blood cells contain twenty to fifty times the tyrosine in human red blood cells.

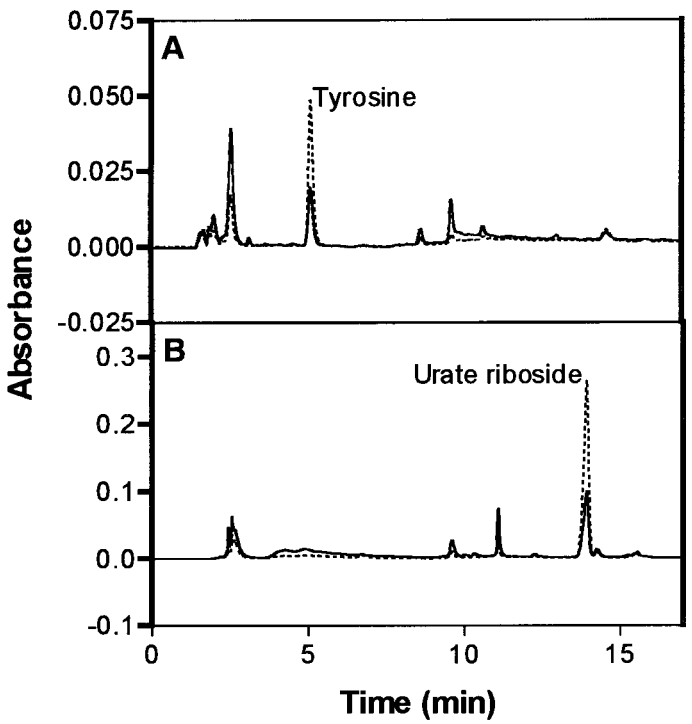

Figure 24. Reverse phase HPLC of PCA extracts from rhinoceros (A) and bovine (B) red blood cells to illustrate the difference in elution position between the rhinoceros tyrosine peak and bovine urate riboside peak.

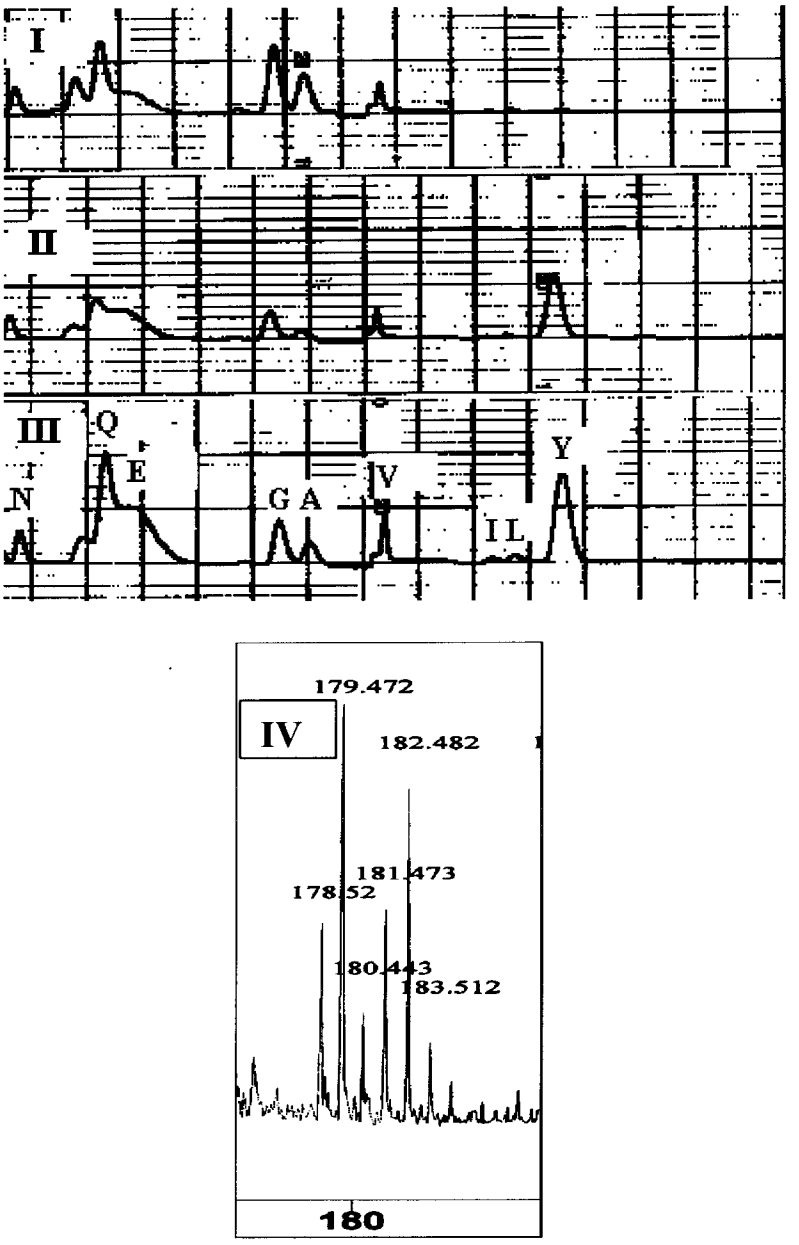

Figure 25. Identification of peak "X". Part of the amino acid chromatogram, of human (I), black rhinoceros (II), and white rhinoceros (III) red blood cells to illustrate the region where tyrosine (Y) elutes. IV; Mass spectroscopy of peak X. Amino acid abbreviations: tyrosine, Y; leucine, L; isoleucine, IL; valine, V; alanine, A; glycine, G; glutamine, Q; glutamate, E; asparagine, N.

3.4 Rhinoceros red blood cell tyrosine

3.4.1 Function of rhinoceros red blood cell tyrosine

In an attempt to determine the function of these remarkably high levels of tyrosine in rhinoceros red blood cells the following possibilities were explored:

(a) its use as an alternate energy source, since ATP levels are so low,

(b) it forms part of an anti-parasitic mechanism,

(c) it has a role as a free radical sump.

3.4.1.1 Tyrosine as an alternate energy source

Tyrosine phosphate could act as an energy source, and acid extraction of such a compound could convert it to tyrosine. So at this stage it was uncertain whether the "tyrosine" peak did not in fact correspond to its phosphorylated form in intact red blood cells. To investigate this, an alternate method of red blood cell deproteinisation, alkaline extraction, was employed to avoid the removal of any possible high energy phosphate groups by the perchloric acid used in the acid extraction technique. This did not produce the shift in the position of the peak in question when analysed on reverse phase HPLC which would be expected if it was phosphorylated. Therefore this possibility was dismissed.

3.4.1.2 Tyrosine as a free radical sump

3.4.1.2.1 Protection against monocyte derived hypochlorous acid (HOCl)

Hypochlorous acid (HOCl), produced by neutrophils during inflammation, permeates red cells freely and preferentially oxidises GSH. This can result in lysis of the red blood cells.

This also places stress on ATP levels since ATP is required for GSH regeneration from GSSG.

The amino acid taurine protects lung epithelial cells from damage by HOCl by reacting with it to form a chloramine by the following reaction scheme:

$$R\text{-}NH_2 + HOCl \longrightarrow R\text{-}NHCl + H_2O$$

Under conditions in which neutrophils are stimulated to produce HOCl, the latter may permeate the red cells. Depletion of GSH could then occur and since rhino cells have very low ATP concentrations, the rate of GSH regeneration might be compromised which could lead to cell lysis. A possibility therefore is that the free tyrosine in rhinoceros red blood cells may act as a protection mechanism against the HOCl by scavenging it before it depletes the GSH.

To determine whether tyrosine could react with HOCl in the same manner as taurine, standard tyrosine solutions were incubated with HOCl. A peak shift with a concomitant decrease in the tyrosine peak was observed indicating that the tyrosine indeed reacted with the HOCl. The new peak formed had a different ratio to that of tyrosine and eluted later than the tyrosine peak. This peak was assumed to be chloro-tyrosine (ring substituted) (data not shown).

To determine whether the free tyrosine in rhinoceros red blood cells would react with HOCl in the same manner, rhinoceros red cell extracts were incubated with HOCl (fig.26) and the same decrease in the tyrosine peak and formation of chloro-tyrosine was seen as with the standard tyrosine. This serves as additional proof that the rhino peak is tyrosine.

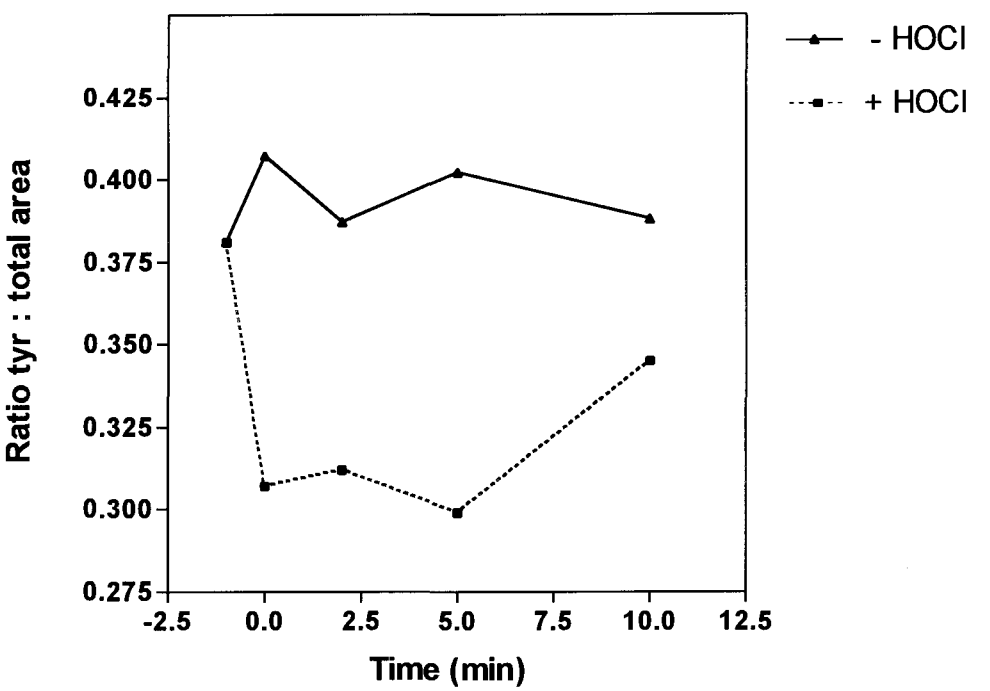

Figure 26. Black rhinoceros red blood cells (10% h.c) were incubated with 2.0μm/ml HOCl and aliquots were extracted and analysed at 0, 2, 5 and 10min by reverse phase HPLC. HOCl was added as a bolus at the start of the experiment.

To evaluate the significance of the above results in a whole cell system, rhinoceros red blood cells were incubated with HOCl for varying times at physiological pH.

Black rhinoceros red blood cells were incubated with HOCl and analysed by reverse phase HPLC for the formation of chloro-tyrosine.

Although the tyrosine content of the cells decreased over the course of the incubation (fig.26), no chloro-tyrosine could be seen. It was evident that haemolysis, as a result of the high HOCl concentration, had taken place and this may account for the decrease in tyrosine content. This experiment was repeated using a lower concentration of HOCl.

To investigate the effect of HOCl on red blood cell GSH and lactate, black rhinoceros red blood cells were incubated with HOCl and assays for GSH and lactate were performed (table2).

Table 2. The effect of 2mM HOCl on GSH and lactate

	- HOCl	+HOCl
GSH (mM)	1.45	1.14
Lactate (mM)	0.017	0.014

The expected decrease in GSH concentration was observed but the absence of chloro-tyrosine on the HPLC trace together with this slight drop in GSH suggests that the concentration of HOCl may be too low. The decrease in the amount of lactate produced in response to HOCl could be explained if more glucose were diverted through the HMP to replenish the GSH levels.

These results suggest that the tyrosine in rhinoceros red blood cells either does not directly react with HOCl in a whole cell system, or that it reacts, but the products are removed too rapidly for them to be observed.

Since no chloro-tyrosine was observed to be formed in the whole cell system it is doubtful whether this proposed role has any physiological significance.

Evaluation of tyrosine acting as a free radical trap for HOCl and its derivatives:

Tyrosine may be acting either

(i) exogenously, by taking the place of taurine in other mammalian cells and protecting against the oxidative effects of HOCl. It is possible that tyrosine could be chlorinated in this reaction, taken up by red cells via the anion transporter, reduced once inside by GSH

to tyrosine (zwitterion) which cannot cross back over the membrane (no nett charge) and therefore accumulates inside red cells, or

(ii) endogenously, by constituting a back up defence for the ATP-GSH system, possibly against $TauCl_2$, an oxidative product of the reaction between taurine and HOCl. The monochloramine derivative of taurine can be reduced by GSH quite easily but this dichloramine derivative is 100 times more reactive and lytic. Reduction of dichloramines by GSH could result in its depletion since low levels of ATP might be insufficient to maintain the balance. It is possible that tyrosine could react with the dichloramine forming TauCl and TyrCl which could more easily be reduced by GSH. Tyrosine has been shown to react with H_2O_2 and NO_2 forming dityrosine and nitrotyrosine respectively. The possibility of tyrosine acting as a free radical trap in rhinoceros red blood cells was therefore explored.

3.4.1.2.2 Protection against hydrogen peroxide

It has been reported that myeloperoxidase, an enzyme secreted by neutrophils during an inflammatory response, together with H_2O_2 catalyses the cross linking of protein bound tyrosine residues to form dityrosine.

To investigate whether rhinoceros red blood cell tyrosine was functioning as a free radical scavenger and hence by protecting the red cell against oxidative stress, black rhinoceros red blood cells were incubated with H_2O_2 and analysed fluorimetrically for the presence of dityrosine (fig.27).

An increase in fluorescence with the same excitation and emission properties as dityrosine i.e. λ_{em} 315 nm, λ_{em} 409, was observed in response to H_2O_2. Human red blood cells incubated under identical conditions showed no indication of the presence of dityrosine. Analysis of a distilled water blank, since distilled water was used to dilute the sample, showed no trace of similar fluorescence. This peak was compared to standard dityrosine prepared according the method of Guilivi et al. and found to have identical fluorimetric characteristics and mobility on HPLC.

In order to investigate the dynamics of dityrosine production in black rhinoceros red blood cells, a timecourse experiment was designed.

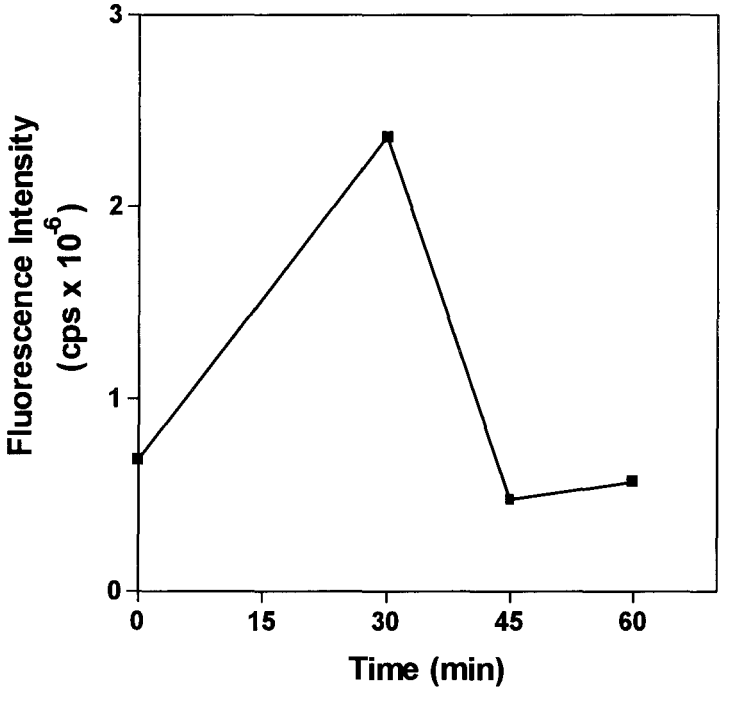

Figure 27. Black rhinoceros red blood cells (10% h.c) were incubated at 37°C in Hanks balanced salt solution (HBSS) containing 4mM H_2O_2 and analysed fluorimetrically for dityrosine. The graph follows the emission intensity at 409nm when excited at 315nm.

Red cells were incubated with H_2O_2 and samples were extracted and analysed at the following timepoints: 0, 30, 45, 60 min. Fluorescence intensity peaked at 30min (fig.27), which was approximately three times above the basal (t=0) level. A subsequent decrease in dityrosine fluorescence back to basal levels in the 45 and 60min timepoints suggests that the dityrosine was either being catabolised within the cell or transported out of the cells. The absence of dityrosine in the supernatant fractions supported the idea that the dityrosine was broken down inside the cell, possibly being reconverted to tyrosine.

3.4.2 Dityrosine production in rhinoceros red blood cells

3.4.2.1 The effect of haematocrit level on dityrosine production

Using the same method as above the timecourse experiment was repeated but now using a haematocrit of 12.5%. The results showed that there was no apparent production of dityrosine in the various timepoints (0, 15, 30, 45, 60min).

It was unexpected that these results were different from the previous experiment. The only difference in methodology was that the haematocrit was 8.4% in the previous experiment and 12.5% in this experiment. The H_2O_2 concentration was the same in both experiments (4mM).

One conclusion is that tyrosine acts as a backup antioxidant mechanism for the glutathione peroxidase in black rhino red cells and therefore dityrosine only accumulates once the glutathione peroxidase is depleted or the H_2O_2 concentration is too high for this enzyme to handle alone.

It would seem therefore that at a haematocrit of 12.5% the cells can adequately protect themselves with the GSH /glutathione peroxidase system and there is no need to use the tyrosine and therefore no production of dityrosine was observed. Alternatively, dityrosine is formed but removed at a rate as fast as its synthesis, so no net accumulation occurs.

The object in the following experiments was to alter the haematocrit or increase the concentration of H_2O_2.

When a haematocrit of 8% was used and H_2O_2 concentration maintained at 4mM, dityrosine production was observed as early as 15min (fig.28). This could be due to GSH being depleted earlier by the H_2O_2.

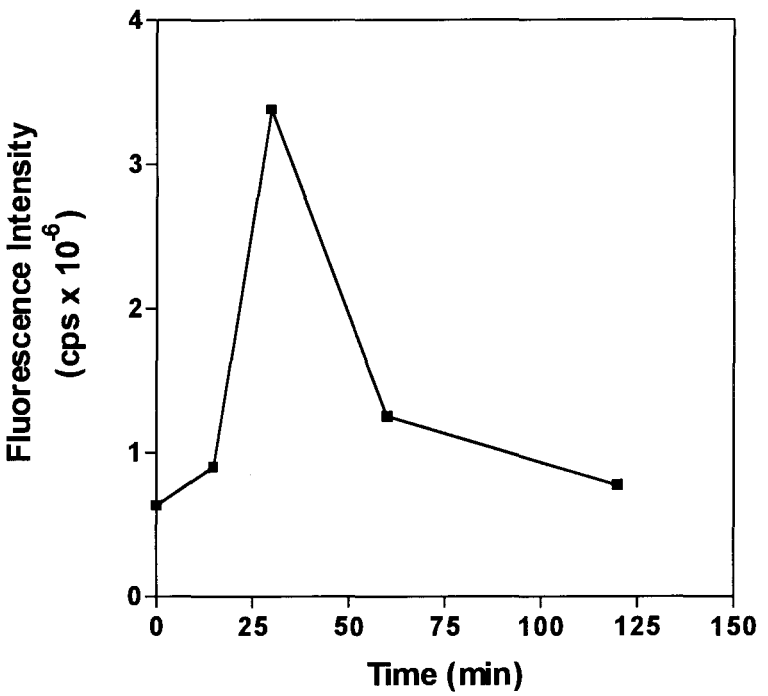

Figure 28. Time course following the production of dityrosine in black rhinoceros red blood cells. Red blood cells (h.c 10%) were incubated at 37°C in HBSS containing 4mM H_2O_2. The production of dityrosine was monitored at 15, 30, 60 and 120 min after addition of H_2O_2. The time 0 aliquot did not contain H_2O_2.

The next step therefore was to find a haematocrit at which a slow increase in dityrosine could be obtained and also to monitor the fate of the dityrosine for a longer period of time. Another experiment was performed and this time a haematocrit of 10% was used and the timepoints at which dityrosine was monitored was 0, 15, 30, 60, 120min (fig.28).

The results obtained showed that dityrosine production peaked at 30min and decreased to baseline levels by 120min. At 15min the level was only slightly higher than basal levels and at 60 min it was back to below half the level at 30min. A haematocrit of 10% has demonstrated the lag in dityrosine production and was therefore used in all subsequent experiments.

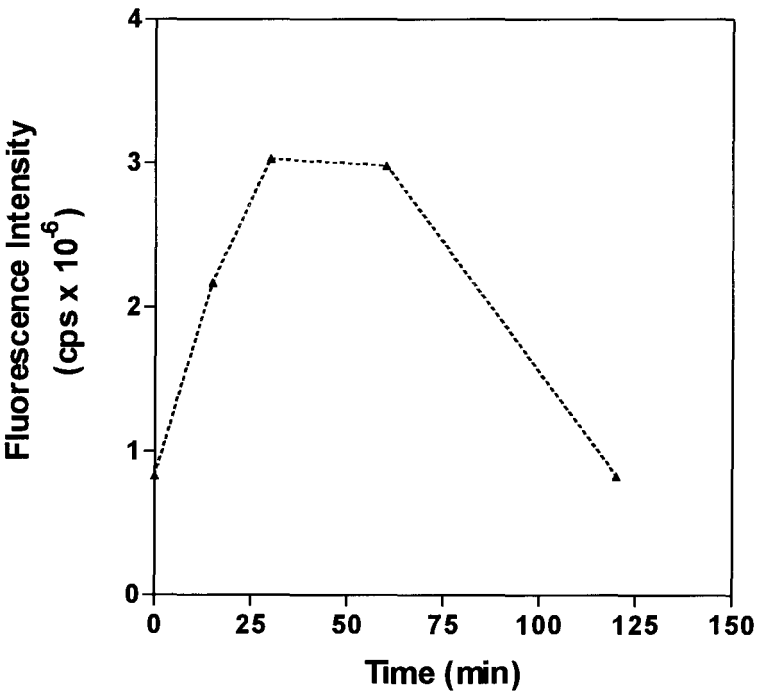

Figure 29. Time course following the production of dityrosine in white rhinoceros red blood cells. Red blood cells (haematocrit 8.5%) were incubated at 37°C in HBSS containing 4mM H_2O_2.

To investigate whether white rhino red blood cells had the same ability to produce dityrosine in response to H_2O_2 these cells were subjected to the same conditions as the black rhino red cells in the above experiments (fig.29). White rhino red blood cells have a higher catalase activity than black rhinoceroses yet the levels are still well below that in human red blood cells.

Significant quantities of dityrosine were present in the basal sample or time zero. By 15 min dityrosine had already increased 2.5 times above basal levels and continued to increase till 30 min (4 times above basal levels). At 60 min the level remained the same at the 30 min timepoint and by 120 min the dityrosine had returned to basal levels.

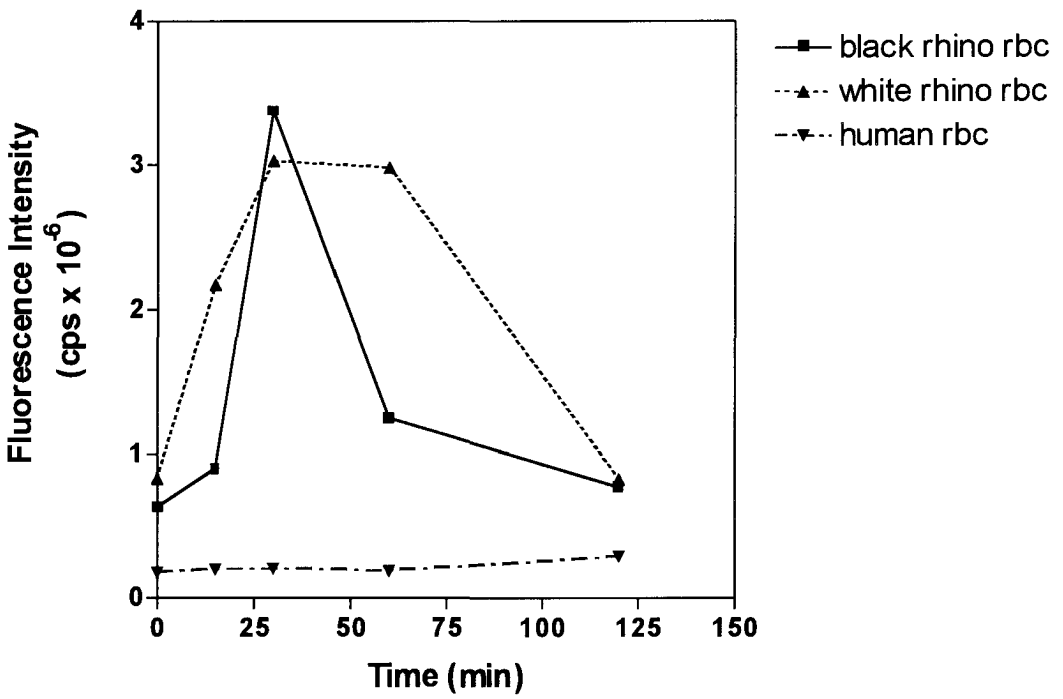

Figure 30. compares the dityrosine production of black and white rhinoceros and human red blood cells (each at 10% h.c) when incubated with 4mM H_2O_2.

Human red blood cells subjected to the same conditions as the black and white rhinoceros red blood cells showed no trace of dityrosine production (fig.30). These experiments (fig.27-29) were pilot experiments and were repeated many times yielding consistently reproducible results.

3.4.2.2 The effect of sodium azide on dityrosine production

To investigate the effect of the inhibition of catalase activity on dityrosine production in black rhino red blood cells, cells were incubated with medium containing H_2O_2 and azide (fig.31). The inhibition of the residual catalase activity in black rhinoceros red blood cells should result in a higher effective H_2O_2 concentration, hence an increased production of dityrosine.

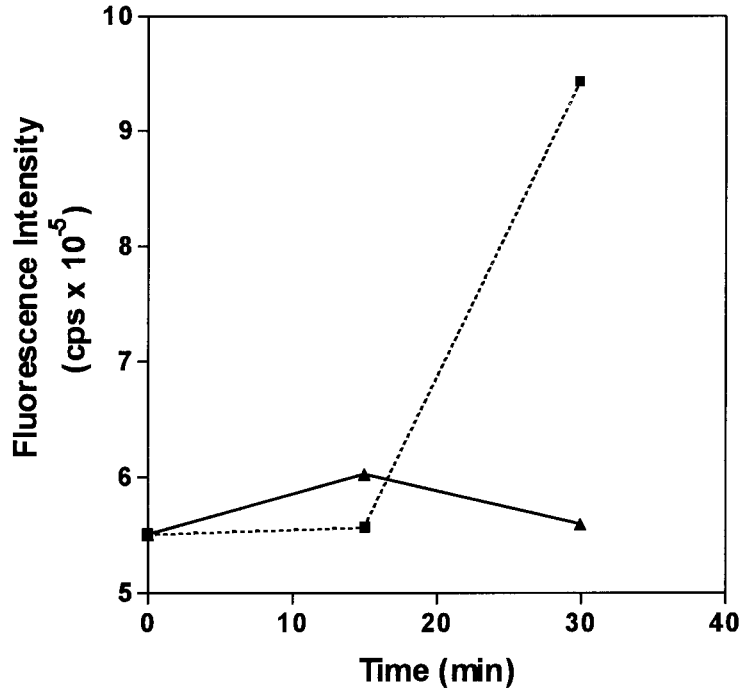

Figure 31. shows the effect of azide on dityrosine production in black rhinoceros red blood cells. Red blood cells (10% h.c) were incubated with 4mM H_2O_2 at 37°C in the presence(—▲—) and absence (—■—) of 2mM azide.

The control samples showed no dityrosine production at 15min (same as baseline/t=0) and approximately a two fold increase in dityrosine at 30min (fig.31). This increase in dityrosine production was expected as it was seen in previous experiments. The samples which contained azide however showed no significant production of dityrosine even at 30min.

The result obtained showed the opposite effect to what was expected i.e. no increase in dityrosine production in the samples which contained azide. This could be due to the azide inhibiting the peroxidase which catalyses the production of dityrosine in red cells.

3.4.2.3 The effect of increasing H_2O_2 concentrations on dityrosine production

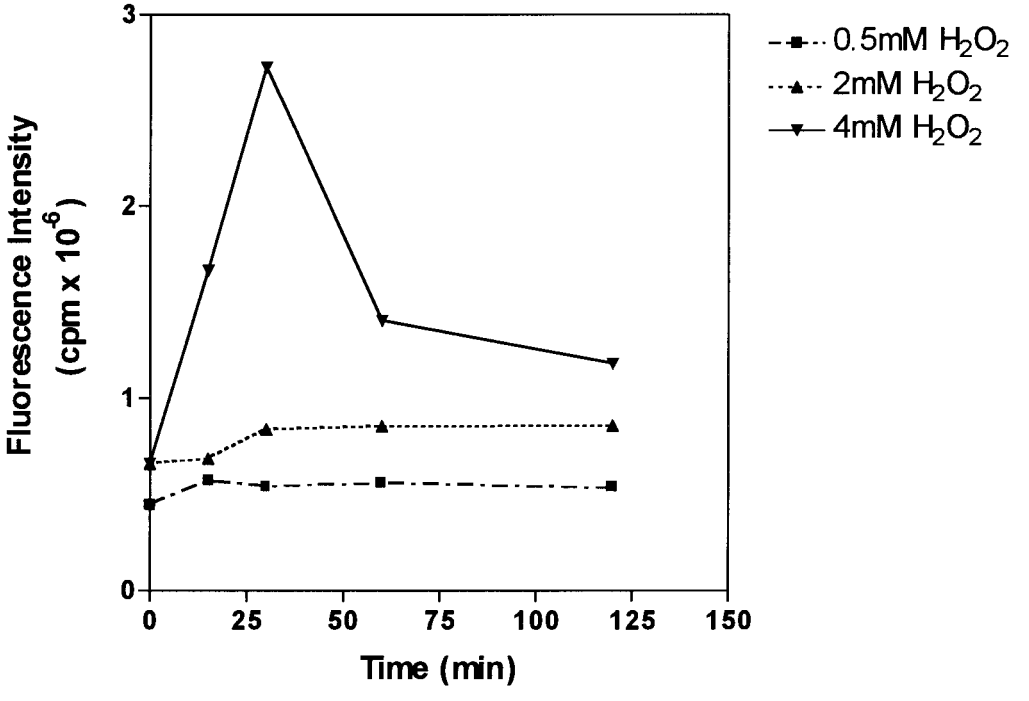

Figure 32. compares the dityrosine production by black rhinoceros red blood cells (h.c 10%) when incubated with 0.5, 2 and 4mM H_2O_2 at 37°C.

Black rhino red blood cells were subjected to 0.5, 2, 4mM H_2O_2 and dityrosine production was determined at the following timepoints : 0, 15, 30, 60, 120 min (fig.32).

It was observed that 0.5mM H_2O_2 produced no dityrosine response and 2mM hydrogen peroxide caused less dityrosine to be produced than 4mM. It would therefore seem that the amount of dityrosine produced increases with the concentration of H_2O_2. This supports the hypothesis that tyrosine acts as a backup mechanism to glutathione peroxidase and any residual catalase. It seems that H_2O_2 concentration needs to reach a threshold before significant dityrosine is produced. With different rhinoceroses it has been observed that dityrosine production peaks at different times and this may be due to different reducing abilities of various animals.

3.4.2.4 The effect of CDNB on dityrosine production

1-chloro-2,4-dinitrobenzene (CDNB) irreversibly depletes GSH by forming 2,4-dinitrophenyl-S-glutathione. This GSH-CDNB conjugate is non-hydrolysable and therefore glutathione peroxidase is effectively inhibited. This would deprive the erythrocyte of a prime antioxidant mechanism, leaving only the tyrosine to deal with any oxidative stress.

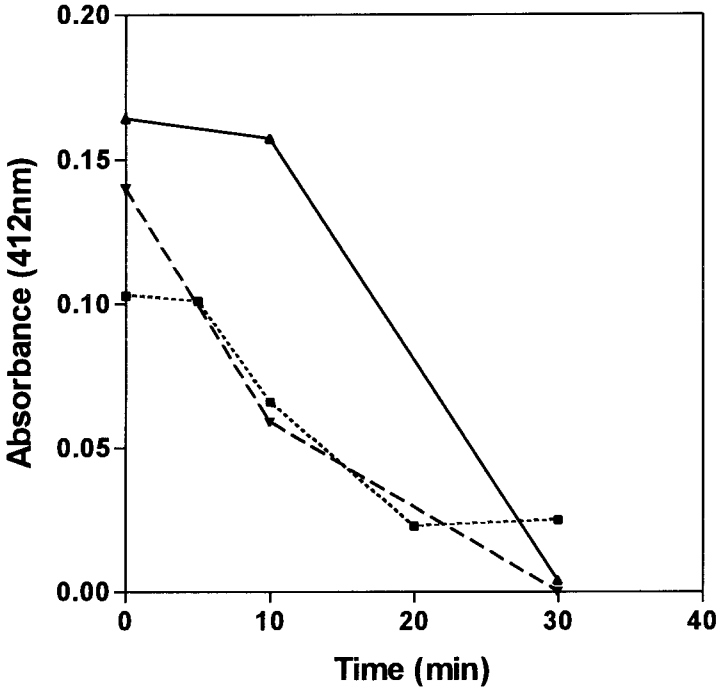

Figure 33 shows the effect of CDNB on the GSH concentration (absorbance at 412nm) of black rhinoceros red blood cells. Red cells were incubated at 37°C in HBSS at 10% haematocrit in the presence of 1mM CDNB. The traces represent 3 different samples of rhinoceros red cells.

To investigate the effect of CDNB on erythrocyte GSH, black rhinoceros red blood cells were incubated with medium containing 1mM CDNB for 30min at 37°C (fig.33). Aliquots were taken at 0, 5, 10, 20 and 30 min and assayed for GSH. Three separate experiments demonstrated the depletion of GSH by CDNB in our experimental system.

To determine whether GSH was resynthesised after depletion by CDNB the red cells were washed after incubation with CDNB-containing medium and incubated with normal medium (CDNB-free) (fig.34). Aliquots were taken at 30, 60 and 120 min and assayed for the presence of GSH.

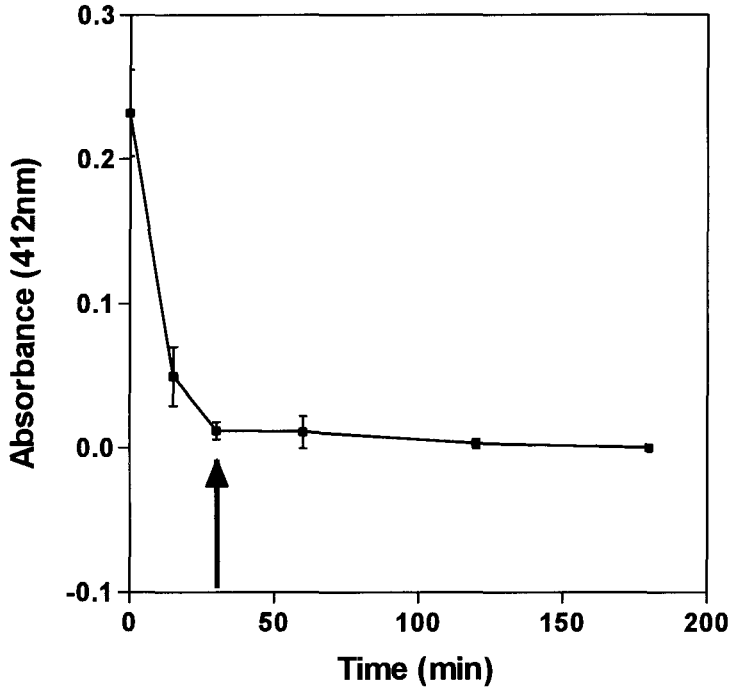

Figure 34. demonstrates the irreversible nature of the CDNB mediated depletion of GSH. Black rhinoceros red blood cells (10% h.c) were incubated at 37°C in HBSS containing 1mM CDNB then washed at 30 min and resuspended in CDNB-free HBBS.

It is clear from this experiment that after incubation with CDNB, a saline wash and incubation in CDNB-free medium GSH remains depleted. It may therefore be assumed that for the entire duration of the H_2O_2 incubations hereafter the GSH is effectively zero. Therefore the glutathione peroxidase is deprived of substrate and plays no role in neutralisation of the oxidative stress (H_2O_2). The residual catalase may also be inhibited by these events as the depletion of GSH also interferes with the maintenance of the NADP/NADPH ratio and NADPH is required for the stability of catalase in the presence of H_2O_2. The only defence therefore should be tyrosine.

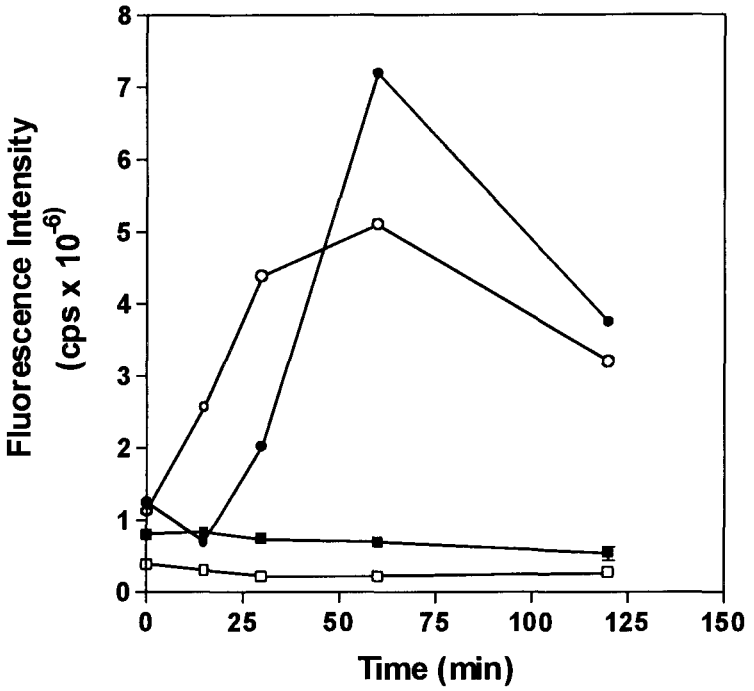

Figure 35. shows the effect of pre-incubation with CDNB on dityrosine production. Black rhinoceros red blood cells (10% h.c) were incubated at 37°C in the presence (—□—,—○—) and absence (—■—,—●—) of 1mM CDNB, with (—●—,—○—) or without (—■—,—□—) 2mM H_2O_2. Data points are the mean of duplicate results.

To determine the effect of CDNB on the H_2O_2-induced production of dityrosine black rhinoceros red blood cells were incubated in medium containing 1mM CDNB prior to exposure to oxidative stress (fig.35).

The control showed a normal increase in dityrosine production, peaking at 60min with 15 and 30min values being less than or equal to a third of the dityrosine in the 60min sample. By 120min the level of dityrosine had fallen to about half maximum. The test showed that at 15 and 30min the amount of dityrosine was approximately double that seen at the same timepoints in the control samples.

It would seem therefore that the 'dityrosine response' had come into effect much earlier because of the presence of the CDNB and absence of GSH.

3.4.2.5 The relationship between dityrosine production and GSH

The next set of experiments have investigated the relationship, if any, between GSH content and dityrosine formation in black rhinoceros red blood cells. This will give insight as to whether tyrosine acts as a backup mechanism or in conjunction with glutathione peroxidase and catalase to deal with oxidative stress.

Black rhinoceros red blood cells were incubated in HBSS containing 2mM H_2O_2 and assayed for dityrosine and GSH at 0, 15, 30, 60 and 120 min.

This concentration of H_2O_2 produced a fivefold increase in dityrosine production above basal levels of approximately 1μM. By 15 min dityrosine production had reached a maximum and tended towards basal levels at later timepoints. This experiment was repeated a number of times with consistent results, of which the results in fig 36 are a representative example. The effect of the oxidative challenge on GSH was significant and revealed an interesting relationship between dityrosine production and GSH concentration (fig.37).

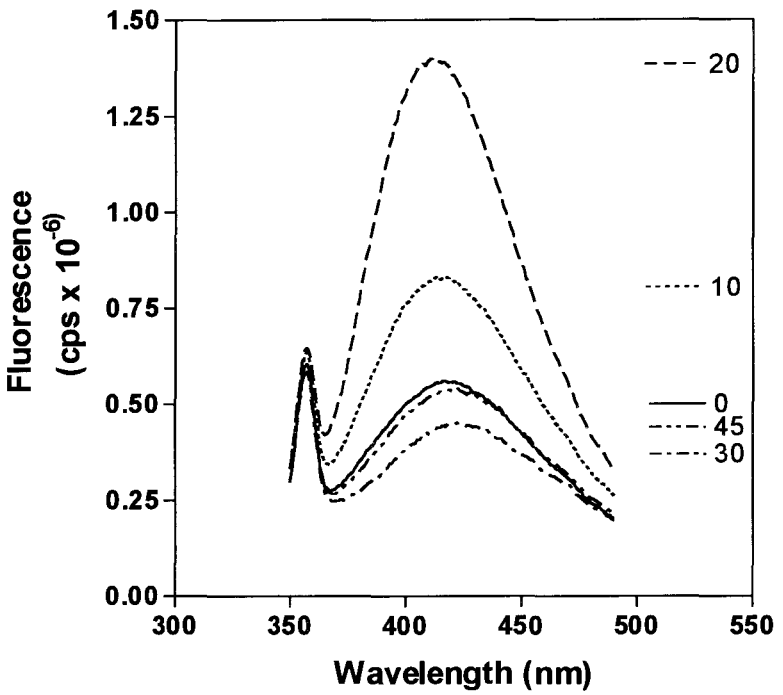

Figure 36. shows fluorimetric emission wavelength scans following the production of dityrosine in black rhinoceros red cells at a haematocrit of 10% incubated with 2mM H_2O_2 at 37°C. λ_{ex} was 315nm. Subsequent measures of dityrosine were at λ_{em} 409nm.

As GSH levels drop due to glutathione peroxidase neutralising the H_2O_2, dityrosine levels initially increase and then decrease as GSH concentration return to initial levels (fig 37). This reciprocal relationship indicates that there is a threshold level of GSH below which either the tyrosine dimerisation mechanism comes into play and dityrosine is formed, or the removal of dityrosine (by presumably a GSH dependent process) is slowed to a rate at which formation exceeds removal, and dityrosine can accumulate.

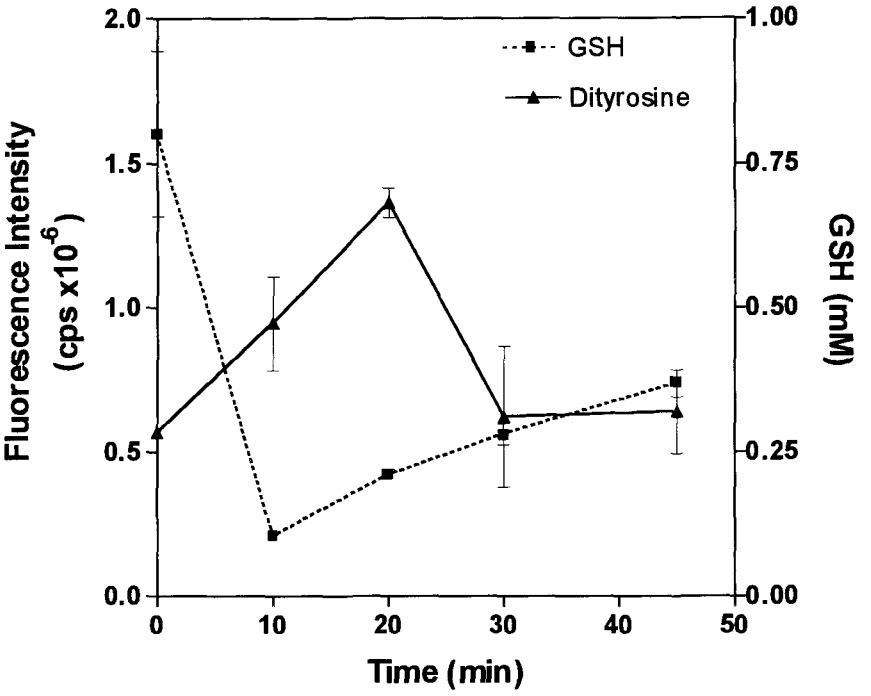

Figure 37. The reciprocal relationship between dityrosine production, measured by fluorescent emission at λ_{em} 409nm, and GSH concentration. Black rhinoceros red blood cells were incubated in HBSS at 10% haematocrit in the presence of 2mM H_2O_2 at 37°C. Points are the mean of duplicate measurements and the bars indicate the standard deviation.

The above experiment was repeated using white rhinoceros red blood cells and assays for dityrosine and GSH were performed for the following timepoints: 0, 15, 30, 60 and 120 min (fig.38).

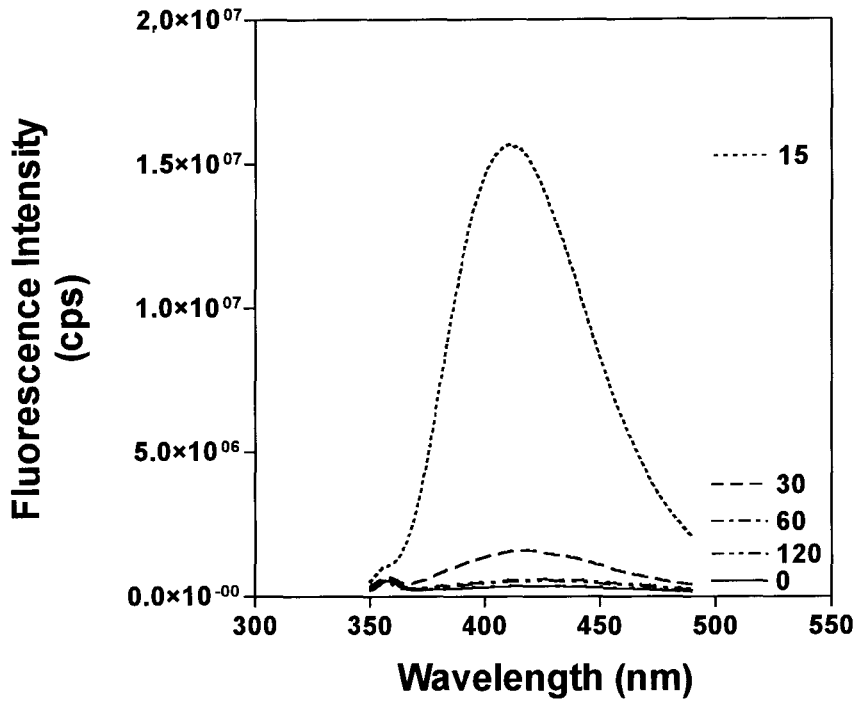

Figure 38. Wavelength scans demonstrating dityrosine production in white rhinoceros red blood cells at 10% haematocrit incubated with 2mM H_2O_2 at 37°C.

The dityrosine response demonstrated by white rhino cells was observed to be much greater than that seen in black rhinoceros red blood cells. Dityrosine levels increase approximately 40 times from basal levels of 0.6 μM to 28 μM by 15 min and returns to baseline by 120 min. Once again, the flourescent data is representative of several experiments conducted with consistent results.

When compared to GSH concentration the same reciprocal relationship is observed (fig.39). Either dityrosine only rises above basal levels once GSH falls below a certain threshold, or there is a more complex non-linear relationship between dityrosine accumulation and H_2O_2 concentration.

Reverse phase HPLC analysis of the samples revealed a decrease in the amount of tyrosine at 15min which is when the dityrosine is formed.

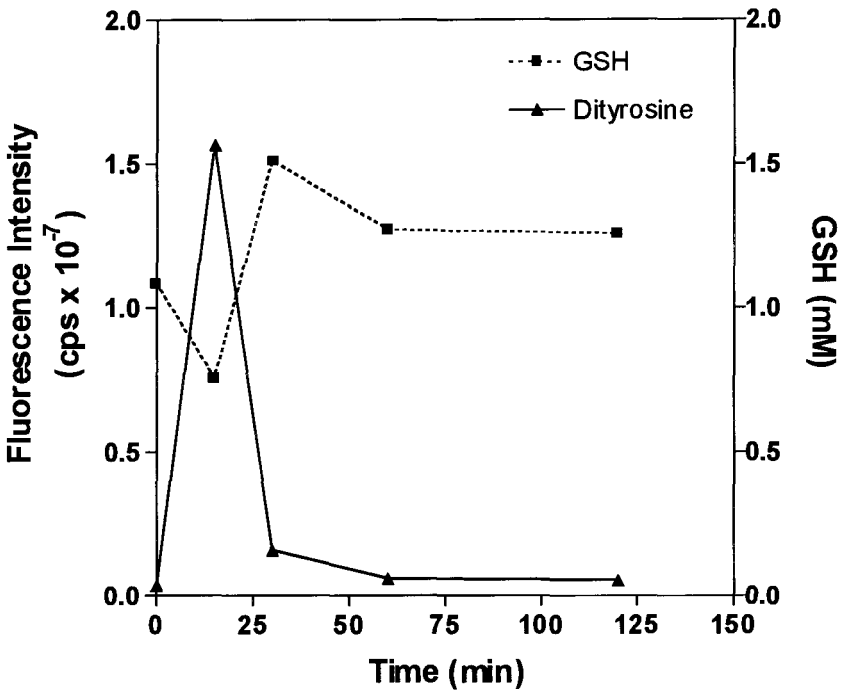

Figure 39. The reciprocal relationship between dityrosine production and GSH concentration in white rhinoceros red blood cells. Rhinoceros red blood cells were incubated in HBSS at a haematocrit of 10% in the presence of 2mM H_2O_2.

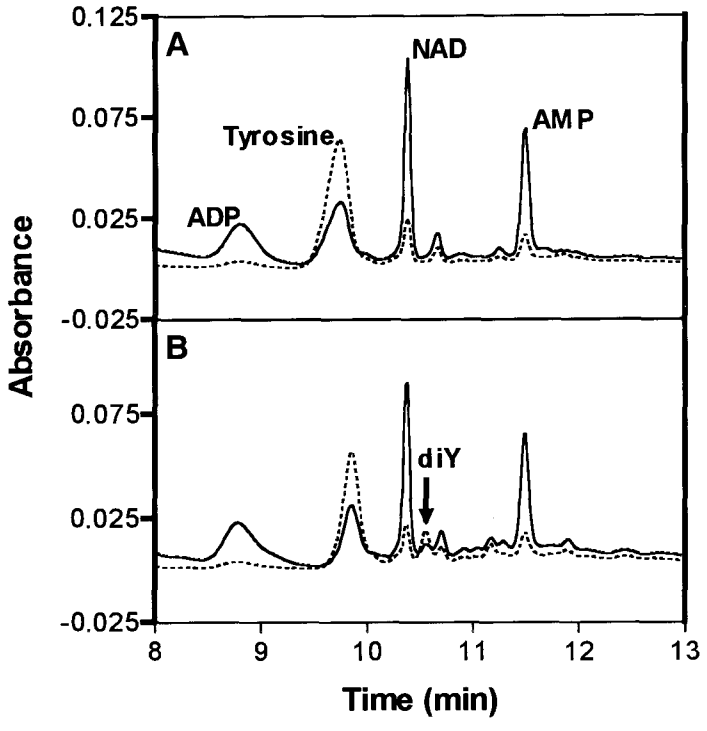

Figure 40. Reverse phase HPLC nucleotide profiles of white rhinoceros red blood cells (10% h.c)incubated with 2mM H_2O_2 at 37°C showing dityrosine. A, time 0 and B, time 15min.

Samples were analysed on reverse phase HPLC to determine the concentration of tyrosine. A new peak was seen in the time 15min sample as compared with the time 0 sample (fig.40). A diode array scan of the peak shows a λmax of 384nm which correlates with the reported wavelength for dityrosine of 383nm. Taken together, these results imply that the free tyrosine is utilised to form dityrosine. The dityrosine may be recycled back to tyrosine after the oxidant challenge has been dealt with or until GSH levels recover and take over the task of neutralising H_2O_2.

3.4.2.6 Repeated exposure of red blood cells to H_2O_2

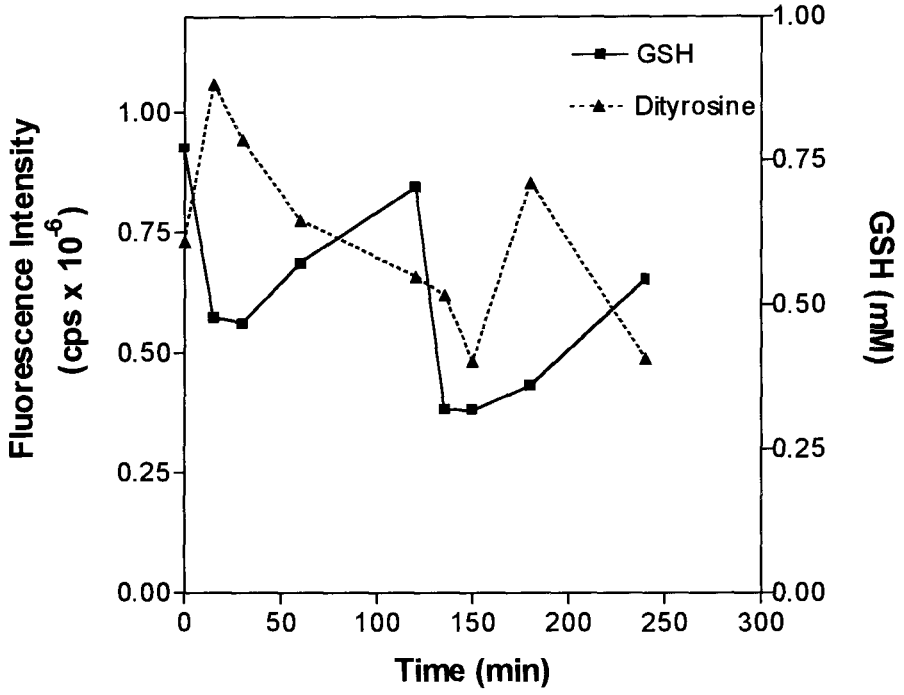

Figure 41. The relationship between GSH and dityrosine production during a double exposure of black rhinoceros red blood cells (10% h.c) to 2mM H_2O_2 at 37°C. After the first period of oxidant exposure (at 120min) red cells were washed and again resuspended in HBSS containing 2mM H_2O_2 and incubated at 37°C.

To investigate whether dityrosine in rhinoceros red cells, formed in response to H_2O_2, is recycled back to tyrosine which may then be used to face another oxidative onslaught, white rhino red blood cells were exposed to successive oxidative challenges in the form of H_2O_2 (fig.41).

The biphasic response of GSH is clearly seen in this experiment. The inversely proportional relationship of dityrosine production to GSH is also demonstrated. During the first 120min it is seen that as the GSH level drops in response to H_2O_2, dityrosine

production increases, then dityrosine levels decrease as GSH is regenerated. This is also observed during the second oxidative challenge.

3.4.3 Cell free analysis of dityrosine production

Dialysed cell-free rhinoceros red blood cell lysates were used to confirm and more thoroughly define the relationship between GSH concentration and dityrosine production. The cell-free system allowed control over the concentration of GSH and also served to isolate the GSH-dityrosine system so that it could be manipulated. Exact concentrations of tyrosine could also be added.

White rhinoceros red blood cells were prepared as described in the methods section and reconstituted as described in table 2.

Table 3. GSH results (mM)

Dialysed cell free lysates were incubated with 2mM H_2O_2 at 37°C in the presence (1 and 2) and absence (3) of 1.5mM GSH and in the presence (2 and 3) and absence (1) of 1mM tyrosine.

Time (min)	(1)	(2)	(3)
30	0.368	0.423	(no GSH added)
60	0.352	0.389	
120	0.311	0.359	

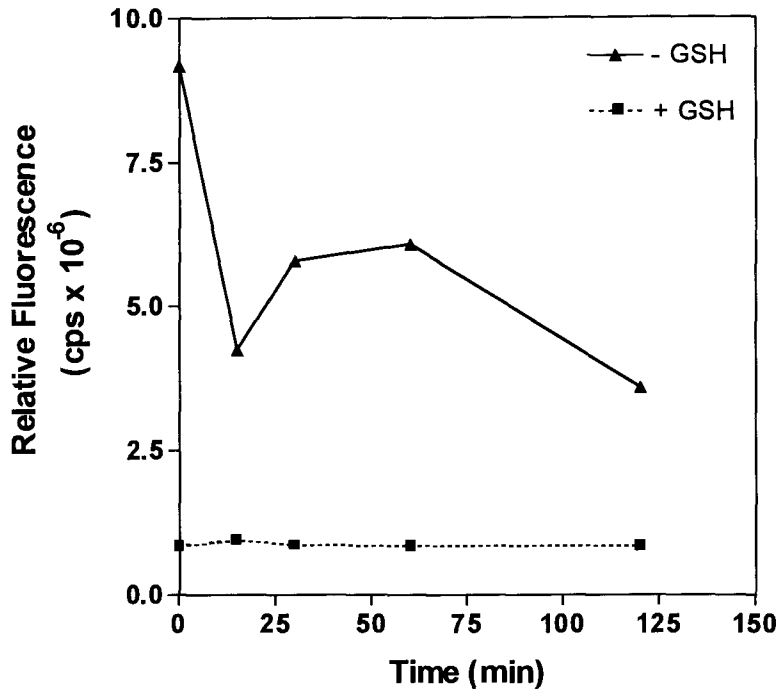

Figure 42. Production of dityrosine in a cell-free system in the absence (—▲—) and presence (—■—) of 1.5mM GSH. White rhinoceros red blood cell lysates were incubated in PBS at 37°C in the presence of 2mM H_2O_2 and 1mM tyrosine.

GSH concentration was observed to decrease in response to H_2O_2 due to its utilisation by glutathione peroxidase (table 2). The presence of tyrosine in (2) seems to spare the GSH since the concentration in this set is higher at all timepoints than in (1). This indicates that a basal level of dityrosine is being produced in red cells even in the presence of adequate GSH.

In the sample which contained 1.5mM GSH, there is very little dityrosine production (fig.42). This may be because there is enough GSH available to neutralise the H_2O_2 via glutathione peroxidase, or that dityrosine removal is as rapid as its production. The

decrease in GSH in the sample as seen in the GSH assay (table 3) supports this. In the sample where GSH was not added we see a substantial amount of dityrosine being formed (fig.42). This would indicate that tyrosine is now the only means of defence against the oxidant H_2O_2. The fact that such a high concentration of dityrosine is present at time zero would indicate that the reaction proceeds quickly since the t=0 sample was taken as soon as possible after the H_2O_2 was added. By time 120min dityrosine levels have decreased considerably.

To investigate the relationship of GSH to dityrosine production and to determine what percentage of tyrosine is converted to dityrosine, a white rhinoceros dialysed lysate was incubated with GSH and ^{14}C-labelled tyrosine at varying specific activities, produced by the addition of unlabelled tyrosine. Aliquots were extracted at 0, 15, 30, 60, and 120 min and assayed for GSH (fig.43) and dityrosine (fig.44). HPLC analysis included in-line radio-detection so that the label could be followed.

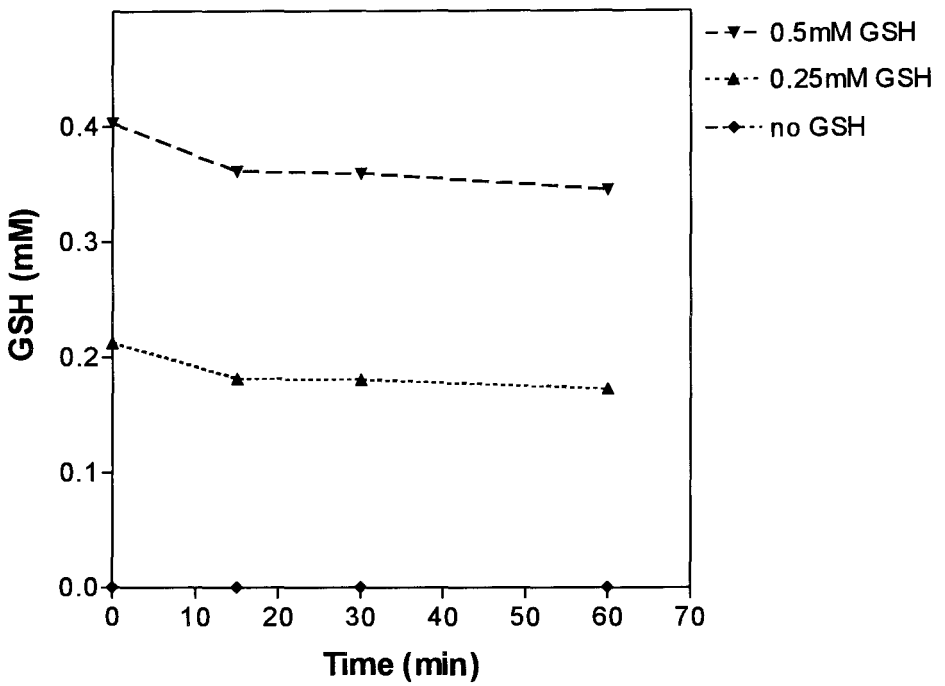

Figure 43. The effect of 2mM H_2O_2 on various GSH concentrations in white rhinoceros red blood cell lysates incubated in PBS at 37°C in the presence of 0.25mM tyrosine.

Significant dityrosine production was only observed in the reaction vessel to which no GSH was added. Those samples that contained 0.25 and 0.5mM GSH showed no production of dityrosine. These concentrations of GSH were therefore sufficient to neutralise the H_2O_2 added (via glutathione peroxidase) without significant dityrosine accumulation. There therefore seems to be a threshold level of GSH above which dityrosine accumulation is not observed. The observation that there is always a basal level of dityrosine present in the red blood cell may indicate a constitutive production at a low level and/or that the enzyme responsible for breakdown of dityrosine has a K_m above this basal level.

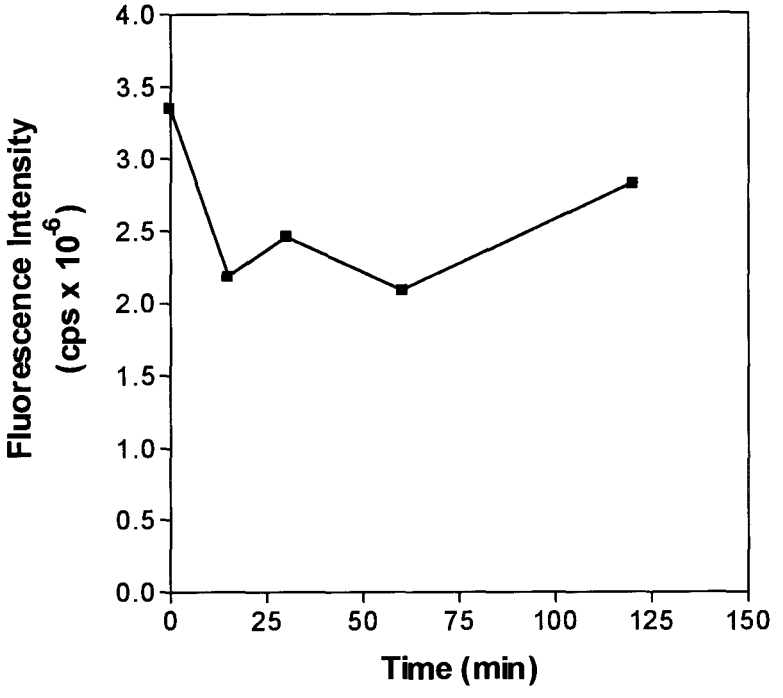

Figure 44. Dityrosine levels in white rhinoceros red blood cell lysates in the absence of GSH. Rhinoceros red blood cell lysate was incubated in PBS at 37^{0}C in the presence of 2mM H_2O_2, 0.25mM tyrosine and 1.5μCi L-[U-^{14}C]tyrosine.

Labelled tyrosine was used in the reaction to confirm the utilisation of free tyrosine in the production of dityrosine in response to H_2O_2 and to determine whether this dityrosine is recycled back to tyrosine. In those samples where GSH was present and no significant production of dityrosine was observed the radiolabelled scan did not differ significantly from that of standard labelled tyrosine. In the sample where no GSH was added, a later eluting peak was observed. Its position corresponded to that of dityrosine as observed on the ultraviolet traces. This peak accounted for roughly 5-7% of the tyrosine label used. This peak was not seen on radiolabel traces of samples in which no dityrosine production

was observed on the fluorimeter. A decrease in the amount of label representing tyrosine was seen in samples in which dityrosine was produced relative to samples in which no dityrosine production was observed. These results therefore prove that dityrosine is produced from free tyrosine, but whether it is recycled back to tyrosine could not be determined from these results.

The level of dityrosine produced was just within the limits of detection by reverse phase HPLC and could therefore be confirmed by this method of detection as well. Although HPLC detection of dityrosine could aid in its quantitation, fluorescence remains the most sensitive method of dityrosine detection.

The dynamics of dityrosine production in black rhinoceros were studied in a cell free system. In the reaction vessel that contained GSH, it was observed that the dityrosine level increased slightly as the GSH level decreased (fig.45). By 30min the dityrosine level decreased and remained low until 60min after which it rose steeply as GSH levels continued to drop. The initial rise in dityrosine can be explained if GSH levels were too low to neutralise all the H_2O_2 added and therefore the tyrosine mechanism was employed to deal with excess H_2O_2. The decrease in dityrosine levels by 30min suggests that all H_2O_2 has been neutralised and therefore the breakdown of dityrosine had begun. The increase in dityrosine levels again at 120min is puzzling. Note however that this increase occurs when GSH levels are at its lowest. This would be consistent with a role for GSH in the breakdown of dityrosine, and could be indicative of a complex situation where GSH is utilised both in H_2O_2 neutralisation and dityrosine breakdown.

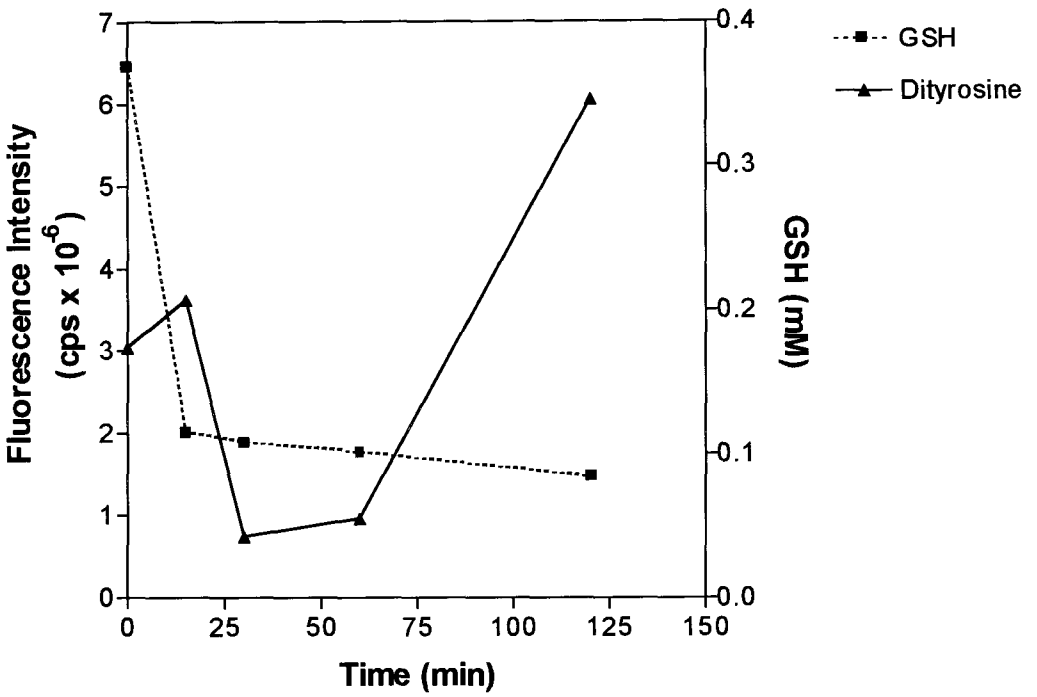

Figure 45. The relationship between dityrosine production and GSH concentration. Black rhinoceros dialysed red blood cell lysates were incubated in PBS at 37°C in the presence or absence of 0.5mM GSH. In both cases lysates were incubated with 4mM H_2O_2, 0.5mM tyrosine and 1μCi L-[U-^{14}C]tyrosine.

To determine whether GSH has a role in the breakdown of dityrosine, black rhino red blood cells were lysed and processed as described in the methods to obtain a dialysed cell-free preparation.

The experiment consisted of three reaction vessels, the first containing GSH but no tyrosine, the second containing tyrosine but no GSH and the third vessel containing tyrosine to which 0.5mM GSH was added after 60min incubation with H_2O_2 (fig.46).

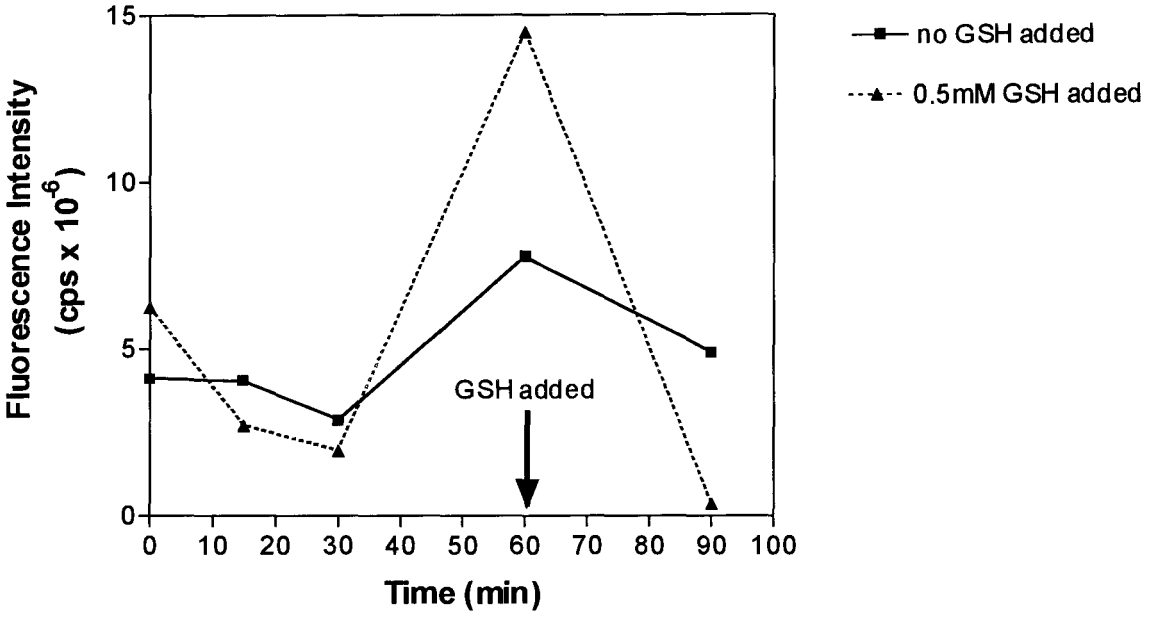

Figure 46. The effect of GSH on dityrosine catabolism in black rhinoceros red blood cell lysates. Dialysed cell free lysate was incubated with 0.5mM tyrosine and 2mM H_2O_2 in PBS at 37°C. To one reaction vessel (—▲—) 0.5mM GSH was added (arrow) at 60min and incubated for a further 30 minutes at 37°C. No GSH was added to the control (—■—) at this time.

No dityrosine production was assayed for in the first reaction vessel since no tyrosine was present. In the second and third reaction vessels, dityrosine was observed to accumulate and peak at 60min. When 0.5mM GSH was added to the third reaction vessel, after the 60min timepoint, a significant decrease in dityrosine was observed at 90min compared to the second reaction vessel to which no GSH was added at this time.

3.4.4 The mechanism of dityrosine production

To determine whether dityrosine production was indeed enzymatic as initially assumed, red blood cell lysates were heat inactivated and exposed to hydrogen peroxide (fig.47).

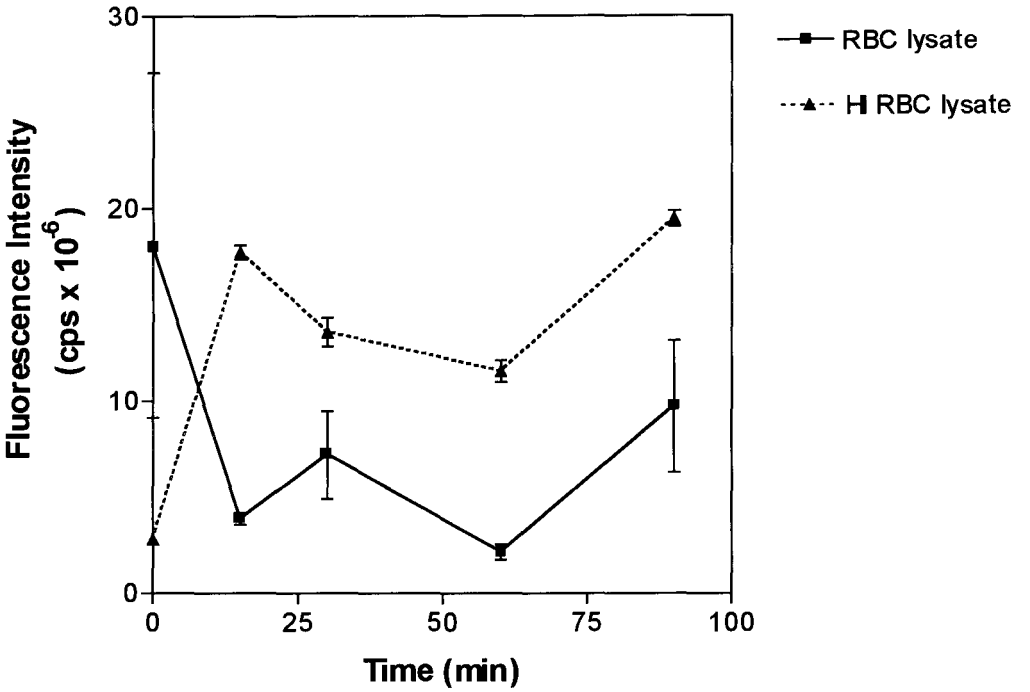

Figure 47. Fluorimetric analysis of dityrosine production at $315_{\lambda ex}$ and $409_{\lambda em}$ by heat inactivated and non-heat inactivated white rhinoceros red blood cells lysates incubated in PBS with 2mM H_2O_2 and 0.5mM tyrosine at 37°C. Each point shows the mean and range of duplicate results. HI– heat inactivated

Heat inactivation served to denature all enzymes in the lysate preparation. The heat inactivated red blood cell lysate, when exposed to H_2O_2, demonstrated the ability to produce dityrosine. At time zero the heat inactivated sample contained a very low concentration of dityrosine. After addition of H_2O_2 dityrosine increased and remained high at subsequent timepoints. In contrast to the heat inactivated sample a high concentration of dityrosine was observed in the non-heat-inactivated sample at time zero, before addition of H_2O_2 (fig.47). Dityrosine did however decrease at later timepoints.

Human red blood cells showed no production of dityrosine in a whole cell system.

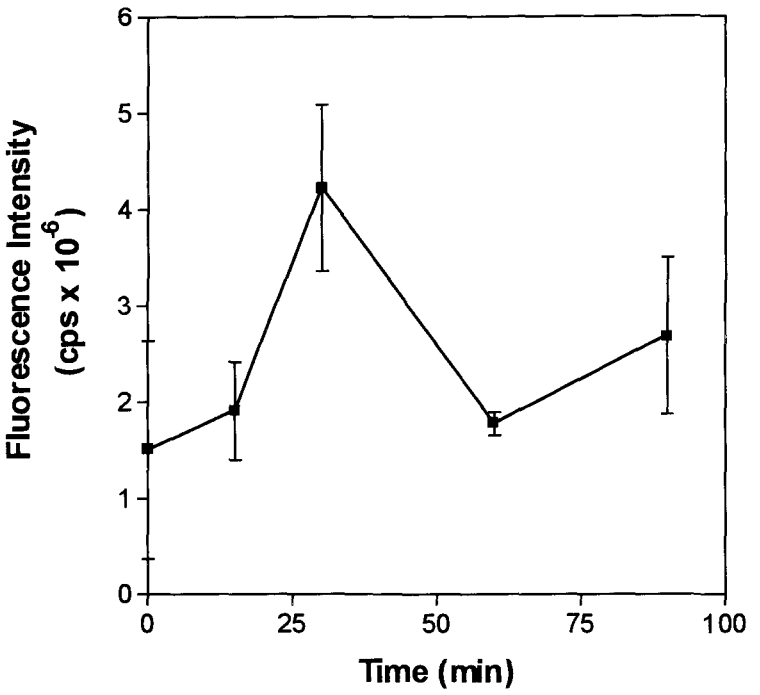

Figure 48. Fluorimetric analysis of dityrosine production at $315_{\lambda ex}$ and $409_{\lambda em}$ in human red blood cells lysates incubated in PBS with 0.5mM tyrosine and 2mM H_2O_2 at 37°C. Data points reflect the mean and range of duplicate results.

Since tyrosine levels are so low in human red blood cells this was assumed to be the reason for the lack of dityrosine production in this system. Tyrosine was added to human red blood cells lysates to determine whether the latter had the same ability to produce dityrosine when provided with adequate quantities of a tyrosine substrate (fig.48).

Despite the presence of a high concentration of catalase, the human lysate demonstrated accumulation of dityrosine in response to H_2O_2 (fig.48). A lag period of 15min was observed before the production of dityrosine peaked at 30min. By 60min the levels had dropped to basal levels. The presence of dityrosine at time zero in the presence of tyrosine

but in the absence of added H_2O_2 is significant and is consistent with that seen in rhinoceros red blood cell lysates albeit to a lesser degree.

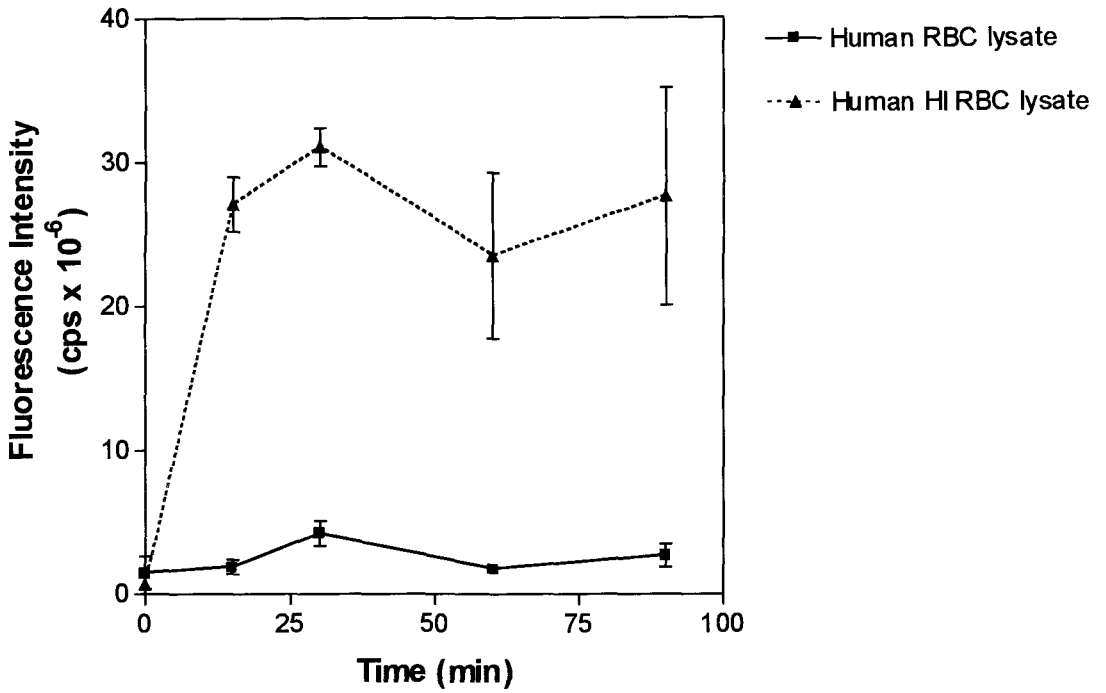

Figure 49. Fluorimetric analysis of dityrosine production at $315_{\lambda ex}$ and $409_{\lambda em}$ by heat inactivated human red blood cell lysates compared to that of non-heat inactivated human red blood cell lysate. Human red blood cell lysates were incubated in PBS at 37°C in the presence of 2mM H_2O_2 and 0.5mM tyrosine. Each point shows the mean and range of duplicate results.
HI - heat inactivated

To determine whether human heat inactivated lysate had the same ability as that of the rhinoceros to produce dityrosine it was incubated with H_2O_2 and assayed for the presence of dityrosine (fig.49).

Human heat inactivated red blood cell lysates showed the same ability as that of the rhinoceros to produce significant quantities of dityrosine. It is interesting to note that while the heat inactivated sample had no dityrosine present at time zero, the non heat

inactivated sample showed the presence of low levels of dityrosine. Human heat inactivated red blood cell lysates produced approximately similar amounts of dityrosine to that of the rhinoceros.

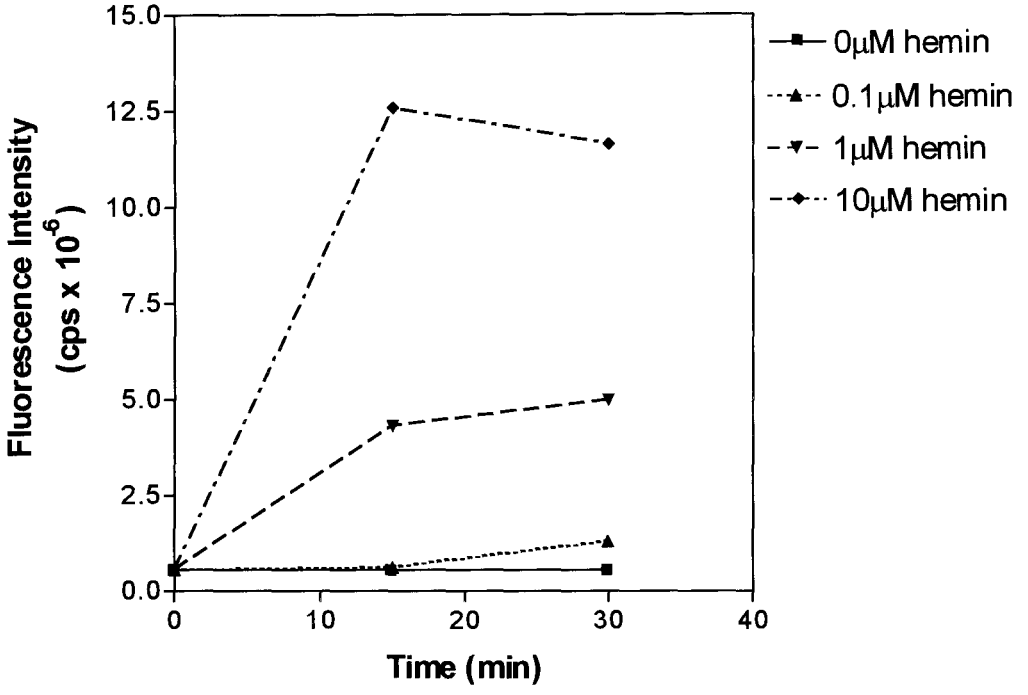

Figure 50. Fluorimetric analysis of dityrosine production at $315_{\lambda ex}$ and $409_{\lambda em}$. Increasing dilutions of hemin were incubated in PBS with 2mM H_2O_2 and 0.5mM tyrosine at 37°C.

The results of the heat inactivation were surprising since they indicated that dityrosine production was non-enzymatic. The possibility then that hemin was the catalytic agent in dityrosine production was therefore investigated (fig.50).

Hemin was found to effectively catalyse the production of dityrosine from tyrosine in the presence of H_2O_2 (fig.50). Dityrosine production increased with the concentration of hemin used. In the absence of hemin there was no production of dityrosine from a mixture of tyrosine and H_2O_2.

Since free hemin was therefore proved capable of producing dityrosine, the next question was whether haemoglobin itself was capable of doing so. Therefore the ability of haemoglobin to catalyse the production of dityrosine was investigated (fig.51).

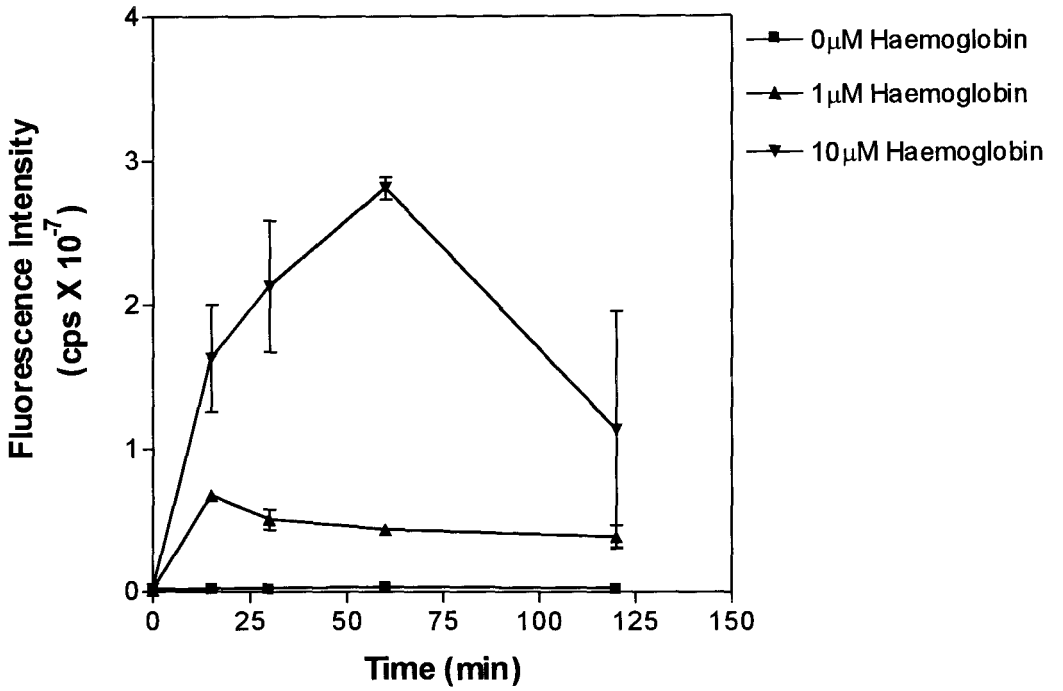

Figure 51. Fluorimetric analysis of dityrosine production by haemoglobin at $315_{\lambda ex}$ and $409_{\lambda em}$. Increasing concentrations of haemoglobin A was incubated in PBS at 37°C with 0.5mM tyrosine and 2mM H_2O_2. Each point shows the mean and range of duplicate results.

Taking into account that haemoglobin contains four haem moeties per molecule and that haemoglobin was used at the same concentration as hemin in the previous experiment (fig. 50), it can be said that these two compounds have equal capacities for dityrosine catalysis. Characteristically, dityrosine levels peaked and then decreased at later timepoints (fig.51). No dityrosine production was observed in the absence of haemoglobin in this *in vitro* system.

Summary of cell-free experiment results:

Table 4. Cell free production of dityrosine

0.5mM tyrosine and 2mM H^2O^2 were incubated with either human or rhinoceros heat inactivated or non heat inactivated dialysed cell free preparations (DCFP) of red cell lysates at 37°C for 0, 15, 30, 60, and 90 min before quantitation of dityrosine by fluorescence spectroscopy. Results are the mean of duplicate assays at the time of maximum dityrosine accumulation, with SEM in parentheses.

Tyrosine	H_2O_2	Addition	Dityrosine μM (SEM)
+	+	Nil	<0.5
+	+	DCFP (rhino)	4.1 (1.1)
+	+	DCFP (human)	7.6 (1.5)
+	+	DCFP (rhino) + GSH	<0.5
+	+	Horseradish peroxidase	180.5 (18.9)
+	+	Heated DCFP (rhino)	149.3 (2.2)
+	+	Fe^{2+}	<0.5
+	+	Hemin	20.5 (5.6)
+	+	Haemoglobin	49.3 (1.3)

(DCFP, Dialysed Cell Free Preparation)

3.4.5 Breakdown of dityrosine

To investigate whether dityrosine was recycled back to free tyrosine after H_2O_2 had been removed, dityrosine was produced as described in the Methods section and incubated with rhinoceros cell free lysates. This was done in the presence and absence of GSH with appropriate controls. Breakdown of dityrosine was followed by ultraviolet and radioactive detection since labelled tyrosine was used in the synthesis of dityrosine.

Results showed that dityrosine was broken down in the presence of the lysate but not to tyrosine as was initially concluded from the intact cell experiments. On both detection systems it was clear that it was broken down to an early eluting substance on reverse phase HPLC which still has to be identified.

4. Discussion

The unusual differences with respect to ATP levels and enzyme activities observed between human and rhinoceros red blood cells prompted a broader comparative red blood cell nucleotide study. This study included cow, rabbit and horse red blood cells. Each nucleotide profile had distinguishing characteristics: the urate riboside in the cow (fig.11), high nucleotide content of the rabbit (fig.8) and the prominent CTP in horse red blood cells (fig.7). The urate riboside previously described in the comparative work by Bartlett (76) has not been observed in the erythrocytes of any other species and may have an antioxidant role in these cells. These differences show that although red blood cells basically serve the same function in any animal i.e. the transport of oxygen to the body's tissues, their metabolism has become modified probably by adaptation to environmental factors, in each of these species. Findings such as the low ATP (15,16) in rhinoceros red blood cells (fig.5) have challenged conventional ideas about red blood cell energy requirements. Questions such as "How do rhinoceros red blood cells survive with such low levels of ATP?" may be countered with the question "Why do human red blood cells contain such high levels of ATP?" This comparative study therefore highlighted the need to characterise the metabolism of rhinoceros red blood cells and then look for a possible metabolic defect or weakness, in the context of its unique metabolism, which would explain the haemolytic anaemia (1-3) experienced by black rhinoceroses in captivity.

Black rhinoceros red blood cells were shown to be particularly susceptible to oxidative stress (25) and therefore much of the metabolic analysis has been concentrated on the hexose monophosphate shunt. This pathway, as mentioned earlier, has the function of

protecting the red blood cell against oxidative stress (fig.1) by providing NADPH for maintaining GSH in the reduced state. Its proper functioning would be essential to the survival of a red blood cell lacking significant levels of catalase (17,18) as is the case in the black rhinoceros. Ascorbate (fig.3) and methylene blue (fig.4) were used as stimulants of the shunt to assess the ability of the cells to deal with an oxidative crisis.

Results show that black and white rhinoceros red blood cells have a low metabolic rate of flux through the HMP under basal conditions compared to human red blood cells (fig.13). Rhinoceros basal metabolic rates of flux through this pathway were approximately half that observed in human red blood cells. This would suggest a relative impaired ability to handle oxidative stress by standard methods (GSH supply) relative to humans.

Despite this low basal rate of flux, when stimulated with ascorbate, the HMP of black rhinoceros red blood cells were stimulated significantly above basal levels (fivefold). Human red blood cells were stimulated even to a greater degree (tenfold) (fig.14). White rhinoceros red blood cells were stimulated poorly under these conditions.

Methylene blue produced a similar increase in flux in the black rhinoceros but this time not proportional to that seen in human red blood cells (fig.15). Human red cells were stimulated approximately fifteen times above basal levels, black rhinoceros red cells three times and white rhinoceros red blood cells only two times above basal levels.

Taken together these results show that black rhinoceros red blood cells are capable of mounting a response to oxidative stress although this response is not proportional to that seen in human red blood cells, being significantly less.

The effect of these stimulants on the Emden Myerhof pathway was also studied. Ascorbate significantly increased lactate above basal levels in rhinoceros red blood cells while methylene blue had no significant effect (fig.16). Since ascorbate stimulates a fourfold increase in flux through the HMP relative to basal levels, an increase in flux through the EMP would also be expected since HMP metabolites enter the EMP after cycling through the shunt. This would therefore indicate an increase in the amount of glucose metabolised in the presence of ascorbate.

Ascorbate has been observed to increase GSH levels (fig.19) in some cases and this would be consistent with reports that ascorbate 'spares' GSH by reacting with free radicals itself. What is significant is that GSH does not decrease relative to basal levels in the presence of ascorbate. Methylene blue however had the effect of decreasing GSH levels relative to basal levels (fig.19). This could be explained in the light of the mechanism of action of methylene blue. Methylene blue increases the ratio of NADP:NADPH. This drop in NADPH relieves its inhibition of G-6-PD and results in an increase in the diversion of glucose-6-phosphate through the HMP. GSH via glutathione peroxidase still has to bear the load of oxidant removal by itself unlike the case with ascorbate.

When black rhinoceros red blood cells were incubated with 2-^{14}C-glucose the amount of labelled 2-$^{14}CO_2$ given off increased considerably in the presence of the shunt stimulants relative to basal levels under these conditions (fig.21). Firstly, cycling is very low under basal conditions. Secondly in the presence of the shunt stimulants these red blood cells

efficiently recycled intermediates through the HMP and therefore have the ability to deal with prolonged oxidative stress. Cells were incubated in the presence of the catalase inhibitor and haem poison sodium azide to see how this affected flux through the HMP. Flux through the HMP increased relative to azide-free controls (fig.22). These results could be explained by the azide inhibiting catalase and therefore with glutathione peroxidase providing an alternative means of oxidant removal, HMP shunt activity would need to increase to provide the reducing equivalents (NADPH and GSH) required.

Although these metabolic studies yielded interesting results and invaluable insights into the oxidant metabolism of black rhinoceros red blood cells, no direct cause for the haemolytic anaemia was identified other than a moderate relative decrease in the ability to mount a response to oxidative stress. It was at this point that it was decided to focus attention on an anomalous and predominant peak in the rhinoceros red blood cell nucleotide profile (peak X, fig.23).

Black rhinoceros red blood cells extracts were analysed on reverse phase HPLC (fig.24) to provide additional information on this compound's physical properties. The elution position and ultraviolet absorbance characteristics (A_{280}/A_{260}) of this "cytidine-like" peak (peak X) did not in fact correspond to that of any cytidine compound or other nucleotide. Although similar to the urate riboside peak of bovine red blood cells on anion exchange (fig.23), reverse phase HPLC clearly showed the difference between these compounds (fig.24). The elution position differed by approximately 10min.

A search for a possible cytoplasmic constituent with similar absorption properties was then made, and the spectral characteristics of the peak were found to be identical to that of the amino acid tyrosine. Diode array scans were performed on the peak and it was found to have 99.99% identity to authentic tyrosine standards. Rhinoceros red blood cell extracts were subjected to amino acid analysis (fig.25) and were found to contain between 30 and 50 times the amount of tyrosine reported to be present in human red blood cells. The peak also co-eluted with tyrosine standards on reverse phase HPLC. Analysis by mass spectroscopy also confirmed this identity. This finding of such high quantities of a free amino acid in red blood cells was highly unexpected and appears to be unique (with one exception, taurine [see below]).

Having positively identified the compound as tyrosine, its function still remained unknown. It would be difficult to believe that the presence of an amino acid in red blood cells at such a high concentration was a chance evolutionary event with no function, so it is most likely that it is playing some significant metabolic role.

The possibility that tyrosine was acting as an alternate energy source was investigated. Since acid extraction would strip the tyrosine of any high-energy phosphate, alkaline extraction was employed and the extract analysed on reverse phase HPLC. From these results it was evident that the tyrosine was not phosphorylated and therefore could not be acting as an energy source.

The other possibility, that tyrosine may form part of a mechanism in which it protects the red cell against oxidative stress, perhaps as part of some anti-parasite defence, was the next option to be investigated. One other example of an amino acid being present at high

concentrations in cells is that of taurine in leukocytes (77), neutrophils (78) and heart epithelial cells (79).

The function of taurine in this context comes to light when one considers the inflammatory response. During inflammation, activated phagocytes secrete myeloperoxidase, a heme enzyme, into the extracellular medium. This generates large amounts of superoxide, which in the presence of SOD, dismutases to form H_2O_2. Myeloperoxidase then catalyses the oxidation of chloride ion (Cl^-) by H_2O_2 to form the potent antimicrobial oxidant HOCl (80,81).

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O$$

HOCl is extremely reactive, irreversibly depleting adenine nucleotide pools and cytochromes in E.Coli cells (82). It has been shown to permeate red blood cells, preferentially deplete GSH (83) in human red blood cells and may mediate red blood cell lysis. These properties make HOCl an effective antimicrobial agent but also means that it may inflict oxidative damage on host proteins, lipids and nucleic acids. In a cell system such as the rhinoceros red blood cell where ATP levels are low and there is a deficiency of catalase, this destruction of adenine nucleotide pools and depletion of GSH might result in red blood cell lysis. HOCl is not the only threat to host cell constituents but is also capable of forming the highly reactive hydroxyl radical by reaction with the superoxide radical (84).

As a self-protection mechanism neutrophils secrete substances containing primary amino groups such as α-amino acids and taurine (85,86). These amines react with HOCl to form monochloramines. These react slowly with neutrophils or target cells and therefore moderate neutrophil toxicity (87). In this way taurine protects these cells against HOCl. Taurine reacts with HOCl to form the monochloramine, taurine chloramine, which may be oxidised further to the dichloramine, taurine dichloramine. These chloramines are taken up into erythrocytes via the anion-transport system and mediate effects such as GSH oxidation and at high oxidant:erythrocyte ratios also haemoglobin oxidation and ATP depletion (88). The dichloramines are much more effective at causing cell lysis. Although this uptake of chloramines by erythrocytes may be a way of protecting other blood cells, plasma components and tissues against oxidative damage, the red cell itself becomes exposed to the toxicity of the taurine chloramines. However, the red cell appears to protect itself from this damage by reducing the taurine chloramine back to taurine, which is then retained in the red cell.

Drawing on the analogy of the amino acid taurine protecting against oxidative stress, we investigated the possibility of the free tyrosine in rhinoceros red blood cells protecting it against various oxidants such as HOCl, H_2O_2 and taurine chloramines.

Work done by Domigan *et al.* (89) and Hazen *et al.* (90) showed that tyrosyl residues in proteins are chlorinated to form 3-chlorotyrosine by HOCl. For this reason the possibility of tyrosine protecting rhinoceros red blood cells against HOCl was investigated first. The results show that while standard tyrosine and free tyrosine in rhinoceros red blood cell extracts reacted with HOCl to form 3-chlorotyrosine, no evidence of this product could be

found when HOCl was incubated in a whole cell system. It therefore seems unlikely that this reaction is relevant to the haemolytic problem in black rhinoceroses.

Besides HOCl another product of myeloperoxidase is the tyrosyl radical (91), which dimerises to form *o,o*-dityrosine (92-95) which is intensely fluorescent.

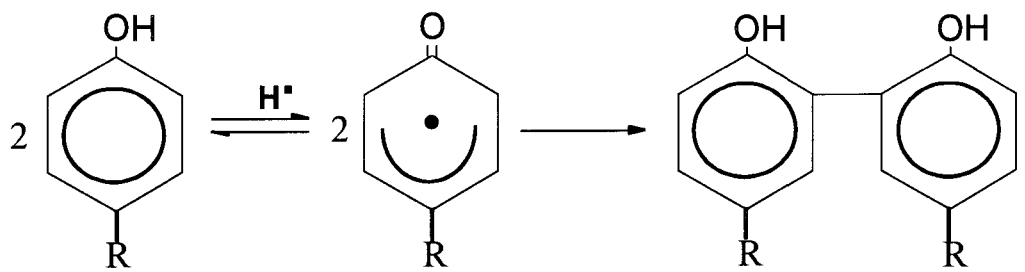

Figure 52. The cross-linking of tyrosine residues to form dityrosine.

Myeloperoxidase catalyses this reaction at physiological plasma concentrations of Cl^- and tyrosine. Dityrosine is normally formed between adjacent protein tyrosyl residues, crosslinking them. Heinecke *et al.* (96) have suggested that protein cross-linking in the form of dityrosine may be an effective method of killing bacteria or injuring normal tissue. It has been proposed that intra-protein dityrosine production oxidatively modifies and thereby marks proteins such as haemoglobin for selective proteolysis (97).

Therefore the possibility that the elevated free tyrosine protects rhinoceros red blood cell proteins during periods of oxidative stress by reacting with excess H_2O_2 and forming free dityrosine was investigated.

We found that when rhinoceros red blood cells were incubated with H_2O_2, free dityrosine was produced in a time-dependant manner (fig.28). Human red blood cells under identical

conditions showed no sign of dityrosine production (fig.30). Production of dityrosine was found to be dependent on the concentration of added H_2O_2 (fig.32). It is significant that a basal level of dityrosine was observed in rhinoceros red blood cells in the absence of added H_2O_2. This implies a steady state production of dityrosine in metabolising rhinoceros red blood cells. This would be consistent with the reaction of free tyrosine with endogenously produced H_2O_2. Labelled tyrosine studies have confirmed that the dityrosine is being produced from the free tyrosine in rhinoceros red blood cells.

Dityrosine formation consistently peaked and then decreased or returned to basal levels at later timepoints (fig.37). This decrease implies that it is either being transported out of the cell after formation, is being metabolised inside the cell to unknown (non-fluorescent) products or is being recycled back to tyrosine. Measurement of flurorescence in the incubating medium after exposure to H_2O_2 revealed no trace of dityrosine, therefore it is not being transported out of the cell after its formation.

To investigate the relationship between GSH concentration and dityrosine production rhinoceros red blood cells were exposed to 1-chloro-2,4-dinitrobenzene (CDNB). CDNB has been shown to irreversibly deplete erythrocyte GSH by forming a non-hydrolysable GSH-CDNB conjugate (98) which cannot be metabolised by glutathione peroxidase. A concentration of 1mM CDNB was found to effectively deplete rhinoceros red blood cell GSH by 30min of incubation at 37^0C (fig.33). This depletion of GSH levels was irreversible with no subsequent increase in GSH levels (fig.34). When red cells were then incubated with H_2O_2, the lag in dityrosine production in the absence of CDNB was abolished (fig.35). This suggests that initially rhinoceros red blood cells are able to remove

H_2O_2 via glutathione peroxidase and only after a certain time employ the tyrosine to deal with the excess H_2O_2. It could also mean that dityrosine is not being broken down so fast in the presence of low levels of GSH. In the absence of GSH however, the glutathione peroxidase pathway is not available and since catalase is virtually deficient, tyrosine forms the only defence against the oxidative challenge hence the early increase in dityrosine production in the presence of CDNB.

In whole cell systems it was found in black (fig.37) and white rhinoceroses (fig.39) that a reciprocal relationship existed between the production of dityrosine and GSH levels in these cells. This suggests that once GSH levels fall below a certain threshold level, dityrosine production becomes the major mechanism for oxidant removal and once GSH levels return to normal or when all the H_2O_2 has been removed, dityrosine levels decrease.

These results imply that a tyrosine antioxidant mechanism works in conjunction with glutathione peroxidase and catalase to protect the cell against oxidant damageHPLC analysis of red blood cell extracts in which dityrosine formation was followed suggested that the free tyrosine in these cells was being used for the production of dityrosine and that possibly the dityrosine was being recycled back to tyrosine after the removal of the H_2O_2.
To investigate the relationship between GSH and dityrosine production and the fate of the dityrosine formed more thoroughly, a cell free system was devised. This enabled precise control of GSH concentration as well as the option of including labelled tyrosine which would enable the fate of the dityrosine to be followed.

Taken together the results from the cell free experiments indicate that no dityrosine is formed in the presence of adequate levels of GSH (fig.42) but if GSH levels drop below a certain level the dityrosine response comes into play. These experiments also suggest that GSH increases the rate of dityrosine removal (fig.46). The exact mechanism of this removal is not yet known.

The concentrations of dityrosine measured in this system i.e. approximately 5μM for black rhinoceroses and 28μM for white rhinoceroses is much lower than the concentrations of GSH in these cells and therefore casts some doubt as to whether this system actually does compliment the GSH dependent pathway to any significant extent. It must be taken into account however that a basal rate of flux has been observed through the dityrosine pathway and that what we refer to as dityrosine "production" may actually be dityrosine accumulation before further metabolism. This would imply that the dityrosine removal system is overwhelmed by the sudden response to the bolus addition of H_2O_2 giving rise to the accumulation observed. This would be consistent with the finding that dityrosine accumulates to a much greater extent in heated cell free preparations (table 4) without decreasing at later timepoints. This suggests that the removal of dityrosine is an enzymatic process which is inactivated in heated cell free preparations.

Lupo *et al.* have reported that TYR1 *Saccharomyces cerevisiae* mutants, which do not have the ability to biosynthesise tyrosine, are highly sensitive to oxidative stress. This sensitivity was removed when this strain was complemented with the *TYR1* gene. They conclude therefore that tyrosine protects *Saccharomyces cerevisiae* from oxidative stress (99) but the exact mechanism has not been elucidated. Although this report supports the

role of tyrosine as an antioxidant, its function as an antioxidant in rhinoceros red blood cells still needs to be tested directly and this will be the subject of further investigation by the author.

Heat inactivated rhinoceros red blood cells lysates were capable of producing dityrosine in the presence of H_2O_2 (fig.47). Dityrosine production was increased in heat inactivated red blood cell lysates relative to non-heat inactivated lysates and did not decrease at later timepoints as was the case in whole cell assays (fig.47). This indicated that while the production of dityrosine may be non-enzymatic, its removal is. GSH therefore may be a co-factor in this reaction since, as mentioned earlier, it increases the rate of dityrosine removal.

Human red blood cell lysates were also capable of producing dityrosine, with the heat-inactivated lysate also producing more dityrosine than the non-heat-inactivated lysate (fig.49). This suggests that the limiting factor in human red blood cells is the low concentration of tyrosine and that it otherwise has the capacity to use tyrosine to produce dityrosine in response to H_2O_2.

Dityrosine production was found to be inhibited in the presence of azide, a known haem protein inhibitor (100) (fig.31). This suggested that the production of dityrosine was being catalysed by a haem containing protein. To investigate this possibility the ability of hemin to catalyse the production of dityrosine was investigated. Experiments with hemin indicated that it is efficient in catalysing the production of dityrosine from tyrosine and H_2O_2 (fig.50). This led to the investigation of the ability of haemoglobin to catalyse the production of dityrosine. When haemoglobin was incubated with standard tyrosine in the presence of H_2O_2 it was also found to catalyse the production of dityrosine (fig.51). The

production of dityrosine was dependent on the concentration of haemoglobin. The concentration of haemoglobin used in the experiment is far below its intracellular concentration. This therefore suggests that haemoglobin is the catalyst of dityrosine production in the rhinoceros red blood cell system. Also interesting to note was the decrease of dityrosine at later timepoints in this lysate-free system, suggesting that the breakdown of dityrosine is not enzymatic in the strict sense. On the other hand, since haemoglobin is a protein, breakdown could be considered as enzymatic in contrast to hemin.

Additional experiments with labelled dityrosine showed that the breakdown of dityrosine was not back to tyrosine but instead to an earlier eluting substance on reverse phase HPLC. This would imply that rhinoceros red blood cells have an uptake mechanism for tyrosine to replenish these levels in order to maintain this response to H_2O_2.

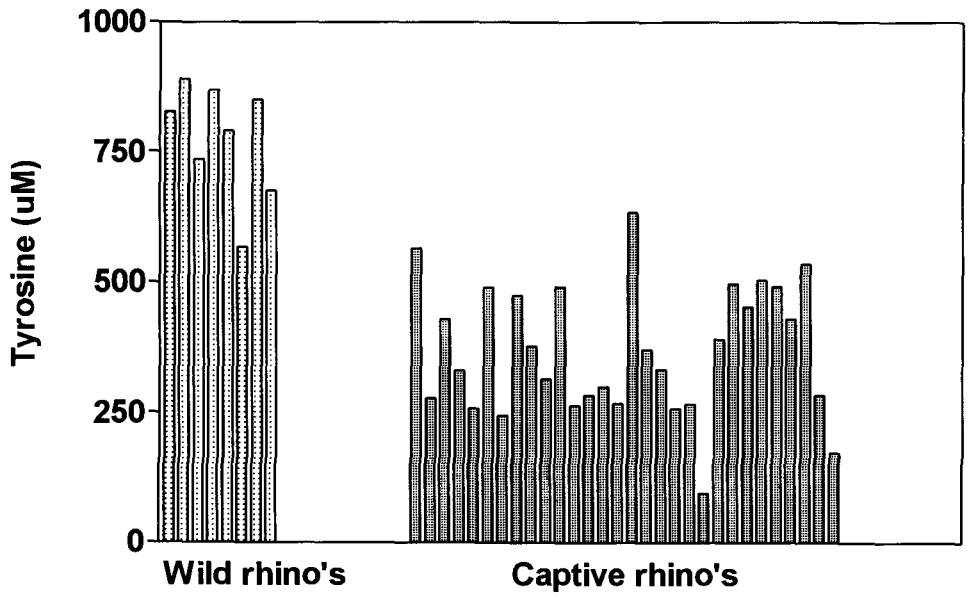

Figure 53. Tyrosine content of wild, free-ranging black rhinoceroses compared to captive black rhinoceroses determined by reverse phase HPLC.

Analysis of tyrosine levels in captive black rhinoceroses show that these animals have significantly lower levels of tyrosine than black rhinoceroses in the wild. Levels in 8 wild free ranging black rhinoceroses had a mean of 0.78 ± 0.11mM while mean levels in 30 captive rhinoceroses averaged 0.37 ± 0.14mM which is significantly lower ($p<0.0001$) than that in the wild rhinoceroses (fig. 53). Plasma levels of tyrosine measured by Dierenfeld *et al.* (101) and confirmed by our own measurements indicate that these do not differ significantly from human levels.

Work done by Paglia *et al.* (102) have shown that black rhinoceroses in captivity apparently suffer from iron overload. Iron levels in captive black rhinoceroses inversely correlated with our measurements of tyrosine levels in these animals (fig.54).

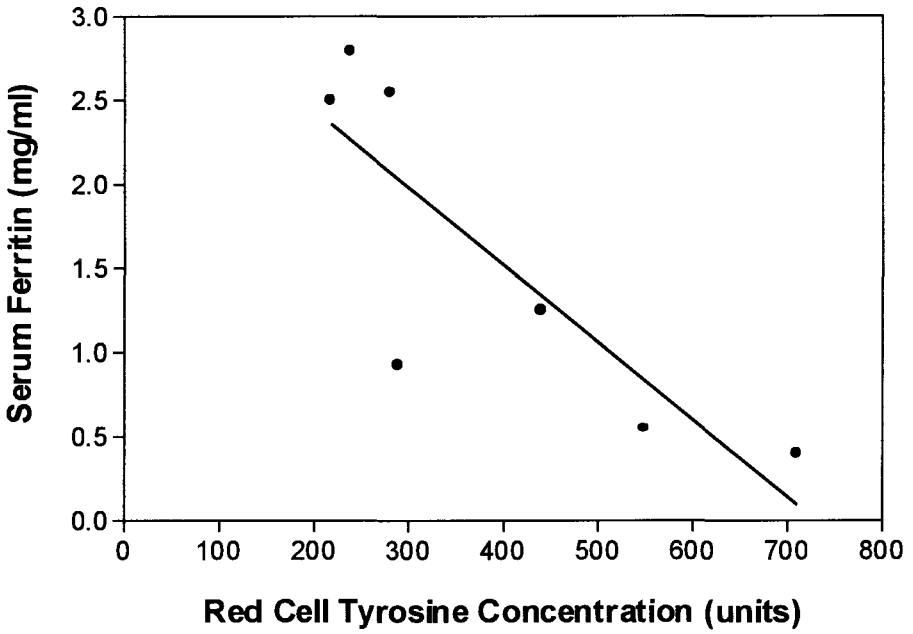

Figure 54. Serum ferritin levels correlated with red blood cell tyrosine concentrations in black rhinoceroses in captivity. The graph was constructed using serum ferritin values provided by Prof. Paglia and tyrosine concentrations determined on the same animals as part of the present work.

This iron load may predispose them to a higher level of oxidative stress than their counterparts in the wild, deplete their red blood cell tyrosine and leave them with primarily the glutathione peroxidase pathway to protect their red blood cells.

In the presence of metal ions such as iron or copper the Haber-Weiss reaction,

$$O^{\bullet}_2{}^- + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2$$

and the Fenton reaction,

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

become significant.

In the absence of sufficient defence against H_2O_2 it becomes clear that the red cell is in danger of undergoing oxidative damage by hydroxyl radicals. Hydroxyl radicals are among the most reactive chemical species known. It reacts with extremely high rate-constants with almost every type of molecule found in cells (103,104). In the presence of free catalytic iron (Fenton reaction) or superoxide radical (Haber-Weiss reaction) and H_2O_2 the production of hydroxyl radicals proceeds autocatalytically generating large amounts of this aggressive radical. Reaction of this radical with other molecules generates secondary radicals which vary in their reactivity. This may therefore result in a chain reaction of radical damage in the red cell.

This underlines the importance of an additional mechanism i.e. the dityrosine response, to deal with the removal of H_2O_2 in a red cell which is deficient in catalase.

Inflammatory derived oxidants such as nitric oxide and taurine dichloramine are potentially harmful to host tissues. It has been reported that tyrosine residues form nitrotyrosine and

chlorotyrosine (105-107) after reaction with nitric oxide derived inflammatory oxidants such as nitrogen dioxide ($^{\bullet}NO_2$) and nitryl chloride (NO_2Cl). The possibility that free tyrosine in rhinoceros erythrocytes protects against these oxidants as well will be the subject of further investigation.

5. Conclusion

Rhinoceros red blood cells have been found to contain concentrations of the amino acid tyrosine approaching 1mM. Haemoglobin catalyses the reaction of this free tyrosine with H_2O_2 in a manner which is inversely proportional to red blood cell GSH concentration to form the highly fluorescent tyrosine dimer, dityrosine. Captive rhinoceroses have lower levels of tyrosine than rhinoceroses in the wild. There is an inverse relationship between serum ferritin and red blood cell tyrosine levels.

One hypothesis to account for this highly unusual metabolism of rhinoceros red blood cells is that rhinoceros red blood cells have developed a unique metabolic strategy to protect themselves against red blood cell parasites e.g. *Plasmodium* species (which cause malaria). They have done so by maintaining low levels of ATP to starve parasites which salvage host purine nucleotides for their own DNA synthesis. The low levels of catalase in rhinoceros red blood cells could also prevent the removal of antimicrobial H_2O_2. Despite these apparent deficiencies, relative to what in human red cells we accept as "normal", rhinoceros red blood cells are still able to mount a significant response to oxidative challenge and are able to deal with prolonged oxidative stress. To compensate for the virtual deficiency of catalase, glutathione peroxidase activity is increased several-fold relative to that in human red blood cells.

This study proposes a potential, novel antioxidant mechanism i.e. the dityrosine response to oxidative stress in the form of H_2O_2. Consistent with this hypothesis is the observation that captive rhinoceroses have significantly lower levels of tyrosine than their counterparts in the wild. A study done by Paglia *et al.* have shown that black rhinoceroses have

elevated serum levels of free catalytic iron. This may be the cause of the low tyrosine in the red cells of captive animals since this may the source of increased oxidative stress which depletes tyrosine stores in the red blood cells.

These severely depleted red blood cell tyrosine levels may be an indication of increased red blood cell free radical production consequent on iron overload, and exacerbated during periods of acute inflammation, which predisposes black rhinoceroses in captivity to haemolytic anaemia.

These findings raise several possibilities for therapy or prevention. It is likely that the wild diet contains either effective iron chelators, or effective free radical scavengers, or both which are missing from the rather bland diet (mostly lucern) which they have been getting in captivity. The addition of appropriate browse or dietary supplements may alleviate the problem.

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