Elevated levels of low molecular weight substances in the red cells of some mammalian species imply unsuspected antioxidant strategies

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Thesis presented for the degree of

DOCTOR OF PHILOSOPHY

In the Department of Clinical laboratory Sciences

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

March 2009

DECLARATION

I, Virginia Davids, hereby declare that the work on which this thesis is based (both in concept and execution) is my original work, and that neither the whole work nor any part thereof has been submitted for another degree, at this or any other university.

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……
March 2009

~

Dedicated to my most wonderful husband, my precious friend and soul mate. Thank you for so much.

~

During a lunch session at the slaughterhouse, a lamb jumped out of its pen and came unnoticed up to some slaughtermen who were sitting in a circle eating some sandwiches; the lamb approached and nibbled a small piece of lettuce that a man was holding in his hand. The men gave the lamb some more lettuce and when the lunch period was over they were so affected by the action of the lamb that not one of them was prepared to kill this creature, and it had to be sent away elsewhere - showing that within each human soul there is an element of pity, compassion and love in varying degrees. It is our duty to encourage the higher qualities to bloom and blossom wherever possible in each individual." Gordon Latto

ACKNOWLEDGEMENTS

To my thesis supervisors: Prof Harley (Chemical Pathology), thank you for providing me with the "ticket" allowing me to get closer to my dream of using my expertise to improve the well-being of animals in some way, but which may also benefit humans. You leave with me the legacy of not shying away from "fishing in the muddy waters". Prof Katz (Medical Biochemistry) (aka Arieh), thank you for your encouragement and belief in my ability to do this from the start. It meant a lot. To my two 'unofficial supervisors', colleagues and great friends, Lester and Dee. You guys are amazing – thank you!!

I wish to thank Professors Pillay and Henderson (current and previous HOD, Chemical Pathology Dept.) for accepting my presence and use of the facilities in their department. Pete and Ingrid, thank you both for allowing me to share space in your lab, use reagents etc., and for the social and intellectual chats. Ingrid, thank you also for your expert culture of the fibroblasts. Trish, and also Felicity and Lisa, your open welcome to use the lab as I needed, and our friendly impromptu chats made me feel welcomed from the start – thank you. Thanks also to Ingrid, Dave and Abdu for all the efficient help placing orders, as well as to Juliet and Mara for their always friendly assistance.

I also gratefully acknowledge the UCT Medical School Library staff for their always efficient and friendly assistance in obtaining the many scientific articles I requested for this thesis.

I am greatly indebted to all the people involved in helping me to obtain the necessary animal samples, in particular Drs Gary Buhrmann, Koos Neuland, Sewellyn Davey, Elzette Jordan and Dave Cooper and Mr John Morris, as well as the UCT Medical School Animal Unit Staff (especially Mr Noel Markgraaf).

I would like to acknowledge and thank the following organizations for their financial support: The National Research Foundation (South Africa) and the Deutscher Akademischer Austauch Dienst (DAAD, Germany) for their award of a doctoral fellowship, and the Medical Research Council (South Africa) for running expenses as part of a Self-Initiated Grant (E.H.Harley).

To my supportive family and friends that lovingly never really understood why it was so endless – at last my reply is "it is done". I promise that I will never be a student again ☺ Mom, I know you were with me all the way. Thank you my very special friend, so far yet so close. To all my 'babies' at home keeping me company while I wrote up…yes, now I will take you for that walk. To my husband and so much more, Lester, I couldn't have done this without you!

Thank you Lord, for getting me through this.

Until one has loved an animal, a part of one's soul remains unawakened

(Anatole France)

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ABSTRACT

Elevated levels of low molecular weight substances in the red cells of some mammalian species imply unsuspected antioxidant strategies

Virginia Davids March 2009

An earlier observation by E.H. Harley (supervisor of this thesis) of curious metabolic anomalies in the red cells of black rhinoceros, and in particular a high free tyrosine level, suggested that a range of unusual, but presumeably physiological, processes might be found in mammalian red blood cells.

As a follow-up to this, low molecular weight metabolites were examined in a range of mammalian species, using HPLC-based methods to compare levels in red cells with plasma levels. A remarkable interspecies diversity in red cell HPLC profiles was observed, with the unprecedented accumulation of substances including tyrosine, tryptophan, urate, and urate riboside occuring within the red cells of some species. Whereas novel evolutionary adaptations may characterise most of these species-specific variations, the ability of red cells to produce urate is proposed to be an inducible feature common to the red cells of many, or possibly even all, mammalian species. A surprisingly high degree of intraspecies genetic heterogeneity was evident in tyrosine and urate levels within horse, and urate riboside levels within cow red cells. This was in contrast with the greater homogeneity seen in levels of these and other low molecular weight substances in red cells from the other species evaluated.

The next phase of investigation addressed the potential function(s) of these soluble substances accumulating within the red cell, particularly relating to a role in antioxidant defense. Using *in vitro* antioxidant assays such as the 'oxygen radical absorbance' (ORAC) and 'ferrous ion oxidation-xylenol orange' (FOX) assays, results were obtained consistent with a role for these substances as endogenous red cell antioxidants against a variety of reactive species produced by pathophysiological processes in the body. The demonstration that haemoglobin is involved in facilitating some of this activity further substantiates the idea that the red cell may be playing a crucial role in maintaining circulatory redox balance, and hence protecting other tissues from oxidative damage. If indeed such low molecular weight substances contribute to systemic antioxidant activity in some mammalian species, then apart from the intrinsic interest of such unexpected biological phenomena, these findings could pave the way for a plethora of further investigations, geared towards potential clinical applications (eg. as biomarkers or therapeutic approaches) in human and/or veterinary conditions associated with oxidative stress.

ABBREVIATIONS

AAPH - 2,2'-azobis(2-amidinopropane) dihydrochloride

AUC - area-under-curve

CuZnSOD - copper-zinc superoxide dismutase

CV - coefficient of variation

DHA - dehydroascorbate

ELISA - enzyme-linked immunosorbant assay

E:P ratio - erythrocyte to plasma ratio

FOX - ferrous ion oxidation-xylenol orange

FL - fluorescein

G6PD - glucose-6-phosphate dehydrogenase

GPx - glutathione peroxidase

GR - glutathione reductase

GSH - glutathione (reduced)

GSSG - glutathione (oxidized)

HAT - hydrogen atom transfer

Hb - haemoglobin

HMP shunt - hexose monophosphate shunt

HBOC - haemoglobin-based oxygen carriers

H_2O_2 - hydrogen peroxide

HOCl - hypochlorous acid

HPLC - high pressure (performance) liquid chromatography

IDO - indoleamine 2,3-dioxygenase

LMW - low molecular weight

MDA - malondialdehyde

metHb - methaemoglobin

Mo – molybdenum

MPO - myeloperoxidase

MR - methaemoglobin reductase

$NO^{\cdot}$- nitric oxide

N/S - normal saline

$O_2^{\cdot -}$ - superoxide radical

$OH^{\cdot}$- hydroxyl radical

$ONOO^-$ - peroxynitrite

ORAC - oxygen radical absorbance capacity

$ORAC_T$ - oxygen radical absorbance capacity of total/whole samples

$ORAC_{LMW}$ - oxygen radical absorbance capacity of acid-extracted samples

oxyHb - oxyhaemoglobin

PCA - perchloric acid

PPRP - 5-phosphoribosyl-1-pyrophosphate

Prx - peroxiredoxin

RCS - reactive chlorine species

ROS - reactive oxygen species

RNS - reactive nitrogen species

RS - reactive species

SD - standard deviation

SDA - semidehydroascorbate

SET - single electron transfer

SOD - superoxide dismutase

TAC - total antioxidant capacity

TAC_{LMW} - total antioxidant capacity of acid-extracted samples

TB - tuberculosis

TE - Trolox equivalents

TEAC - Trolox equivalent antioxidant capacity

TRAP - total radical trapping antioxidant parameter

UMP synthase - uridine monophosphate synthase

XO/XD - xanthine oxidase/xanthine reductase

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* The peak purity index, information which is provided by the HPLC Beckman System Gold for every profile, but not shown in the figures above, indicates if more than 1 substance is responsible for every peak. Therefore, the first-eluting peak in (A) would have been shown to represent multiple co-eluting substances. On the other hand, peaks h, i and l in (A) would have shown peak purity i.e. each representing a single eluting substance.

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[#]Black rhino red cell tyrosine concentrations are taken from a previously published report [2].

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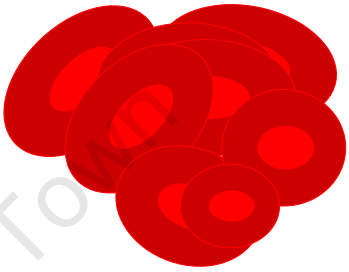
CHAPTER 1

Clarifying and investigating differences in low molecular weight metabolites in red cells from different mammalian species

(A) LITERATURE REVIEW AND BACKGROUND WORK



Until the 1940's, the red blood cell/erythrocyte was viewed merely as an inert bag of haemoglobin. Due to the absence of a nucleus or any other normal cellular organelles, no new protein synthesis takes place in the mature red cell of most vertebrates and all mammals, and there is an unusual paucity of intracellular pathways compared with other cell types. For example, ATP generation via the Kreb's cycle and oxidative phosphorylation does not occur in the red cell because it lacks mitochondria. The only biochemical pathways known to be retained are those necessary to preserve its own cellular integrity and shape, and to allow it to efficiently perform its well known functions, namely as a reversible O_2/CO_2 carrier and in the control of blood pH [10]. Whilst on the one hand this makes the red cell worthless for the study of unrepresented intracellular pathways, on the other hand it has facilitated the study of the represented pathways because interference by unwanted pathways is minimal [11]. Moreover, despite the red cells relatively long lifespan in circulation with no new protein synthesis, the only enzymes that appear to decline in activity during aging of the red cell are pyrimidine-5'-nucleotidase and AMP-deaminase [12]. Certainly no other eukaryotic cell is as readily available for study as the red blood cell, making up almost half of the whole blood volume and being easily separated from other circulating cells and plasma in sufficient quantities for most analytical methods. Consequently, today the red cell membrane is the most thoroughly studied, and remains the paradigm for ongoing studies of the membranes from other cell types [13]. Similarly much of today's knowledge on numerous ubiquitous major metabolic pathways has been gained through study of the human red blood cell [11]. However, despite the abundant literature available on such studies, very little further exploration of red cell function has been undertaken more recently, and this

apparently simple and unassuming oxygen transporter may still be quite underestimated.

The first part of glucose metabolism, namely glycolysis (or the Embden Meyerhoff pathway), is a good example of a major ubiquitous metabolic pathway which has been well studied in the red cell as a model for other cell types (although the formation of 2,3-diphosphoglycerate is specific to the red cell) (Fig 1A).

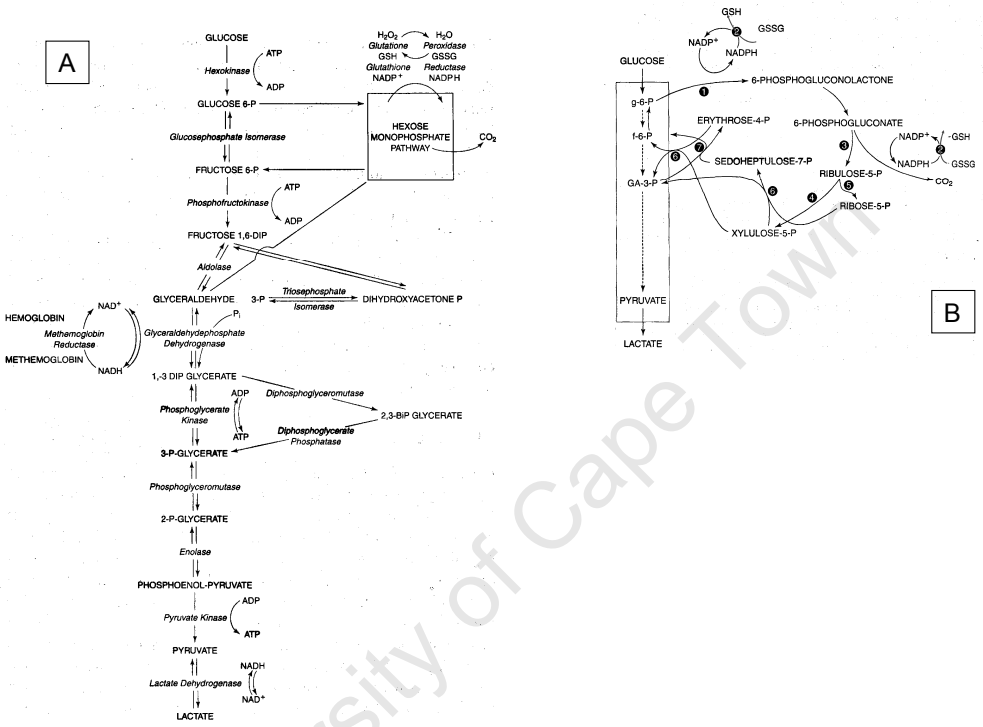


Figure 1. (A) Glucose metabolism, and (B) the hexose monophosphate (HMP) shunt/pathway of the red blood cell in more detail [1].

During glycolysis, each glucose molecule leads to the net formation of two ATP molecules, this being the only source of energy in most red cells. Interestingly, glucose is unable to enter the pig red cell, which uses inosine for ATP production, [14-16]. Glycolysis also recycles the reduced form of nicotinamide adenine dinucleotide (NADH) from its oxidized form (NAD^+). Similarly, NADPH is recycled from $NADP^+$ via the hexose monophosphate (HMP) shunt (Fig 1B), which branches off from glycolysis at glucose-6-phosphate. Both of these purine dinucleotide coenzymes play vital but different roles in redox reactions and maintenance of an active reducing potential within cells, particularly within the red cell.

Much about the purine metabolism (Fig 2) has also been learnt through study of the human red cell [17]; however differences do exist between red cells and nucleated cells, such as:

- No *de novo* purine synthesis occurs in the red cell, which relies instead on the salvage pathways for AMP and GMP synthesis from adenine (and also adenosine) and guanine respectively [1].
- The nucleotidase isozyme present in red cells is uniquely restricted to pyrimidine substrates (pyrimidine nucleotidase), and this presumably preserves AMP levels [18-20].
- The dephosphorylation of IMP to inosine occurs by action of a different red cell nucleotidase isozyme, namely deoxyribonucleotidase (dNase) [21].
- Red cells reportedly have no xanthine oxidase/xanthine dehydrogenase (XO/XD) enzyme activity [22].

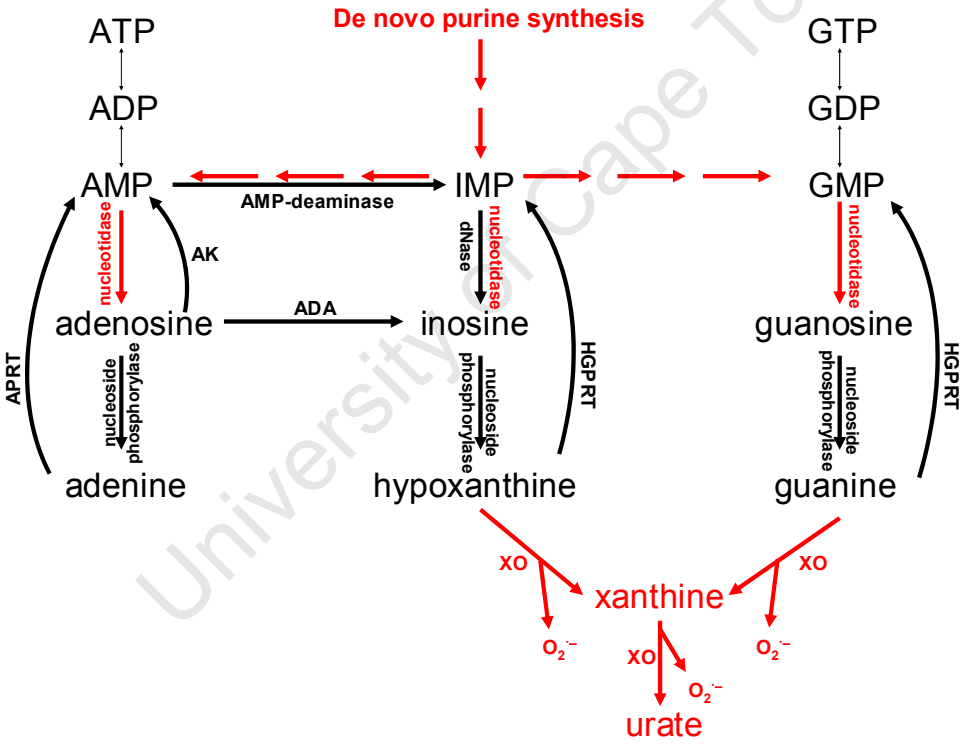


Figure 2. Basic pathway of purine metabolism in mammalian cells. Red annotations indicate steps that are reportedly absent in the red cell. AK=Adenosine kinase; ADA=adenosine deaminase; APRT=adenine phosphoribosyltransferase; dNase=deoxyribonucleotidase; HGPRT=hypoxanthine-guanine phosphoribosyltransferase; XO=xanthine oxidase; $O_2^{\cdot-}$ =superoxide radical.

Evaluation of the highly UV-absorbant nucleotides present in cells has been greatly facilitated by the development and refinement of ion-exchange chromatography (circa 1950), and then high performance/pressure liquid chromatography (HPLC)

(circa 1970) [17]. After precipitation of all proteins via an acid-extraction step from a blood or tissue sample, UV-absorbing nucleotides/sides and their bases, as well as any other UV-absorbant acid-(and water-) soluble low molecular weight (LMW) substances in the neutralised extract can be rapidly and accurately quantified simultaneously, as long as they don't co-elute. Anion-exchange HPLC of human red cell acid extracts reveals that the major component detectable is the highly anionic purine nucleotide ATP, with smaller peaks of AMP, NAD(H), ADP and NADP(H) being easily detectable, although the last two often co-elute (Fig 3A).

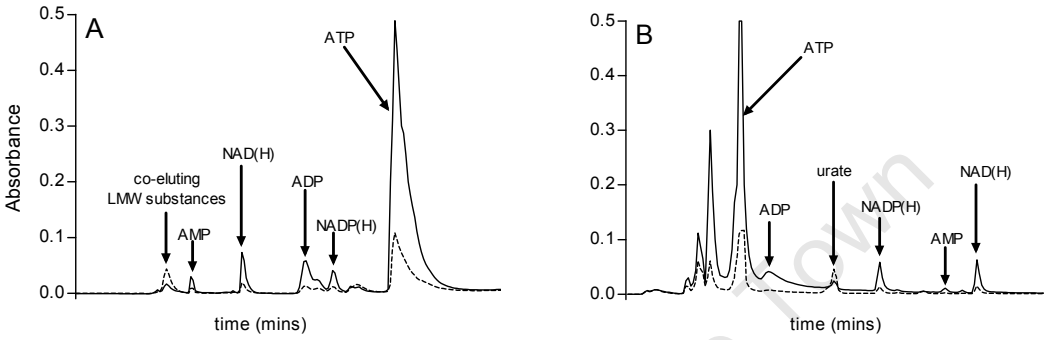


Figure 3. (A) Anion-exchange, and (B) reverse-phase HPLC absorbance profiles, monitored at 260nm (solid line) and 280nm (dashed line), of a neutralised, acid-soluble human red cell extract.

A small amount of the purines GTP and GDP, as well as the pyrimidine nucleotide UDPG, may also be evident with some HPLC systems [23]. Uncharged, positively charged and non-polar substances are not retained on the anion exchange column – their peaks thus co-elute and their identities cannot be determined. However, use of reverse-phase HPLC (Fig 3 B), which separates substances based on their polarity (with the more polar substances eluting earlier), usually allows the determination of the identity of substances not retained on anion-exchange.

The human red cell HPLC profile is well documented [23], but does not reflect the profile of all other mammalian species. Distinct differences in the ion-exchange chromatographic profiles of the red cells from various animal species has long been reported [24, 25], but given surprisingly little attention since first described. Such differences are not unexpected given the fact that significant interspecies differences in purine metabolism and also transport of substances into red cells have been described [26]. The most noticeable difference on chromatography is the large variation in levels of ATP, with human, rat, mouse, pig and rabbit red cells having relatively large amounts of ATP in comparison to the low levels present in the red cells of cow, sheep, and horse [15, 25, 27-29]. The high levels found in humans and pigs may be superfluous - at low ATP levels, the generation and utilization rates

remain the same; it is just the turnover which is faster. Only about 15% of the available ATP is necessary to power the known energy requirements of the Na-K pump [15]. Moreover, in the anucleate mammalian red cell all the substrate utilizing reactions (ion pumps etc) are low Km (micromolar for ATP) reactions; therefore, as long as ATP levels exceed about 5-10μM, the rate of these reactions is not increased by higher levels of ATP. Furthermore, none of the allosteric effects that exist in nucleated cells, and for which millimolar amounts of ATP are required, are operative in the red cell. High levels of ATP may therefore merely reflect levels originally found in red cells when they were nucleated. In fact, it has been proposed that low red cell ATP may be an evolutionary adaptation in some species conferring a selective advantage against common red cell parasites [30].

Besides the striking variability in ATP concentration, another very distinct HPLC profile difference seen is the large amount of urate riboside present in cow red cells. Urate riboside was first observed in the red cells of cattle in 1922 [31], and subsequently shown to be present in buffalo red cells too [32]. It was found to be undetectable in the red cells of all other species evaluated, including humans [25, 32, 33]. Urate riboside is formed through condensation of the purine degradation product, urate, with 5-phosphoribosyl-I-pyrophosphate (PPRP). This reaction is catalysed by the enzyme uridine monophosphate synthase (UMP synthase, also known as orotate phosphoribosyl transferase), to form 3-N-ribosyluric acid 5'-monophosphate (also termed urate ribotide). The phosphate moiety is subsequently removed to form 3-N-ribosyluric acid, commonly termed urate riboside [32, 34]. Bovine red cells have much higher levels of UMP synthase than most species investigated, having about 50 times more than is present in human red cells [35]. This may explain the presence of large amounts of urate riboside in cow red cells, yet apparent absence in human red cells.

In an attempt to elucidate the role for urate riboside in the red cells, Smith and co-workers found urate riboside to have a similar antioxidant capacity as its precursor urate, a well-established antioxidant. They showed that both urate riboside and urate were able to protect human red cells against haemolysis by the reactive species t-butylhydroperoxide [36, 37] and sodium nitrite [38]. In the process, urate riboside (as for urate) is oxidized by these oxidants. These investigators also showed that *in vitro* exposure of bovine red cells to sodium nitrite leads to a decrease in red cell urate riboside level [38]. They therefore hypothesized that the high levels of urate riboside were providing antioxidant protection to the bovine red cells, in a fashion similar to

that proposed by Ames et. al. (1981) for the high plasma levels of urate in humans [39] (see also Results and Discussion, section iv).

In addition to the above-described variations in mammalian red cell profiles, Brown et. al. (1972) also detected large, but variable early-eluting peaks reflecting elevated levels of LMW substance(s) in the red cells of some species (eg. horse and sheep). Because a number of non-ionic LMW substances could have been contributing to these peaks (NAD(H), nucleosides and bases all co-eluted early on their ion-exchange HPLC system), they were unable to determine the identity of the species-specific peaks. Although these variable early-eluting peaks all contributed to the reproducible and distinctive whole blood chromatographic profile evident for each species [25], these interspecies differences were never clarified since no significant further comparative HPLC studies on mammalian red cells were performed subsequently. That is until collaboration between E.H. Harley and D.E. Paglia led to the evaluation of the HPLC nucleotide profile from healthy black rhinoceros red cells.
Emeritus Professor Eric H. Harley, supervisor of this thesis, has published widely in the field of purine/pyrimidine nucleotide metabolism [2, 4, 23, 40-59]. Harley and co-workers (1988) also reported that a characteristic human erythrocyte nucleotide pattern is identifiable in certain inherited purine and pyrimidine enzyme defects where there is clinical evidence of cellular toxicity [23], and which may provide a useful diagnostic tool.

On comparison of human and black rhinoceros red cell anion-exchange HPLC profiles, Harley and co-workers found that the rhinoceros red cell acid extracts show a number of very unusual features [2]. Besides confirming an extremely low red cell ATP level previously noted by Paglia et. al. (1986) (approximately 2-5% relative to humans) [30, 60, 61], Harley and co-workers detected a large amount of uncharged UV-absorbant LMW material, this peak dominating the anion-exchange HPLC profile.


White rhinoceros (photographed by V Davids)

As for Brown et. al., they were initially unable to determine the identity of the uncharged substance(s) from the anion-exchange HPLC profile alone, although the peak had a similar 260:280nm ratio and early elution time to that seen in the human red cell profile (Fig 3A). By using reverse-phase HPLC technology, Harley and co-workers determined that the substance responsible for the large peak in the rhinoceros red cell extracts was not the same as that responsible for the peak in

human red cells, as these were differentially retained on reverse-phase HPLC. The absorption spectrum of the substance present in the rhinoceros red cells could then be visualized on the HPLC's built-in diode array spectrophotometer (whilst the extract eluted), and its array was determined to be identical with that of commercially-available L-tyrosine. Confirmation that the peak in rhinoceros red cell extracts was indeed produced by large amounts of free tyrosine was then achieved by co-elution with a tyrosine standard, by ninhydrin positivity on cation-exchange amino acid analysis, by excitation and emission properties on fluorescence spectrophotometry, and by mass number identity with tyrosine on mass spectrometry [2].

Harley and co-workers (2004) were thus first to report that the red cells from black rhinoceros (n=8) are highly unusual in containing very large amounts of the free amino acid tyrosine, with a mean level of 780 $\pm$ 110μM [2]. In comparison, the reported mean level of tyrosine in human red cells is 59 $\pm$ 9 μM [3] (Fig 4). This finding of such extraordinary free tyrosine levels in red cells from any species (being up to 20 times greater than is present in human red cells) was unprecedented.

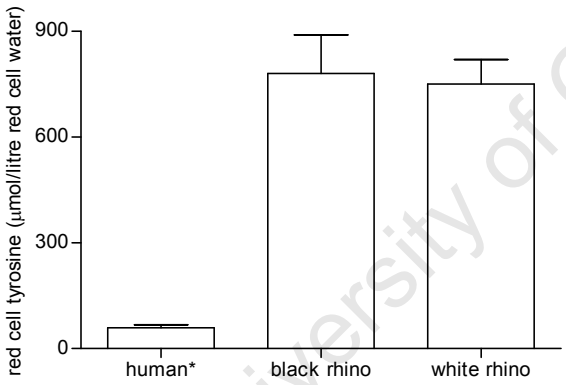


Figure 4. Modified representation of previously published data on rhinoceros red cell tyrosine levels [2]. The human red cell tyrosine level* is as reported by Beutler et. al (2001) [3].

The mean tyrosine level in the red cells from white rhinoceros (n=3) was similar to the level in black rhinoceros, but somewhat lower in Sumatran (n=3), and in one Asian greater one-horned rhinoceros [2]. In contrast with these high red cell levels, plasma levels of tyrosine in all these rhinoceros were low and very similar to human plasma levels of tyrosine (unpublished [62]), as had also previously been reported by Dierenfeld et. al. (1995) [63]. Primary cultures of rhinoceros fibroblasts also showed very low levels of intracellular tyrosine (personal communication [64]). These results taken together suggest that the red cells specifically, from rhinoceros but not from humans, accumulate high levels of free tyrosine.

In preliminary investigations the same investigators found that horses, being of the same mammalian Order as rhinoceros, namely *Perissodactyla*, did not seem to have elevated red cell free tyrosine levels compared with humans. They reported a mean

level of 68.18 ± 4.1μM free tyrosine in the red cells from 6 horses [2], although some unpublished results suggested that higher levels could sometimes be found [5]. Zebra (n=4), also *Perissodactyla*, had levels of 54.57 ± 2.77μM.

The Harley group began to investigate the possible role(s) for such elevated levels of the free form of tyrosine in red cells. They first considered whether this might be providing an alternative energy source in the form of tyrosine phosphate, particularly since rhinoceros red cells have such low levels of ATP – they however dismissed this as a possibility by showing that this red cell tyrosine was not phosphorylated (unpublished [62]). They then began to investigate the possibility that free tyrosine in the rhinoceros red cells was providing antioxidant protection for these cells. Their proposition was supported by the fact that:

(1) human spermatic fluid has even higher levels of free tyrosine, 2000μM [65], and that this tyrosine is hypothesized to play a moderating antioxidant role due to its relatively slow but continuous ability to quench oxygen radicals [66, 67].

(2) the accumulation of the β-amino acid taurine in various cell types has been suggested by some to be providing crucial antioxidant protection. Taurine is present at very high concentrations in neutrophils (10-50mM) [68, 69] and heart epithelial cells (6-35mM) [70], where it may protect against the respiratory burst oxidants released by the neutrophils during acute inflammation and protect the epithelial cells against the reactive species generated during reperfusion-associated phenomena in the heart [71] respectively. Notably though, whilst taurine has been shown by some to have antioxidant activity [72, 73], others have found that hypotaurine, although present at lower concentrations than taurine in leukocytes, is a much better antioxidant [74] and may be providing more effective antioxidant protection than the taurine in these cells [8].

(3) the *in vitro* antioxidant activity of the phenolic amino acid tyrosine, like the well-known polyphenolic antioxidant substances such as resveratrol [75-77] has been reported more recently (see also Chapter 2). Harley et. al. (2004) also reported that tyrosine seems to have similar *in vitro* antioxidant activity to that of the well known physiological antioxidant urate, and that it is oxidized by hydrogen peroxide (H_2O_2) in the presence of haemoglobin (Hb). They proposed that the unusual accumulation of large amounts of free tyrosine in the red cells of rhinoceros, analogous to the high levels of urate riboside found exclusively in red cells from cows and buffalo (see

above), may have evolved as a species-specific red cell antioxidant defense strategy [4].

It is possible that, as for rhinoceros red cells, some of the 'as yet unidentified' large early-eluting peaks previously noticed in the red cells of other mammalian species on ion-exchange HPLC may also be produced by an elevated level of tyrosine, or else reflect evolutionary adaptations in other biochemical pathways that are likely to be specific and critical to red cell function for that species.

(B) AIMS

To produce and compare anion-exchange and reverse-phase HPLC profiles of the acid-soluble extracts from the red cells (and plasma) of various mammalian species in order to further clarify baseline data on UV-absorbant LMW substances within these red cells, with a particular interest in urate riboside, tyrosine, and any other LMW substance(s) found to be elevated in the red cells from any of the species compared with humans.

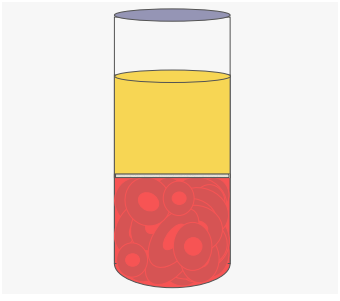
(C) MATERIALS & METHODS

(i) Samples

Blood samples were obtained from human donors and various animals (in collaboration with veterinarians, either opportunistically or from the local abattoir). Approval from the Human and Animal Research Ethics Committees (Health Sciences Faculty of the University of Cape Town) was obtained prior to commencement of the study, and written, informed consent was obtained from every human volunteer prior to venepuncture. All blood donors were healthy, and blood samples were obtained whilst at rest and non-fasting - thus data obtained from these blood samples were presumed to reflect baseline/normal levels for each species.

Blood was collected into sterile, plastic, lithium-heparin vacutainers (17 IU spray-coated LH/ml blood, Beckton Dickinson, UK). Heparin is recommended as anticoagulant because of the high UV absorbance of EDTA [78]. For all human donors, needle bore size was not smaller than 21 gauge and not larger than 23 gauge in order to minimise haemolysis [79, 80], particularly since plasma levels of LMW substances were also to be determined. Blood-filled vacutainers were immediately inverted 8-10 times, as prescribed, to ensure complete anticoagulation. The blood samples were processed as soon as possible within 2 hours of phlebotomy, except where this was not possible for some wild animal samples (and as indicated in the results section). Until processing was begun, the whole blood samples were maintained at ambient temperature - such sample handling has been shown to improve the stability of red cell nucleotides [78, 81, 82] and minimise *in vitro* haemolysis [80] when compared with storage of whole blood at either 4°C or 37°C.

(ii) Preparation and acid extraction of red cells and plasma



The method that was used is a modification of the basic protocol previously described by Bartlett et. al. (1986) [17]. Whole blood samples were centrifuged at 4000rpm (1350g) for 10 minutes at 22°C (if the extent of red cell lysis was thought to be more than minimal, by visual inspection of the plasma colour, the sample was discarded). An amount of 200μl of plasma was transferred to a 5ml tube already containing 300μl of normal (physiological) saline (N/S) at ambient temperature. The residual plasma, entire buffy coat and very top layer of the red cells in the vacutainer were carefully removed with a plastic pipette. The remaining packed red cells were then washed once[(a)] (see appendix) by adding an excess of N/S to the packed red cells, inverting the closed

vacutainer 5-10 times until all the red cells were well resuspended, and centrifuging this suspension at 4000rpm for 10 minutes at 22°C. The N/S plus very top layer of red cells were then removed using a plastic pipette. The remaining washed, packed red cells were well mixed (by 'finger-flicking' the bottom of the blood tube) so as to prevent selective sampling based on size (and hence maturity) of red cells. A volume of 200µl of packed red cells was then transferred to a 5ml tube already containing 300µl N/S[(b)] - a blue disposable pipette tip which had its very tip cut off at an angle was used, this facilitating transfer of an accurate amount of the highly viscous packed cells.

For most of the earlier samples in this thesis, two milliliters of ice-cold 0.9M perchloric acid (PCA, Merck, USA) were then added drop-wise to the 500µl red cell and plasma suspensions, whilst vortexing constantly on high. This gave a final PCA concentration of 0.72M, Bartlett having recommended at least 0.6M PCA [17]. A thick brown protein precipitate is formed as the pH decreases. The acidified samples were immediately centrifuged at 4000rpm for 10 minutes at 22°C to sediment the protein precipitate, leaving behind a volume of 2300µl of acid-soluble extract. Two milliliters of this protein-free extract were transferred to a fresh 5ml tube, and neutralized by the slow addition[(c)] of between 150µl-200µl of freshly made 5M K_2CO_3 until the extract pH was approximately 7, as determined using Whatman's pH paper. However, towards the end of this thesis, use of a novel, alternative neutralizing method was developed[(d)].

The resultant $KClO_4$ is extremely water insoluble, and this precipitate is easily removed by centrifugation, leaving behind a volume of 2100µl of supernatant. As per the recommendation of Bartlett, the neutralized extract was left on ice for 1-2 hours in order to ensure complete crystallization of the $KClO_4$. The extracts were stored at -80°C[(e)] prior to HPLC analysis.

The resultant extracts were a 17-fold or a 12-fold dilution of red cell water and plasma respectively:

$$\text{Red cell concentration (µmol/litre red cell water)} = \text{extract concentration (µM)} \times \left[\frac{2100 \times 2300}{2000 \times 146^*}\right]$$

(*0.73 X 200µl = 146µl)

$$\text{Plasma concentration (µmol/litre plasma)} = \text{extract concentration (µM)} \times \left[\frac{2100 \times 2300}{2000 \times 200}\right]$$

These factors take into account both the dilution during extraction, as well as that red cells contain 0.73kg water/litre (with the rest being made up by the protein mass [83]), whereas protein content is negligible in plasma.

(iii) Acid extraction of the buffy coat

The buffy layer, isolated as above from approximately 100ml cow blood, was washed with N/S and then centrifuged at 2500rpm for 10minutes at 22^{o}C. As much of the white cell pellet was then transferred to a fresh tube, leaving behind most of the underlying residual red cells. This wash step was repeated 4-5 times, until no red cells were visible in the white cell pellet, which was then extracted as above for red cells. The extract was stored at -80^{o}C.

(iv) Culture and acid extraction of fibroblasts

A small section of skin obtained from a slaughtered cow's ear was scrubbed in hibitane and the hair scraped off with a scalpel. The skin was then immersed in 70% ethanol for a few seconds. The clean tissue was dissected into sections of < 2mm in a drop of culture medium (DMEM plus 10% fetal calf serum, both from Invitrogen, NZ). The dissected pieces were transferred, with some medium, to a few 25X10mm Petri dishes (Corning, USA) and coverslips placed over the tissue. The dishes were filled with 2.5ml medium and placed in a 37^{o}C, 10% CO_2 incubator, to allow dermal fibroblasts to grow out from the explants. Confluent cultures were transferred to larger culture dishes. When the fibroblasts were optimally confluent, the medium was discarded and the adherent cells washed gently with N/S (repeated three times). Then 0.9M PCA was added directly to the adherent cells so as to form a thin film over the cells, and the dishes were left on ice for 5 min. The PCA from the first dish was transferred to the next dish in order to minimise the dilution factor of the extracts. Notably, the PCA causes the adherent cells to release their contents without lifting the cells, so microscopic evaluation of the dishes after acid extraction is deceiving because the cells still look intact. The resultant acid-soluble extract was neutralized with K_2CO_3, the $KClO_4$ precipitate allowed to form completely and then removed by centrifugation as above for the red cells. The extract was stored at -80^{o}C.

(v) HPLC analyses

Each extract was thawed and filtered through a 0.45μm syringe filter just prior to HPLC injection. The HPLC system consisted of a Beckman System Gold, model 126 programmable solvent module connected to a 168-detector, providing a spectrophotometric diode array profile (200-350nm) of each peak as the extract eluted. UV absorbance was monitored at 260nm and 280nm. For anion-exchange

chromatography, extracts were fractionated on a 250X4.6mm Hypersil APS-2 5μm column (Thermo Electron Corporation), while reverse phase chromatography was performed using a 250X4.6mm Hypersil ODS 5μm column (Thermo Electron Corporation). Buffers were made with deionised water, and were filtered/degassed. Phosphate buffers older than 48hours were discarded. HPLC gradient conditions were as follows:

For anion-exchange chromatography, buffers were 5mM KH_2PO_4 (pH 2.8) and 0.5M KH_2PO_4/1M KCl (pH 3.5). Initial conditions were 99% buffer A and 1% buffer B. After 1min these conditions changed over 15min to 1% buffer A and 99% buffer B. Conditions were maintained as such for 1min, and then returned to the initial conditions over 5min. These baseline conditions were then maintained for another 18min, by which time no more UV-absorbance (from specific substances or buffer mixing) was detectable on the diode array screen (200-350nm). Total running time was 40min.

For reverse-phase chromatography, buffers were 10mM KH_2PO_4 (pH 5.6) and 50% HPLC-grade methanol (Sigma, USA). Running conditions were identical to those for anion-exchange, except that the final conditions were maintained for only 8min, by which time no more UV-absorbance was detectable on the diode array screen. Total running time was therefore 30min.

(vi) *Determination of the identity of substances responsible for each peak*

A reverse phase (Fig 5A) and anion-exchange profile (Fig 5B) was produced of a 'standards mix' of 12 commercially-available LMW substances (in phosphate buffer, pH 7.4). The UV-absorbing substances chosen to make up the mix were those known to be present (or inducible) within red cells. This mix was used as a rapid reference guide for the initial determination of the identity of substances in the acid extracts that appeared as pure* peaks on HPLC.

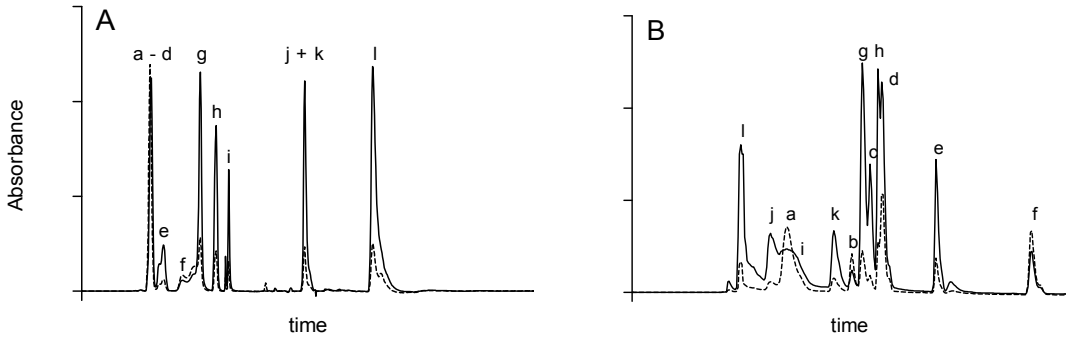


Figure 5. (A) Anion-exchange, and (B) reverse-phase HPLC profiles of the standards mix. The substances in the mix are as follows: a-uric acid, b-tyrosine, c-hypoxanthine, d-xanthine, e-inosine, f-tryptophan, g-AMP, h-NAD^+, i-IMP, j-ADP, k-$NADP^+$ and l-ATP. The substances are not at equimolar concentrations.

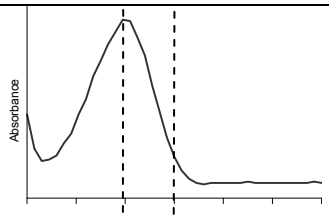
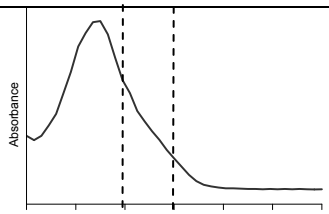
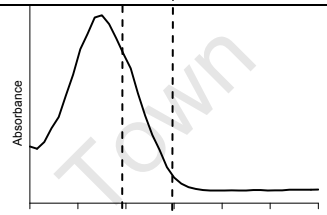
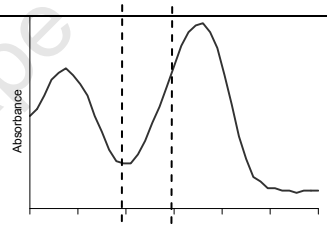
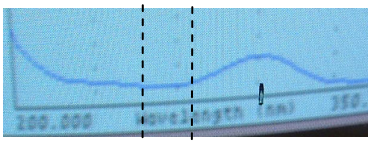
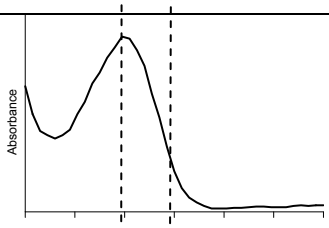
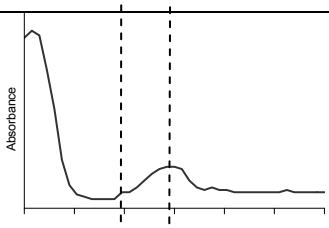
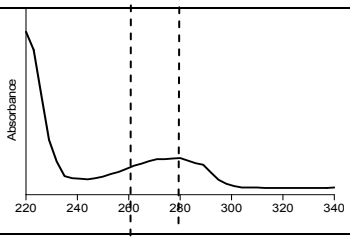
*The peak purity index, information which is provided by the HPLC Beckman System Gold for every profile, but not shown in the figures above, indicates if more than 1 substance is responsible for every peak. Therefore, the first-eluting peak in (A) would have been shown to represent multiple co-eluting substances. On the other hand, peaks h, i and l in (A) would have shown peak purity i.e. each representing a single eluting substance.

An HPLC profile for each of the standards substances making up the mix (in phosphate buffer, pH 7.4) was first produced in order to determine its reverse-phase and anion-exchange HPLC elution positions, its 260:280nm absorbance ratio, and to visualise its absorbance spectrum on the HPLC's built-in diode array spectrophotometer. Table 1 shows the 260:280nm ratios and absorbance spectra of some of these LMW substances (in phosphate buffer, pH 7.4).

Note that absorbance spectra for some substances differ with varying pH; for example, whereas the 260:280nm ratio for xanthine in buffer pH 7.4 = 0.87, on reverse phase HPLC (where the exact pH would be < 7) the 260:280nm ratio is = 1.6. An HPLC profile of the standards mix was also produced regularly as a control for HPLC column retention.

Confirmation of the identity of substances in the extracts was obtained when a solution of the commercially-available LMW substance suspected to be responsible for the peak was spiked into the extract, and this co-eluted with the unknown peak whilst maintaining purity of the peak.

Table 1. Absorbance ratios (260nm:280nm) and spectrophotometric diode array patterns of some LMW substances of interest, dissolved in phosphate buffer (pH 7.4). The dotted lines indicate 260nm and 280nm on the x-axes.

LMW substance	260:280nm ratio	Diode array patterns
ATP, ADP, AMP	5.8	
IMP, inosine	4.0	
hypoxanthine	10.0	
urate	0.3	
urate riboside	0.3	Urate riboside in cow red cell extract, as seen on diode array of HPLC 
NAD^+, $NADP^+$	4.1	
tyrosine	0.5	
tryptophan	0.7	

(vii) Determination of concentration in red cells and plasma

The concentration of a LMW substance in an extract (μM) was determined from the HPLC profile by multiplying its peak area with a specific constant unique for each of the commercially-available substances. Peak areas for each HPLC profile were retrieved from the System Gold (1992) package (Beckman Coulter, USA). The constant for each substance to be evaluated was derived off a pre-determined standard curve (Fig 6), as the ratio between concentration (μM) and peak area. For example, the constant for tyrosine was 7.6 (see standard curve below), and that for urate was 1. The correct concentration of the highest standard solution from which the serial dilutions were then made for each standard curve was confirmed spectrophotometrically.

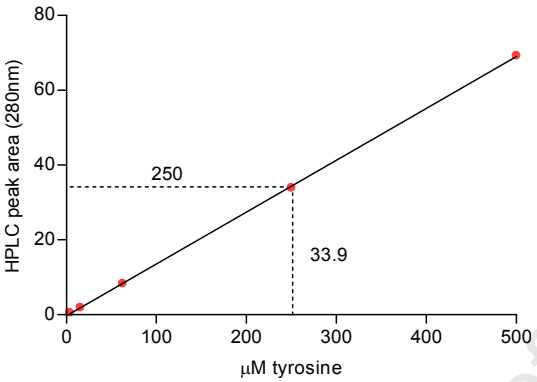


Figure 6. L-tyrosine standard curve to determine tyrosine concentration in red cell and plasma extracts evaluated on reverse-phase HPLC.

The intra-erythrocytic concentration of each LMW substance (μmol/litre red cell water) was then determined by multiplying the neutralised red cell extract concentration for that substance by a factor of 17 (see section ii above). For plasma, this factor was 12 (see section ii above). The concentrations of LMW substances present in human red cells as determined in this study were always comparable with previously reported data for human red cells.

If a small degree of lysis of a blood sample was noticed, the validity of the determined plasma levels of LMW substances was always confirmed by ensuring that insignificant amounts of the dominant red cell peaks were present in the plasma extract.

Since commercially-available urate riboside preparations are not available, red cell concentrations of this LMW substance had to be determined differently. Smith et. al. (1981) previously described how to determine urate riboside concentrations spectrophotometrically [84]. Accordingly, the absorbance at 298nm of K_2CO_3-neutralised cow red cell extracts was measured, and urate riboside concentration in these extracts calculated using a molar absorbance of 16.4 X 10^3 (absorbance X

10^6/16 400 = μM concentration). The red cell concentration was then determined by multiplying the extract concentration by 17, as for other red cell substances (see above). Although most of the cow red cell extracts evaluated earlier on in the current study were only evaluated on HPLC, and not with spectrophotometry, a standard curve of urate riboside HPLC peak area versus spectrophotometric absorbance (at 298nm) (r^2=0.9975) was produced using extracts for which both measurements were available (n=10). This standard curve gave a ratio (constant) of 54 for HPLC pea k area:absorbance (298nm), which was then used to extrapolate absorbance measurements from HPLC peak areas, were necessary.

(viii) In vitro exposure of horse red cells to H_2O_2 or AAPH to determine haemolysis

A suspension of red cells from different horses was each incubated with 50mM H_2O_2 or 20mM AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride)(Sigma-Aldrich, USA) in 10mM phosphate buffered saline, at 37°C. AAPH decomposes spontaneously in solution at 37°C to release a constant flux of peroxyl free radicals. The reaction tubes were incubated with continuous, gentle rotation, on a mini labroller rotator (Labnet International, USA), in order to maintain the red cells in suspension. The supernatants were evaluated at different time points for haemolysis by diode array spectrophotometry. No inhibitors of red cell antioxidant enzymes were added. The diode array profile of a triton-X treated sample (no oxidant) of the red cell suspension from each horse was also evaluated, as control for Hb content.

(ix) Statistical considerations

Unless otherwise indicated, data are presented as mean ± SD. When comparing means, the Unpaired t test was used to determine statistical significance, unless the data were skewed, in which case the Mann Whitney test was used. The Paired t test was used when comparing plasma and red cell levels within the same species, except where the data were skewed, in which case the Wilcoxon signed rank test was used. The Pearson test was used to determine statistical significance of correlation, unless the data were skewed in which case the Spearman r was determined. The Goodness of Fit test was used to determine linear regression. A *P*-value < 0.05 was considered statistically significant. Five repeat extractions and HPLC analysis of red cell NAD^+ peak area showed a coefficient of variation (CV) of 1.78%.

(D) RESULTS AND DISCUSSION

(i) Comparative HPLC profiling

In figure 7, typical anion-exchange (left side) and reverse-phase (right side) HPLC profiles of red cell extracts from human (A,B), rhinoceros (C,D) and cow (E,F) are depicted.

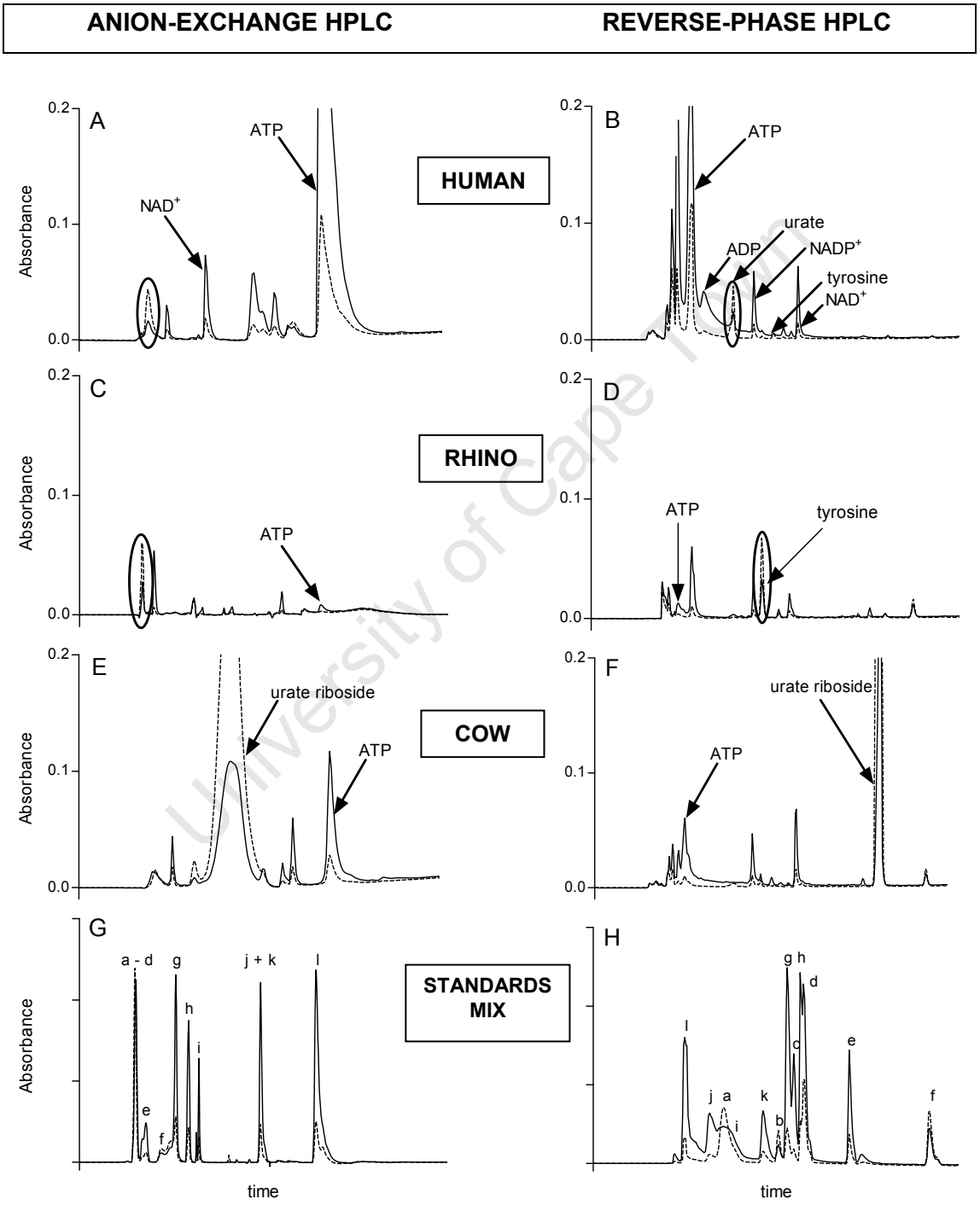


Figure 7. Anion-exchange and reverse-phase HPLC absorbance profiles at 260nm (solid line) and 280nm (dashed line) of neutralised, acid-soluble red cell extracts from a human (A,B), a white rhinoceros (C,D), a cow (E,F), and a standards mix (G,H). The standards in the mix are a-uric acid, b-tyrosine, c-hypoxanthine, d-xanthine, e-inosine, f-tryptophan, g-AMP, h-NAD^+, i-IMP, j-ADP, k-$NADP^+$ and l-ATP.

By comparison with Brown et. al. (1972) who produced comparative (ion-exchange) HPLC profiles for extracts from whole blood extracts [25], the use of red cell extracts eliminates the influence of variable haematocrit and allows determination of red cell levels of LMW substances that are present in both red cells and plasma.

One of the more notable red cell profile differences evident in figure 7 is that the ATP level in healthy rhinoceros red cells is strikingly low – this was also shown in previous investigations by Paglia et. al. (1986) [60]. In addition, as shown by the reverse-phase profile of figure 7D and as previously reported by the Harley group [2], rhinoceros red cells have a markedly greater amount of free tyrosine when compared with human red cells (Fig 7B). It is clear that use of anion-exchange chromatography alone would have made it very difficult to elucidate the identity of the substance(s) accounting for the large amount of early-eluting UV-absorbing material in the rhinoceros red cell extract (Fig 7C, oval), which appears similar to the peak seen in the human anion-exchange profile (Fig 7A, oval). Complimentary use of reverse-phase HPLC, however, revealed that whereas in humans the main component of this peak is urate (Fig 7B, oval), in rhinoceros red cells it is tyrosine (Fig 7D, oval).

Comparative HPLC, as opposed to targeted analysis, is therefore useful to screen for unsuspected differences in the range and level of UV-absorbing LMW substances present in the red cells from different species. Limitations of the protocol should however be noted: The use of acid-extraction to remove proteins including the membrane lipoproteins will remove lipophilic LMW substances such as Vitamin E. Ascorbate is unstable in aqueous solutions [85], and is likely to be degraded in the freeze-thawed acid-extracts. Any LMW substance that is not UV-absorbing, for example glutathione (GSH), will go undetected.

The very large and dominant UV-absorbing peak seen in cow red cell profiles (Fig 7E,F), and which was undetectable in human and rhinoceros red cell profiles, displayed HPLC characteristics consistent with previously described urate riboside [84]. Since the discovery of urate riboside in 1922, fewer than 25 publications have appeared on this substance (pubmed search), with R.C. Smith and co-workers being the authors of all the more recent ones. Further investigations of this potent LMW endogenous red cell antioxidant were undertaken.

(ii) Urate riboside: inter- and intraspecies variation

It is known that large, yet variable amounts of the antioxidant urate riboside are present in the red cells from cow and buffalo (Order *Artiodactyla*, Family *Bovidae*, Subfamily *Bovinae*, and Tribe *Bovini*) [86, 87]. It is absent in the red cells from all other species evaluated, including the only other Artiodactyls investigated to date, namely pigs (Family *Suidae*) and even sheep (Family *Bovidae*, Subfamily *Caprinae*) which belong to the same Family as cow/buffalo [32]. The exact phylogenetic position of the evolutionary mutation for urate riboside formation has however never been determined. In order to address this, red cells from cow, buffalo, sheep, as well as 3 further *Artiodactyls* not investigated before, namely one grysbok (Family *Bovidae*, Subfamily *Caprinae*), one bontebok (Family *Bovidae*, Subfamily *Caprinae*), and two kudu (Family *Bovidae*, Subfamily *Bovinae*, and Tribe *Strepsicerotini*) were evaluated. Because processing of these wild animal blood samples was delayed by 48 hours post phlebotomy, it was first confirmed that urate riboside levels remain unchanged in a blood sample, whether processed within 2 hours or kept at room temperature for 48 hours prior to processing.

HPLC profiles revealed that urate riboside was absent in the grysbok and bontebok red cells, but present in both kudu. As expected, urate riboside was present in the red cells from all cows and buffalo sampled, but undetectable in sheep. Hence urate riboside is present in the red cells of both major Tribes of the *Bovinae* Subfamily (namely *Bovini* and *Strepsicerotini*), but not in any of the other *Bovid* Subfamilies evaluated. This almost certainly proves that the evolutionary mutation for urate riboside formation within red cells is specific to the *Bovinae* Subfamily, and is thus predicted to have occurred in the position indicated by the arrow in figure 8.

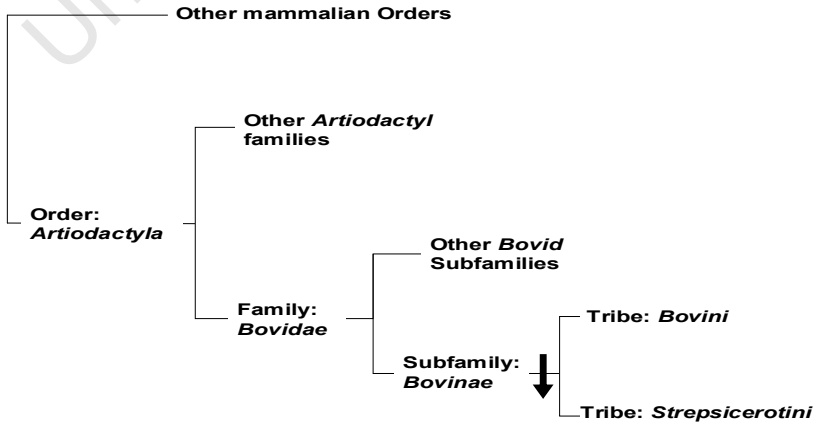


Figure 8. Phylogeny of the *Artiodactyla*. The arrow indicates where the mutation responsible for red cell urate riboside production is predicted to have occurred.

As previously reported [33], urate riboside was undetectable in the plasma from cows. Since urate riboside is resistant to the action of uricase [84], the liver enzyme specific for urate degradation, its absence in plasma implies that once formed (presumably inside the red cell) that it is trapped within the cell, possibly by virtue of the large ribose moiety.

Levels of urate riboside present in the red cells of the 3 different *Bovinae* were then determined. The red cells from the two kudu had levels of 530μM and 237μM. The mean level of urate riboside in cows (n=24) was 2560 ± 735μM, and in Cape buffalo (*Syncerus caffer*) (n=6) it was 665 ± 245μM (Fig 9A). Levels in cows were significantly higher than levels in buffalo ($P < 0.0001$).

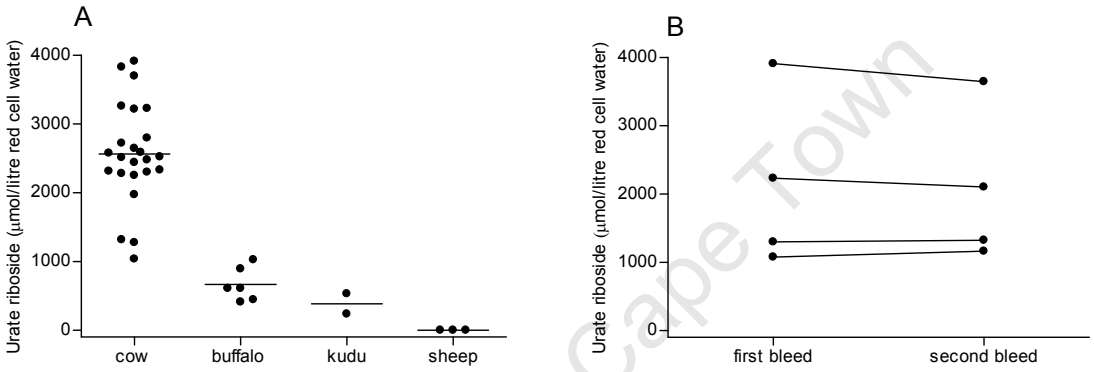


Figure 9. (A) Scatter plots of red cell urate riboside concentration in cow, buffalo, kudu and sheep. The Unpaired t test was used to determine *P* values. (B) Red cell urate riboside concentration in four cows that were bled once, and then again 30 days later

A study by Smith et. al. (1981) comparing urate riboside concentrations in cows and buffalo reported a much lower mean urate riboside level in both cows and buffalo, with a greater concentration in the red cells from buffalo than cow: 700μM ± 360μM versus 920μM ± 210μM in cow (n=115) and buffalo (n=10) respectively [86]. In the only other study to report urate riboside levels in cows (n=50), Smith et. al. (1985) found a mean level of 1210μM ± 190μM [87].

Urate riboside levels are known to vary widely. In this study, levels in cows ranged between 1000μM and 4000μM i.e. a 4-fold difference within the same species. In order to determine if the highly variable red cell urate riboside concentrations fluctuate within an individual, or are stable and thus specific to each cow, two blood samples from the same four cows were obtained with an interval of 30 days between the two bleeds. The cows were all healthy, all came from the same herd and the environment of the cows remained essentially unchanged within this interval.

Figure 9B shows that urate riboside level varied very little in each cow over 30 days – this finding, together with the report by Smith et. al. (1985) of a positive correlation for urate riboside concentration in the red cells of calves with that of their dams, is consistent with the likelihood that such normal/baseline variability in urate riboside level is genetically determined. One possibility to account for such normal variability is that there is intraspecies heterogeneity in UMP synthase enzyme level. In support of this, it has been shown that urate riboside levels in cows with inherited deficiency of UMP synthase fall within the lower range of normal levels [87]. Smith et. al. (1981) offered another explanation, suggesting that such variation may result from variable permeability of red cells to urate riboside [84]. However, this possibility is unlikely considering that no urate riboside is detectable in plasma, as it should be if leaking out of the red cells. Extrinsic factors (physiological/non-pathological) could theoretically also influence baseline/normal urate riboside levels. For example, variable levels of physiological oxidant production could theoretically lead to variable levels of urate riboside oxidation (see also Chapter 4).

Interestingly, although it is known that the mature red cell of the cow has active UMP synthase as well as the substrate PPRP [88], urate (the other substrate) is actually undetectable by HPLC in cow/buffalo red cells, and plasma urate level in cows is negligible (see later, section iv). It is probable that any small amount of urate entering the red cell from these low levels present in plasma is immediately incorporated into urate riboside. But is this enough to account for the high levels of this LMW substance trapped within the red cells? It is possible that urate riboside production in the red cell occurs only under certain conditions of elevated urate substrate levels. The enzyme xanthine oxidase/xanthine dehydrogenase (XO/XD), which produces urate (see also section iv), is present at very high levels in cow milk [89]. Lactation may lead to an increased level of plasma urate, which may then provide the red cells with sufficient substrate to form urate riboside. However the presence of urate riboside in bulls as well as cows [84] would make this possibility less likely.

As previously mentioned, bovine red cells have a much higher level of UMP synthase than the red cells from other species [35]. But there is no record of whether other bovine cell types also have such elevated levels of this enzyme, and the possibility that urate riboside is also made by other cell types in the cow has to date not been reported on. This was investigated, and figure 10 shows that, unlike cow red cell acid extracts (Fig 10A), extracts from the buffy coat (white blood cells and platelets) (Fig 10B), and cultured primary fibroblasts (pinnae dermis) (Fig 10C) of a cow had no detectable urate riboside.

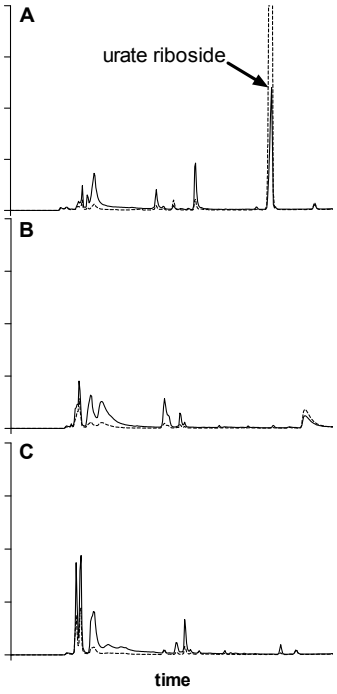


Figure 10. Reverse-phase HPLC absorbance profiles at 260nm (solid line) and 280nm (dashed line) of a neutralized, acid-soluble extract of (A) washed red blood cells, (B) buffy coat, and (C) cultured primary dermal fibroblasts from a cow.

This suggests that the mutation leading to the formation of urate riboside finds expression only in the red cells of these animals, and raises a number of questions. For example, is it made in most cells, but rapidly degraded in cells other than red cells?

(iii) Free tyrosine: inter- and intraspecies variation

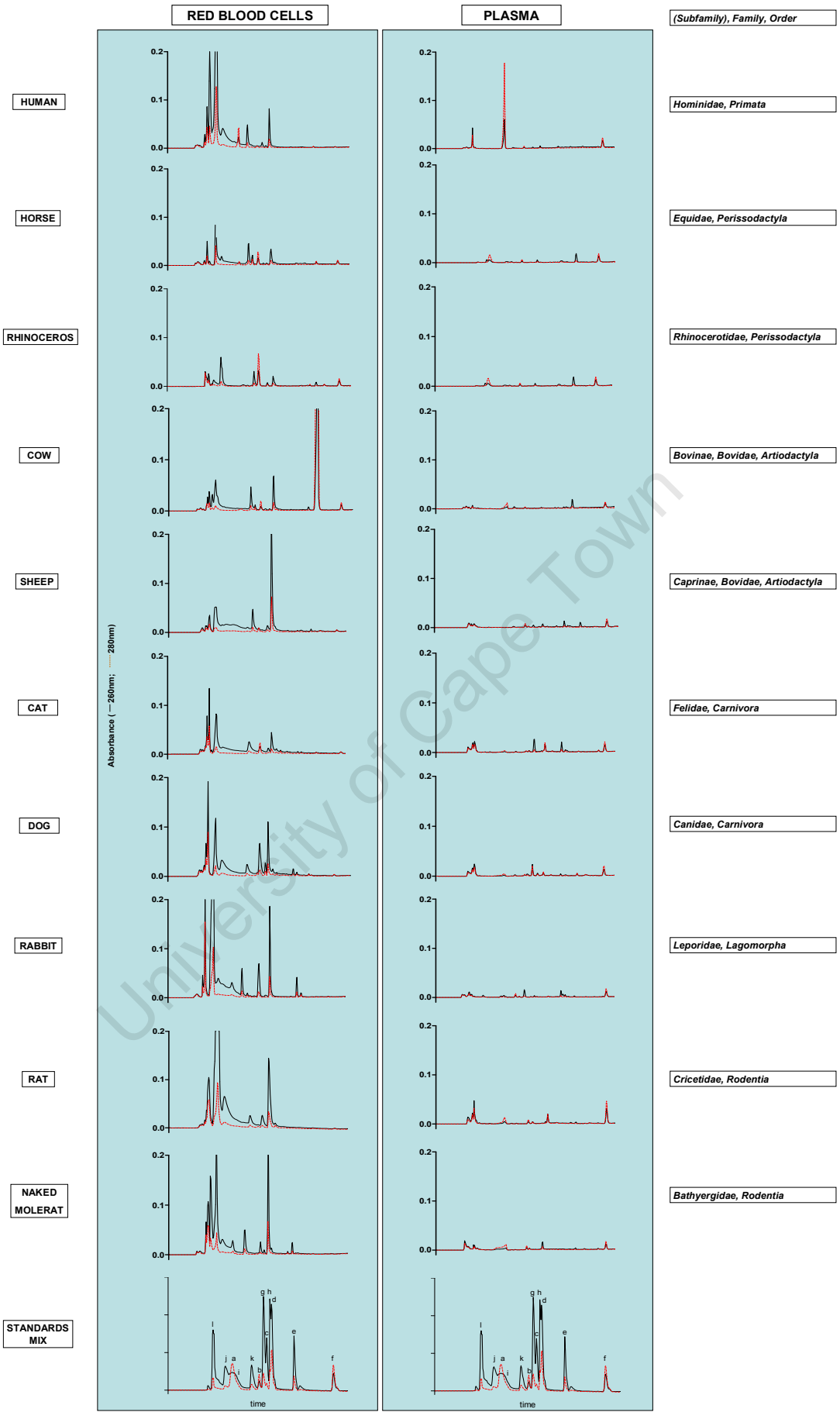
Harley and co-workers (2004) previously reported that rhinoceros red cells are unusual in having very high levels of free tyrosine. They suggested that this ability to accumulate such large amounts of free tyrosine may have evolved in rhinoceros to provide antioxidant protection to their red cells [2, 4] in the same way that the accumulation of free tyrosine in human seminal plasma has been suggested to have arisen in order to provide antioxidant protection to the spermatozoa. Although *in vitro* assays show that tyrosine does have antioxidant activity (see Chapter 2), the possibility that elevated levels of free tyrosine (exogenous source, or as a naturally occurring phenomenon in some tissues) may provide antioxidant protection *in vivo* has been virtually ignored.

Such an evolutionary adaptation may not be limited to rhinoceros if indeed it plays an important baseline physiological role. In order to determine if other mammalian

species also have elevated levels of red cell tyrosine, levels in the red cells (and plasma) from 20 horses (*Equidae*), being the closest evolutionary relative to the *Rhinocerotidae* (both being members of the Order *Perissodactyla*), and from at least 2 individuals of various other mammalian species were compared with levels in 25 humans. A blood sample from 1 white rhinoceros was also obtained for comparative purposes (this blood sample was processed 48 hours post-phlebotomy).

Values were determined from reverse-phase HPLC profiles (an example HPLC profile for each species that was evaluated is shown in figure 11 below). These profiles were later also used to evaluate other differences in red cell LMW substances between species. The paucity of, and lack of much variability between UV-absorbant LMW substances in the HPLC profiles of plasma from the different species (other than for humans) was in stark contrast with what was seen in the red cell profiles.

Figure 11 (see below). Reverse phase HPLC profiles (260nm black solid line and 280nm red dotted line) of a standards mix (bottom) and the red blood cell and plasma acid extracts from animal species belonging to 6 different mammalian Orders. The HPLC profiles for buffalo red cells and plasma are not shown, but other than for a smaller urate riboside peak they were essentially identical with that from cows. The standards in the mix are a-uric acid, b-tyrosine, c-hypoxanthine, d-xanthine, e-inosine, f-tryptophan, g-AMP, h-NAD^+, i-IMP, j-ADP, k-$NADP^+$ and l-ATP.



The mean tyrosine level in human red blood cells was determined to be 67.5 ± 23.8μM (Fig 12A), which is in accordance with previously published levels [3, 83, 90, 91]. By comparison, the mean red cell tyrosine level in horses were 279.0 ± 199.3μM. Levels in horses were significantly higher than in humans ($P < 0.0001$), and also significantly higher than previously reported for 6 horses by this group [2]. One possible reason for this discrepancy is that the red cells in this earlier cohort of horses were washed three times in N/S prior to extraction, a procedure which has been found to wash out significant amounts of tyrosine from red cells [83] (see also Appendix, section (a)). Confirmation that this peak in horse red cells was indeed due to tyrosine was achieved by visualization of its absorption profile on diode array scan, which was identical for that of a commercial tyrosine preparation, by its co-elution with the peak produced from a solution of tyrosine spiked into a horse red cell extract (and purity of the peak was maintained), and by disappearance of the peak in question on exposure of a horse red cell extract to the tyrosine-specific enzyme tyrosinase (Sigma-Aldrich, USA)(0.1IU per ml of extract for 1hour at 37°C), with no other profile changes.

Although red cell tyrosine levels in horses were significantly lower ($P = 0.0004$) than that previously reported for black rhinoceros red cells (778.8 ± 40.0μM) [2], and for one white rhinoceros sampled for the current study (1010μM), the range in horses was very large (137.4μM - 876.1μM), and the data skewed to the right. Thus a few horses had levels as high as that seen in rhinoceros, and the median [and interquartile range] for horses was 194.7 μM [168.8 μM – 300.7μM].

The finding of elevated levels of red cell tyrosine in members from both major Families of the *Perissodactyla* Order (*Rhinocerotidae* and *Equidae*) relative to humans was initially suggestive that this was a feature peculiar to this Order of Mammalia. Unexpectedly however, cats, which are of a completely different Order (*Carnivora*) to rhinoceros and horses, were also found to have high red cell tyrosine concentrations, with a mean level (365.1 ± 55.8μM) even higher than for horses. Dogs (also *Carnivora*) and sheep (*Artiodactyla*) also had significantly higher red cell tyrosine levels compared with humans, but significantly lower than in cats and horses. Humans, however, were not alone in having relatively low levels of red cell tyrosine - rats (*Rodentia*) had levels similar to humans, while naked molerats (also *Rodentia*) had no HPLC-detectable tyrosine in their red cells. Levels of tyrosine in the red cells of cow and buffalo eluted very closely with another small peak, this most likely being urate ribotide, the precursor to urate riboside (see Literature Review) because of their identical diode array scans. Tyrosine in cow and buffalo red cell

extracts could therefore not be accurately determined using HPLC, although it was apparent that levels were similar, if not less, than in human red cells.

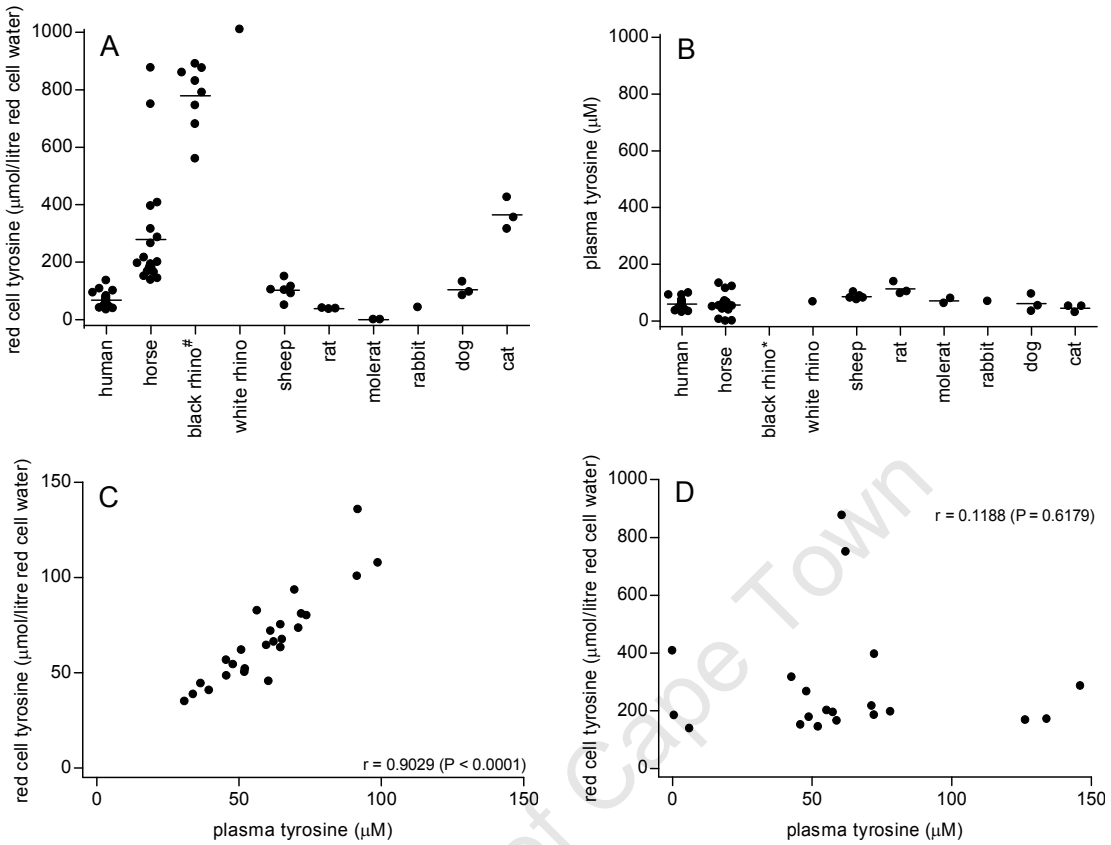


Figure 12. Tyrosine concentrations in the (A) red cells, and (B) respective plasmas from various mammalian species, including humans. Comparison of the tyrosine concentration within red cells and in plasma for (C) humans and (D) horses. Horizontal bars indicate means. The Mann Whitney test for non-parametric data was used to determine *P* values between horses and other species in figure A; otherwise the Unpaired t test was used. The Paired t test was used to determine *P* values when comparing plasma and red cell tyrosine levels within species, except for horses where the Wilcoxon signed rank test was used. The Pearson test was used to determine statistical significance of correlation in figure C, and the Spearman test in figure D.

#Black rhino red cell tyrosine concentrations are taken from a previously published report [2].

*Individual black rhino plasma tyrosine levels were not available. However the mean plasma tyrosine level was similar to that of humans [4].

Human plasma tyrosine levels (60.0 ± 17.6μM) (Fig 12B) were very similar to the red cell levels. Red cell and plasma tyrosine levels showed a strong correlation, with an 'erythrocyte to plasma' (E:P) tyrosine ratio ± SD of 1.12 + 0.15 (and ranging between 0.75 to 1.48 for individuals) (Fig 12 C). Hagenfeldt et. al. (1980) previously reported a mean human plasma tyrosine level of 54 ± 11μM, and an E:P tyrosine ratio of 1.22 ± 0.17 [83], but no reports on whether the red cell and plasma values within individuals correlate, or not, have to date been reported.

In humans, the presence of tyrosine in the red cell, and its correlation of effectively 1:1 with plasma level, is consistent with non-concentrative, facilitated diffusion transportation of tyrosine, from plasma, across the red cell membrane via both the L-system and T-system of amino acid transporters [92-94]. As has been shown *in vitro*, tyrosine is also easily able to exit the human red cell [5, 83]. But this system of tyrosine movement/transport across the human red cell membrane cannot explain the very high red cell tyrosine levels found in some of the other mammalian species.

The mean E:P tyrosine ratio was clearly highly variable between species, with plasma tyrosine levels showing much less interspecies variability than red cell tyrosine levels.

Only rats and sheep had plasma tyrosine levels that were significantly higher than human plasma tyrosine levels ($P < 0.0001$ and $P = 0.004$ respectively) - this may relate to diet. In contrast, horses, rhinoceros, cats and dogs - the species that actually had a significantly higher red cell tyrosine level compared with humans - had mean plasma tyrosine levels that were very similar to human plasma levels, being 56.6 $\pm$ 35.6μM, 61.1 $\pm$ 31.2μM and 44.9 $\pm$ 12.4μM for horses, dogs and cats respectively. As a consequence, plasma tyrosine levels in these species were all significantly lower than the respective red cell levels of tyrosine ($P < 0.0001$). And in contrast with humans, red cell and plasma tyrosine levels showed no correlation in horses (Fig 12D). This demonstrates that tyrosine is accumulating in the red cells of some species by some active process(es).

Is it possible that tyrosine is being made within the red cells of some species? Tyrosine is a non-essential amino acid, being formed by hydroxylation of the essential amino acid phenylalanine by the enzyme phenylalanine hydroxylase. This enzyme is present at high levels in liver tissue, and has also been identified in human kidney, pancreatic and brain tissue [95]; its presence in red cells, however, has not been reported (pubmed search and [1, 3]). It is therefore unlikely that tyrosine is being formed *de novo* in the red cell.

The more likely explanation, however, is that as for human red cells, in horses, rhinoceros, cats and dogs the tyrosine in their red cells originates from the plasma; but that unlike in humans where red cell tyrosine essentially equilibrates with plasma tyrosine levels, in these other animals tyrosine may be actively pumped into the red cells against a steep concentration gradient, and is then trapped within the red cells. Although much is reported on the variable transportation of dibasic and neutral amino

acids across the red cell membranes of different species, very little comparative data is available regarding tyrosine transportation.

The very different tyrosine gradients evident between the plasma and red cells of these different species with elevated red cell tyrosine also indicates that different processes/mechanisms may be responsible for its accumulation (or removal) in each species. Alternatively, heterogeneity in levels of the same protein transporters for tyrosine within the red cells of different species may have evolved. The large variability of red cell tyrosine levels just amongst horses themselves (ranging from 140μM to 880μM), as well as within the rhinoceros species (560μM to 890μM), was particularly interesting, and rather reminiscent of the highly variable levels of urate riboside present in the red cells of cows (Fig 9A, section ii above). However, the fact that horse red cells in particular are reported to show heterogeneity in the transportation of the amino acid L-alanine [96-98] may be indicative that heterogeneity in the transportation of the aromatic amino acid tyrosine may also exist, particularly amongst horses. Since all the horses were being housed and fed identically (as reflected by very consistent plasma tyrosine levels), it is probable that the significant range in baseline tyrosine level found in these horses is because of genetically-determined variable tyrosine transport across the red cell membranes of different horses.

In further support of this variability being genetically-determined, analysis of blood samples from four horses repeated on three different occasions over a month showed that these wide-ranging red cell tyrosine levels were highly stable within each individual horse (Fig 13A), this being similar to the results obtained for urate riboside in cow red cells (see Fig 9B).

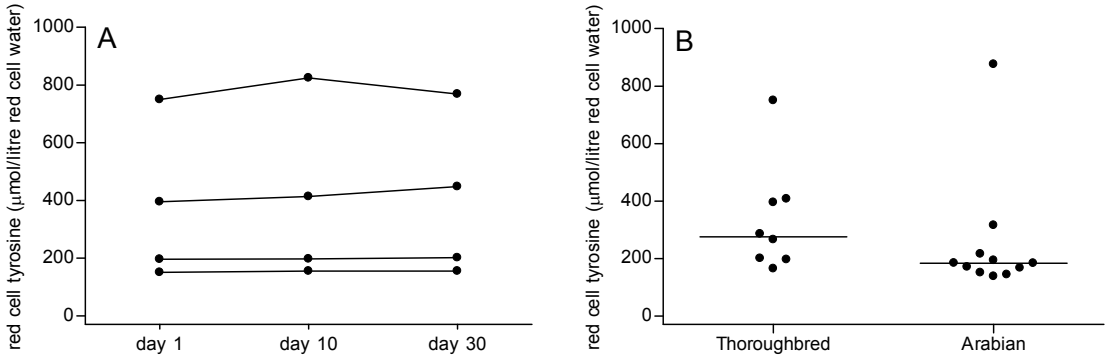


Figure 13. (A) Red cell tyrosine concentrations in three blood samples taken on different days from the same four horses, illustrating the significant stability of tyrosine within a period of one month. (B) Red cell tyrosine levels in Thoroughbred (n=8) and Arabian Cross bred (n=11) horses. Horizontal bars indicate medians. The Mann Whitney test was used to determine that *P* = 0.0632.

Since most of the horses evaluated were of two different breeds (Thoroughbred and Arabian Crossbred), red cell tyrosine levels in these two breeds were compared. Although levels in the Thoroughbred horses tended to be higher than in the Arabian Crossbred horses, this difference did not reach statistical significance (Fig 13B). Gender had no influence on tyrosine red cell level (data not shown). Hence genetic variability in red cell tyrosine cannot be linked to gender or breed alone.

Extrinsic factors may however also be playing some part in the variable tyrosine levels found in horse red cells. The fact that all the horses sampled happened to be competitive athletes may be of some relevance. Physical exertion in horses and humans, particularly when exhaustive, is associated with a significantly increased production of reactive species, measurable by an increased degree of oxidative modification to various molecules [99-108]. Tyrosine residues within proteins may react with some reactive species both *in vitro* and *in vivo*. It is possible that free tyrosine molecules similarly would be degraded by such oxidants, and levels of tyrosine may be decreased (see also Chapter 2, Literature Review). Although none of the horses had taken part in any racing event for at least 1 month prior to blood sampling, it is possible that the large variability in red cell tyrosine level seen in these horses was because some of them had been exercised more than others prior to the quarantine period (but for no longer than a period equivalent to the red cell's lifespan), accounting for lowered levels in some of these horses. If so this would constitute not a genetically-determined variability, but variability arising because of a 'non-pathological' extrinsic influence. Red cell tyrosine levels for athlete horses may therefore not be representative of baseline levels in non-athlete horses. It is possible that non-athlete horses have a higher mean red cell tyrosine level, and a lot less variability, compared with athlete horses, but this remains to be tested.

Finally, it is necessary to consider that other explanations for the elevated levels of tyrosine found in the red cells of some species may need to be sought. Harley and associates had found that in the presence of exogenous tyrosine, whereas washed human red cells accumulate tyrosine (which can subsequently also exit the red cells), that the red cells evaluated from one rhinoceros and from one horse showed no significant tyrosine uptake under the same conditions [2, 5] If tyrosine is unable to enter the red cells of these species (or some individuals at least) *in vitro*, then how might tyrosine have accumulated in the red cells of these animals? One possible explanation is that tyrosine may be able to enter the red cell of these animals in a different form, in a manner analogous to how taurine has been shown to accumulate

in human red cells under certain conditions. Although taurine has no transport system in human red cells, it can enter in the hypochlorous acid (HOCl)-oxidized form (taurine chloramine) [70, 72] produced on reaction of neutrophil-released HOCl and taurine within the surrounding plasma. At higher HOCl levels taurine dichloramine is formed which is very toxic but can enter red cells. Amines are readily regenerated by reducing agents such as thiols [109], and in the red cell they are readily reduced by GSH back to taurine. It is believed that in this way the red cell is able to remove the potentially toxic taurine derivatives from sites of imflammation. Taurine is then trapped and accumulates within the red cell [71]. This may be the reason why reported red cell taurine levels are so discrepant, with one very early report finding a red cell concentration of 30μM [110](this value being referenced by a more recent publication [71]), while a later report found a 5-fold higher level of taurine in human red cells [83](this value being referenced in a current hematology text book [3]). This compares with human plasma taurine concentration reported as 50-70μM [70, 83].) On *in vitro* exposure of red cells to activated neutrophils (which release HOCl and taurine during the respiratory burst, see Literature Review and Background Work), red cell taurine content rises significantly (300-fold) [71]. Tyrosine may similarly be able to enter the red cells of some species only in its oxidized form, and once reduced back to tyrosine, it would then be trapped in these red cells. If this was the case, then recent, excessive neutrophil activation, such as occurs with a bacterial infection, but also in association with strenuous physical exertion [111-114] could theoretically lead to a high level of red cell tyrosine in an individual animal. Halliwell et. al. (2007) also indicate that tyrosine may be formed from the hydroxyl radical-mediated oxidation of phenylalanine [8]. This is however unlikely to explain the elevated levels of tyrosine found in the red cells of some healthy mammalian species.

(iv) *Urate: inter- and intraspecies variation*

Whilst investigating tyrosine levels in the red cells of horses, very noticeable was the presence of what appeared to be extremely variable levels of urate in the red cell extracts of the different horses, to the extent that it became the dominant peak in some horses (Fig 14A), yet was undetectable in almost 50% of the horses (Figs 14C and D). This peak had the same anion-exchange and reverse-phase elution times, 260:280nm ratio and diode array spectrum to that produced by the distinct peak of urate detectable in human red cells and human plasma (Figs 14E and F), and also that of a urate standard. The identity of the substance accounting for this HPLC peak in horse red cells was confirmed to be urate by co-elution with a solution of urate spiked into the red cell extract shown in figure 14B (purity of the peak was maintained, spiked profile not shown), and by disappearance of this unknown peak

on exposure of the red cell extract to the urate-specific enzyme uricase (Sigma-Aldrich, USA)(0.1IU per ml of extract for 1hour at 37°C), with no other profile changes. The levels of red cell urate present in some of the horses in the current study were unprecedented.

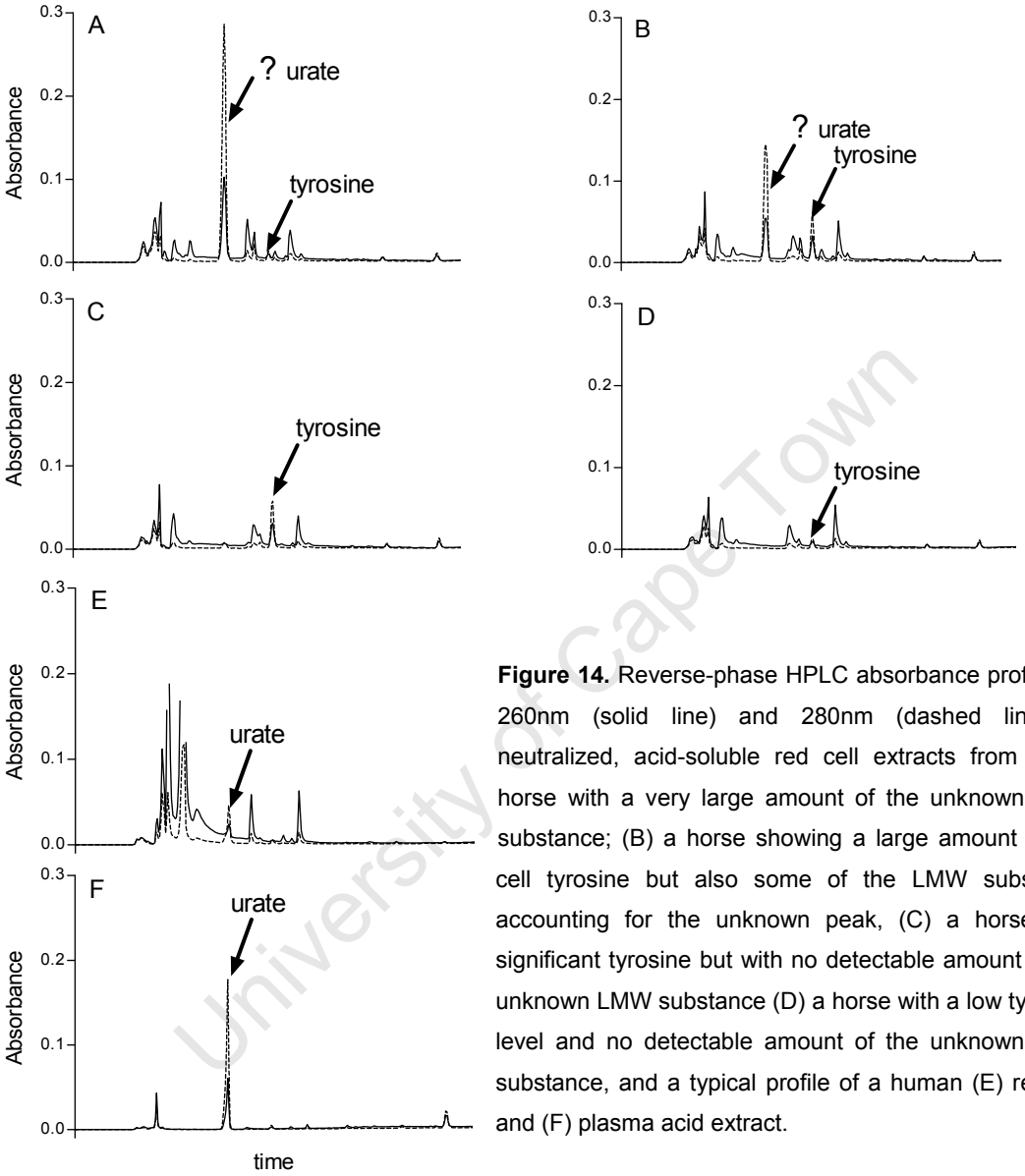


Figure 14. Reverse-phase HPLC absorbance profiles at 260nm (solid line) and 280nm (dashed line) of neutralized, acid-soluble red cell extracts from (A) a horse with a very large amount of the unknown LMW substance; (B) a horse showing a large amount of red cell tyrosine but also some of the LMW substance accounting for the unknown peak, (C) a horse with significant tyrosine but with no detectable amount of the unknown LMW substance (D) a horse with a low tyrosine level and no detectable amount of the unknown LMW substance, and a typical profile of a human (E) red cell and (F) plasma acid extract.

Harley and coworkers had previously observed that a few horses sampled by them had small amounts of urate in their red cells (unpublished [5]). Although these levels (20.2 $\pm$ 38.5μM) were within the lower range known to be present in human red cells [3], this in itself was intriguing to the investigators, considering the near absence of urate in the plasma from horses [115, 116], and there being no previous reports on the presence of urate in horse red cells. Moreover, when eight of these horses took part in an endurance racing event, Harley et. al. (2004) found that red cell urate level 1 hour after the race had increased significantly (P = 0.0313) over baseline levels in

many of these horses (the median level increasing from 0μM to 35μM), with levels in one horse rising from 0μM to 824μM (Fig 15). These elevated levels of red cell urate, in most cases, dropped back to baseline levels by 24 hours. Surprisingly, the median level was increased again at 72 hours (to 78μM); however levels at this time point were not significantly different to levels at baseline (*P* = 0.0938) [4].

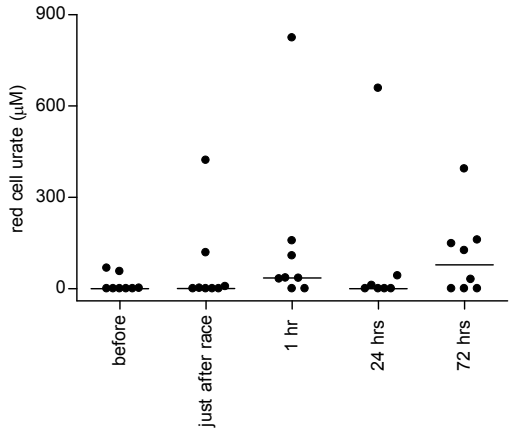


Figure 15. Modified representation of data previously reported by Harley et.al. (2004) [4]. Red cell urate levels in eight horses bled before, and then immediately, 1 hour, 24 hours and 72 hours after an endurance race of 80km, with a mean duration of 5.1 hours. The horizontal bars indicate median values. The Wilcoxon signed rank test was used to determine *P* values.

The production and disappearance of uric acid was also observed over short time periods in rhino, horse, and human erythrocytes incubated on the bench (unpublished). This was a chance and sporadic observation seen during their HPLC analyses of UV-absorbing compounds in red cell extracts (personal communication [64]).

Now, in the current study, although none of the 20 horses sampled had undergone any significant exertion for at least one month prior to sampling, just by viewing the HPLC profiles it was clear that some of these horses had baseline red cell urate levels exceeding even human plasma urate levels (Fig 14F above). All of these novel findings in red cell urate metabolism were unprecedented, not readily explainable, and of particular interest because of the well-established physiological role of urate as an important LMW antioxidant in human plasma.

Urate is a product of purine nucleotide degradation (see Fig 2, Literature Review and Background Work). The enzyme required to make urate is xanthine oxidase/xanthine dehydrogenase (XO/XD), also known as xanthine oxidoreductase (XOR). It catalyses the hydroxylation of hypoxanthine to xanthine, and then of xanthine to urate. XO/XD

activity has been detected in all species examined, including bacteria. As the name implies, it exists in two interconvertible active forms: the oxidase form (XO) and the dehydrogenase form (XD). Most of the cell-bound enzyme exists in the dehydrogenase form *in vivo*, whilst circulating (plasma) enzyme is mostly in the oxidase form due to conversion by serum proteases. The electron-acceptor for the XD form is preferentially NAD^+, whereas for XO it is solely O_2. Reduction of O_2 by either form of the enzyme yields the superoxide free radical. In mammalian tissues, although activity is widely distributed, degree of activity is very tissue-specific [117]. In humans very low levels of XO/XD activity are present in blood, and the highest levels are found in liver, intestine and mammary tissue [118]. The urate formed in these cells enters the plasma and is present as the sodium salt at biological pH 7.4 (uric acid has a pKa = 5.8). Most (95%) of it exists as the free form, but a small proportion is bound to albumin [119]. In most mammalian species, the hepatic enzyme urate oxidase (uricase) then rapidly converts urate into allantoin as the final step in purine degradation, keeping plasma urate levels to a minimal. Humans and apes have however lost uricase; a number of distinct mutations are known to have occurred over about 60 million years of evolution to eventually make uricase non-functional [39, 120]. In addition, in humans the kidneys reabsorb > 90% of the filtered urate. As a consequence humans have a significantly higher plasma urate level, being at least 10-fold higher than in animals with uricase [116, 121]. Normal human plasma levels range between 50 and 900μM [122], reaching a mean of about 350μM in men. Since the solubility limit of sodium urate is about 420μM [119], such an elevated level of urate in humans is associated with a significant risk of precipitation (gout, and urinary uric acid crystals). Yet it is believed that the evolutionary changes leading to an elevated plasma urate level occurred despite these risks because of the significant advantages associated with the potent antioxidant activity of urate. Urate is non-enzymatically degraded by various oxidants and is well known to be a potent antioxidant (see also Chapter 2). In 1981, Ames et. al. first suggested that by virtue of this antioxidant activity, the evolution of high plasma urate levels was advantageous by contributing to human longevity and a reduction in cancer rates [39].

Unlike for plasma urate levels, data on urate levels in red cells is not available for any animal species other than for humans – *in vitro* experiments have shown that urate can enter the human red cell in the anionic form [123], most likely via the non-selective band-3 protein membrane transporter [124]). Therefore, in humans, the presence of urate in the red cells is presumed to arise from entry of some of the high level of plasma urate into the red cells. Whereas in all mammals with uricase, the

paucity of plasma urate, together with the fact that the red cell supposedly lacks the enzyme XO/XD necessary to make urate (see below) seems to justify the assumption that urate levels would be negligible in the red cells from most mammalian species [125].

In 1965, Al-Khalidi et. al. reported that the red cells of mammals lack the enzyme XO/XD necessary to make urate [22], and to date no-one has challenged this conclusion. These investigators measured XO/XD activity in both whole blood and serum samples, separately, from various vertebrate species including humans (n= 40). In all the mammals that showed whole blood activity, they found that the serum activity was always approximately double that found in whole blood. Given that the haematocrit in most animals is very roughly almost 50%, they concluded that red cells of all mammals including humans do not contain substantial amounts of the XO/XD enzyme. Nucleated red cells from chicken, tortoise and turtle, in contrast, had significant XO/XD activity with their detection method.

In view of the above points, the detection of urate in the red cells of horses is clearly fascinating. Investigation of urate levels in the red cells and plasma of horses and other mammalian species (as evaluated for tyrosine) was therefore undertaken.

Humans (n=25) had a significant (although relatively variable) amount of urate in their red cells (Fig 16A), levels ranging from 23.7μM to 214.5μM, with a mean of 117.5 ± 39.7μM, which is in agreement with the text book values of 113μM [3] and 130μM [126]. Although red cell urate levels in horses (n=20) did not differ significantly from levels in human red cells (P = 0.5423), the range of urate levels in horses was huge, and skewed to the right. Thus whilst 50% of the horses had a red cell urate level of less than 50μM, the other 50% had levels ranging between 50μM and 750μM, giving a mean of 181.4 ± 231.3μM, and a median [and interquartile range] of 73.7 μM [8.3 μM – 284.4μM]. None of the other mammalian species evaluated had levels significantly greater than their respective plasma levels (Fig 16B).

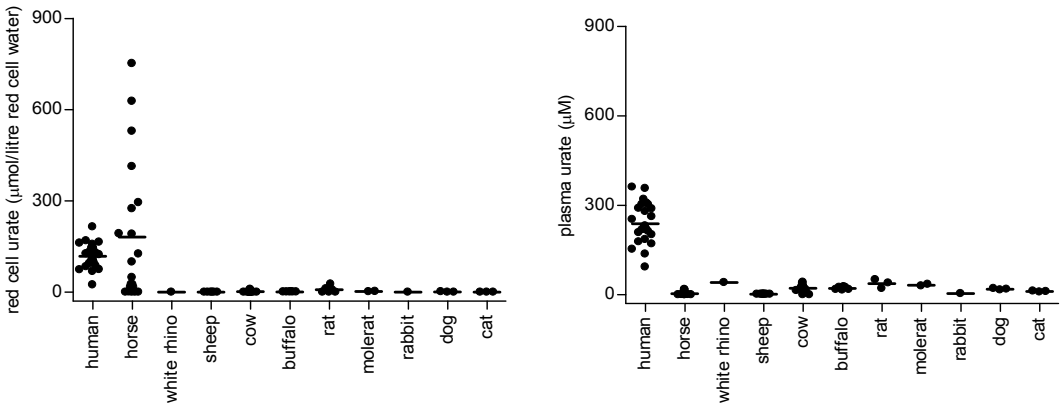


Figure 16. (A) Urate concentrations in the red cells from various mammalian species, including humans. Horizontal bars indicate means. It should be noted that due to the highly skewed distribution of red cell urate in these horses, the median for red cell urate in horses was 74μM, being much lower than the mean. The Mann Whitney test for non-parametric data was therefore used to determine *P* -values between horses and other species; otherwise the Unpaired t test was used. (B) Urate concentrations in the plasma from the same individuals as in (A). The Unpaired t test was used to compare plasma urate levels between the different species The Paired t test was used to determine *P* values when comparing plasma and red cell urate levels within a species.

Furthermore, analysis of blood samples from four horses repeated on three different occasions over a month showed that baseline (i.e. not whilst racing) red cell urate levels in these horses, similar to tyrosine levels, were stable over time (Fig 17).

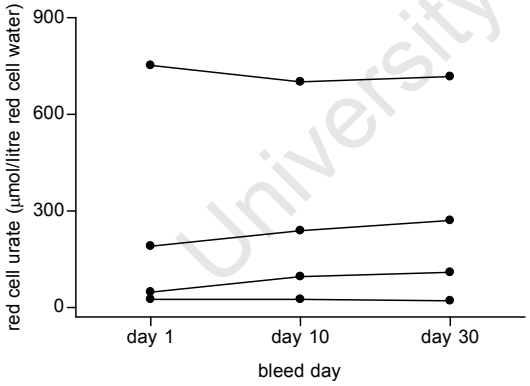


Figure 17. (A) Red cell urate concentrations in three blood samples taken on different days from the same four horses, illustrating the significant stability of urate within a period of one month.

Because the skewed pattern of distribution and stability of red cell urate was reminiscent of the skewed yet stable red cell tyrosine levels in horses (as reported in section iii above), levels of each of these two substances were compared in each horse; however no correlation was evident between red cell urate and tyrosine levels (Fig 18). Gender had no influence on red cell urate level (data not shown).

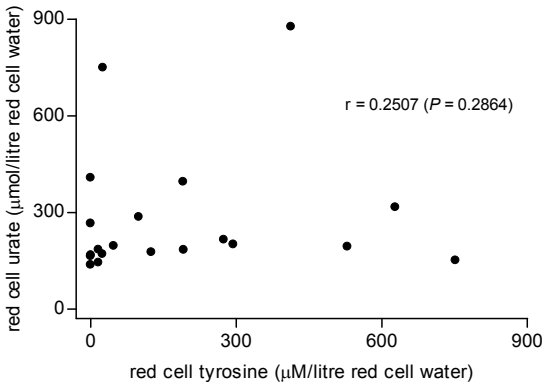


Figure 18. Comparison of urate and tyrosine in horse red cells (n=20). No correlation was evident using the Spearman test.

Evaluation of plasma urate levels showed that, as expected, humans had a significantly higher mean level than that found in any other animal species evaluated. Human plasma levels ranged from 92.9μM to 360.7μM, with a mean of 238.1 ± 66.6μM (Fig 16B above). As shown in figure 19, plasma and red cell levels of urate were well correlated in humans (open circles), with an average E:P ratio of 0.49 - this ratio and correlation have not been reported on before.

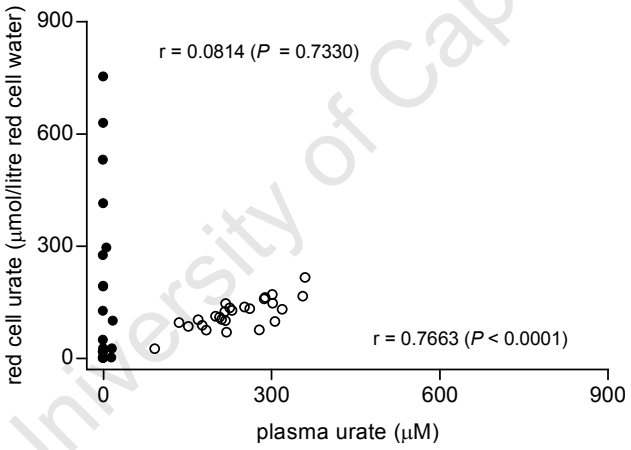


Figure 19. Comparison of the urate concentration within red cells and plasma for humans (open circles, n= 25) and horses (black circles, n=20). The Pearson test was used to determine statistical significance of correlation for humans, and the Spearman test for horses.

In contrast with this correlation in humans, the extensively ranging red cell urate levels in horses showed no correlation whatsoever with plasma urate levels (Fig 19, black circles). As expected, because of the presence of uricase, the mean plasma urate level in horses, as for all the other species evaluated (Fig 16B above) was negligible, being 3.2 ± 5.9μM, with a range of 0-17μM in horses, which is in accordance with a previously published range of 7.3-17.4μM [115] and mean level of 9.0 ± 1.6μM [116]. Similarly, the increases in red cell urate level found by Harley et.

al. in some horses post-exertion (Fig 15 above) could not be explained by the lesser increases in plasma urate level found in the same horses [64], and that are reported by others to be associated with physical exertion in horses [115, 127].

It is possible that, as hypothesized to account for the elevated red cell tyrosine levels in horses, urate is being actively pumped from the plasma into the red cells of horses. However this would be occurring against a concentration gradient of more than 1mM in some horses, and would have to proceed very rapidly to account for the increased levels detected 1 hour post-exertion, as seen in the exercised horses of figure 15. Such a mechanism to explain the presence of high red cell urate levels in the horses is further unlikely since Harley and associates found that, in the presence of 500μM urate in the incubation medium, whereas human red cells accumulated urate to a concentration of approximately 170μM, the red cells from a horse showed no significant uptake [5]. Therefore, whilst all of the urate found within human red cells is by all accounts entering from the plasma, in horses significant amounts of urate are accumulating in the red cells by an active process which is highly unlikely to be related to plasma urate levels, particularly considering the negligible plasma urate levels in horses.

The only other plausible explanation for the presence of urate in the horse red cells is that urate is actually being produced and then trapped within the red cells. For this to occur, active XO/XD enzyme (see also above) would have to be present in these red cells. Considerable variation in the distribution and level of XO/XD in tissues exists between species [118], and it is possible that as for urate riboside which is detectable exclusively in the red cells from bovines, that horses may be a unique species in having XO/XD enzyme activity in their red cells (horse red cells were not actually evaluated by Al-Khalidi). However, an alternative explanation is suggested by observations in various other tissue types: it was found that measures of XO/XD activity often do not reflect protein levels (as measured using antibodies or ELISA) because inactive forms of the enzyme exist *in vivo* – two forms of naturally-occurring inactive XO/XD enzyme are described - demolybdo-XO/XD lacks molybdenum (Mo), and in desulfo-XO/XD the Mo=S grouping, essential for catalytic activity, is replaced by Mo=O [128-130]. As much as 60% of purified bovine milk XO/XD is inactive towards xanthine [129]. In other tissues, the ratio of inactive to active forms varies significantly, and depends on the animal species and the specific tissue investigated [117]. However mammalian red cells were never re-evaluated for the presence of inactive forms of XO/XD. It is therefore possible that horses, and even other

mammalian species, may similarly have significant protein levels of inactive XO/XD within their red cells.

The production of urate in the red cells is then proposed to occur when the inactive form of the enzyme in the red cells becomes activated via post-translational modifications under specific conditions. This is supported by studies using cell culture experiments which show that upregulation of XO/XD activity occurs not only at the transcriptional, but also at that post-transcriptional level in nucleated cells, (depending on the stimulus and tissue), and that such post-translational modifications may include the incorporation of Mo and also sulfuration reactions [117, 131].

A variety of factors such as hypoxia, various inflammatory cytokines, hormones etc. have been found to lead to increased levels of XO/XD activity in nucleated cells *in vitro* [117]. Increased XO/XD activity was reported in the lung tissue of a mouse model of acute viral pneumonia [132], and a 20-fold increase of enzyme activity (and 30-fold increase in urate) was found in the brains of rats with induced bacterial meningitis versus healthy controls [133]. Urate levels were also elevated in the cerebrospinal fluid from patients with bacterial meningitis and other inflammatory brain diseases such as multiple sclerosis, viral meningitis and stroke [117]. The production of urate in skeletal muscle was shown to increase after submaximal exercise in fit humans [105]. The production of urate in brain and skeletal muscle is unexpected considering that normal human brain tissue and skeletal muscle have been shown by Sarnesto et. al (1996) to have no detectable XO/XD by western blotting [134], yet Hellsten et. al. (1997) showed that the number of xanthine oxidase structures observed by immunohistological methods in exercised muscle was up to eightfold higher than control from day 1 to day 4 after exercise. The increase was attributed to an enhanced expression of xanthine oxidase predominantly in microvascular endothelial cells - this again illustrates the potential for some tissues that don't normally have detectable XO/XD to upregulate enzyme level/activity when required.

What would be the advantage of having such a carefully regulated system of inducibility for XO/XD activity? Certainly compared with many other animals, only low baseline levels of XO/XD are present in most human tissues [117, 134]. XO/XD activity leads to the production of potentially damaging reactive species (superoxide and thus H_2O_2, nitric oxide and even peroxynitrite [117, 135]), and has been implicated in ischemia-reperfusion injury [136-139], although others feel that this is probably unjustified [117, 140], and also various other clinical conditions [141].

Conversely, its involvement in normal physiological processes has been much less studied, and the fact that XO/XD activity is inducible infers some useful role of this enzyme under particular conditions. The fact that XO/XD is the only metabolic source of the protective antioxidant urate, the presence of which can limit oxidative damage [142, 143](see also Chapter 2), has generally been overlooked by investigators in the field [144]. As such, it is not surprising therefore that many of the above-mentioned conditions, where an increased XO/XD activity or urate level have been measured in tissues, are known to be associated with oxidative stress, and where an increase in a potent antioxidant would be clinically advantageous.

Certainly it is known that organisms will often increase levels of their antioxidant defense systems in response to oxidative stress in an attempt to restore the prooxidant/antioxidant balance. For example, some yeast cultures exposed to H_2O_2 for 30min showed significantly increased activities of the antioxidant enzymes catalase and superoxide dismutase (see also Chapter 2, Literature Review), although another strain of the same species showed no activation and its survival was much lower. Notably, whereas a certain concentration of H_2O_2 caused an increase in catalase activity, a higher concentration of H_2O_2 resulted in a decreased catalase activity in the same strain [145]. A single bout of exhaustive physical exertion is well known to induce *in vivo* oxidative stress and damage in various species including humans and horses [99, 101-103, 108, 146-149]. An increase in antioxidant enzyme activities has been observed in humans in association with acute physical exertion. For example, red cell catalase and glutathione reductase (GR) activities have been shown to increase during a long-distance cycling event in humans [108], although another study showed no increase in antioxidant enzyme activity [114]. Post-exertion increases in red cell antioxidant enzyme activities, including that of plasma XO/XD, have also been shown in horses [102, 150], although in other studies increases in red cell antioxidant enzyme activity were not found [151, 152]; this may be due to variations in study protocol. Plasma levels of urate are well known to increase after exhaustive exertion both in humans [105, 108, 153] as well as in horses [115, 127, 150]. The increases in antioxidants all tend to return to pre-exertion levels within a few hours after the exertion. Of note is that the reported increase in plasma urate levels reported in horses (these being similar to that observed by Harley and coworkers (personal communication [64]) was considerably lower is highly unlikely to be able to account for the much higher post-race increases in horse *red cell* urate observed by Harley et. al. (2004) [4]. Rather, it is proposed that post-exertion increase in red cell urate levels seen in some horses occurred via increased activation of red cell XO/XD (induced by the associated acute exercise-induced

oxidative stress) [4]. The significant increase in circulating hypoxanthine which is associated with physical exertion [154], would most likely be an important contributing factor to the increased level of urate observed (both in plasma and red cells), by providing the substrate for XO/XD. The subsequent decrease in red cell urate level (usually within 24 hours) could be attributed to continued oxidation of the urate (to form allantoin) by the residual levels of circulating oxidants post-exertion.

Baseline levels of antioxidant enzymes may also be increased with more prolonged or repeated *in vivo* exposure to mild-moderate oxidative stress, a process termed 'adaptation'. For example, rats exposed to daily bouts of restraint stress show elevated baseline red cell catalase and glutathione peroxidase activity [155]. The red cells from smokers (smoking induces oxidative stress [156, 157]) have higher catalase [158], superoxide and GPx activity [159] than red cells from nonsmokers. The increased antioxidant levels may allow individuals to better tolerate mild-moderate oxidative stress, although the increased level of antioxidant defenses are often not enough to completely inhibit oxidative damage. An 'overcompensating'/protective adaptive response is suggested to be the mechanism behind "body hardening", defined by Siems et. al. (1994) [160] as "exposure to a natural, e.g., thermal stimulus, resulting in an increased tolerance to stress e.g. diseases". The amount of oxidative stress induced by the stimulus would be such so as to not induce any significant oxidative damage, yet enough to stimulate an antioxidant response. An example of hardening is that induced by exposure to repeated intensive short-term cold stimuli, which increases oxidant production [8, 160] – this is often applied in hydrotherapy. Siems et. al. showed that the baseline concentration of reduced GSH was increased and the concentration of oxidized GSH was decreased in the red cells of subjects who swim regularly in ice-cold water during the winter (winter swimming) as compared to those of nonwinter swimmers [160]. Ischaemic preconditioning would be another example of oxidant-stimulated hardening which protects against subsequent severe ischaemia-reperfusion oxidant-induced damage [161].

Relevant to the current study is that all the horses sampled in the current study, unlike the other mammalian animals sampled, were 'professional' athletes, and therefore extremely fit individuals. Regular controlled physical exercise/training, unlike strenuous/exhaustive exercise, may also induce 'body hardening'. Increases in baseline antioxidant defenses are evident in the blood and/or muscle cells in humans [114, 162-164] and animals, including horses that undergo fitness training [165-170], and an increased level of fitness is universally accepted to have beneficial effects in

terms of general health. It is therefore possible that high, stable levels of red urate found in these horses, which had not taken part in any racing event for at least 1 month prior to sampling, reflects an adaptive/hardening response in antioxidant defenses, via an increased level of baseline XO/XD activity in these fit individuals compared with the sedentary animals sampled. This could be the basis of future investigations.

Notably, red cell urate levels in the 20 athlete horses of the current study were significantly higher (P = 0.0023) than pre-race levels in another 20 horses previously sampled by Harley and coworkers (unpublished work [5], as shown in figure 20).

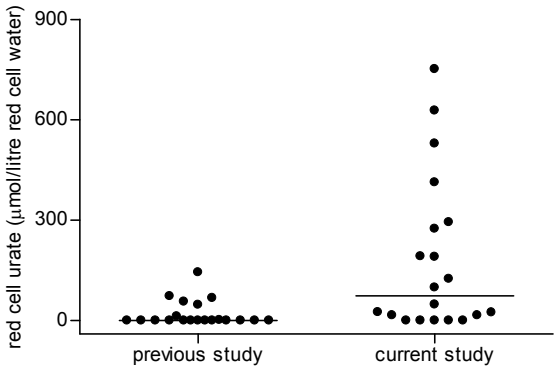


Figure 20. Baseline urate concentrations in the red cells from the athlete horses, as sampled by Harley and co-workers in a previous study (n=20) (unpublished [5]), and those sampled in the current study (n=20). Horizontal bars indicate means. The Mann Whitney test for non-parametric data was used to determine that there was a statistically significant difference between these two groups of horses (P = 0.0023).

The only difference between the horses from the previous study, and the horses of the current study was that the latter were 'elite'-athletes (highly trained and competing race/endurance horses, awaiting exportation after having been sold for high prices). It is possible that the elite-athlete horses from the current study had a higher mean red cell urate level because they had a higher level of red cell XO/XD activity, perhaps induced by their special fitness regime.

Despite the very high red cell urate levels in some of the elite athletes, the variance was large, with some of these horses having almost undetectable levels of red cell urate. Since they were all being housed in the same quarantine facility, with the identical diet, such environmental contributing factors could be excluded. Genetic factors were considered. Horses are a species that seem to show much heterogeneity in the transport of substances across their red cell membranes, for example, as mentioned earlier, in amino acid transport. Relevant here is the report by Jarvis et. al. (1998) that horses show significant heterogeneity in the inward transport of hypoxanthine into their red cells, with 15% of Thoroughbred horses tested (n=25) failing to transport hypoxanthine [26]. This could be a reason why some of the elite-athlete horses had no detectable urate in their red cells. It is also possible that transport of urate *out* of the red cell may also show individual variation. Alternatively,

heterogeneity in protein levels of red cell XO/XD may exist. Kirschvink et. al. (2006) evaluated blood oxidant/antioxidant status in thoroughbreds, standardbreds and jumping horses and concluded that breed, gender and age were all influencing factors [171]. Red cell urate levels in the Arabian-Crossbred versus the Thoroughbred horses were thus compared in order to determine what influence, if any, horse breed had on the variability seen (Fig 21), but levels of red cell urate between these two breeds were not significantly different.

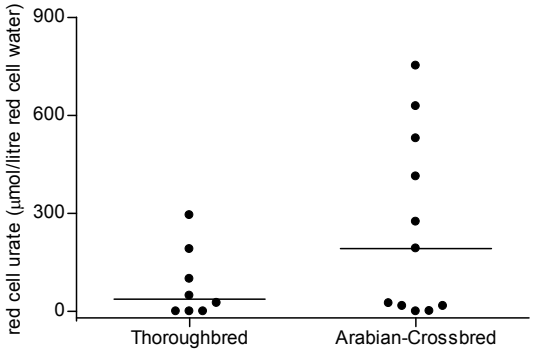


Figure 21. Red cell urate levels in the elite athletes horses of figure 20, now separated by breed into Thoroughbred (n=8) and Arabian-Crossbred (11). Horizontal bars indicate medians. The difference between the two groups was not statistically significant (Mann Whitney P = 0.2153).

In order to determine if red cells with higher levels of urate were more resistant to H_2O_2-lysis, the red cells from horses with varying levels of urate were exposed *in vitro* to H_2O_2- and AAPH-induced oxidative stress - no correlation was found between urate level and resistance to lysis (data not shown). However, an unexpected and striking difference in red cell susceptibility to lysis was found between the Arabian-Crossbred and Thoroughbred horses, with the red cells from the Arabian-Crossbred horses being much more resistant to H_2O_2- and AAPH-induced lysis than the red cells from the Thoroughbred horses (Fig 22).

Notably, the Thoroughbred horses were all trained for sprinting events (racehorses), whilst all the Arabian-Crossbred horses were endurance-trained athletes - this seems to fit in with the study by Kinnunen et al. (2005) reporting that, compared with trotters, endurance-trained horses had higher baseline activity of some red cell antioxidant enzymes and higher baseline total antioxidant capacity. These investigators reported that, unlike the trotters, the endurance horses did not show evidence of increased oxidative stress after exhaustive exertion, although they had higher baseline levels of oxidative stress compared with the trotters [172]. Marlin et. al. (2002) also found endurance horses to exhibit much lower signs of oxidative stress and oxidative damage than anticipated after a prolonged bout of exhaustive exercise [115]. It is possible that endurance training induces a greater adaptive antioxidant response than the training used for racehorses. However, since endurance-trained horses are most often Arabian-Crossbred horses, it remains an interesting question as to which

factor is a greater influence in the resistance of this group of horses to episodes of acute oxidative stress: genetics or training mode?

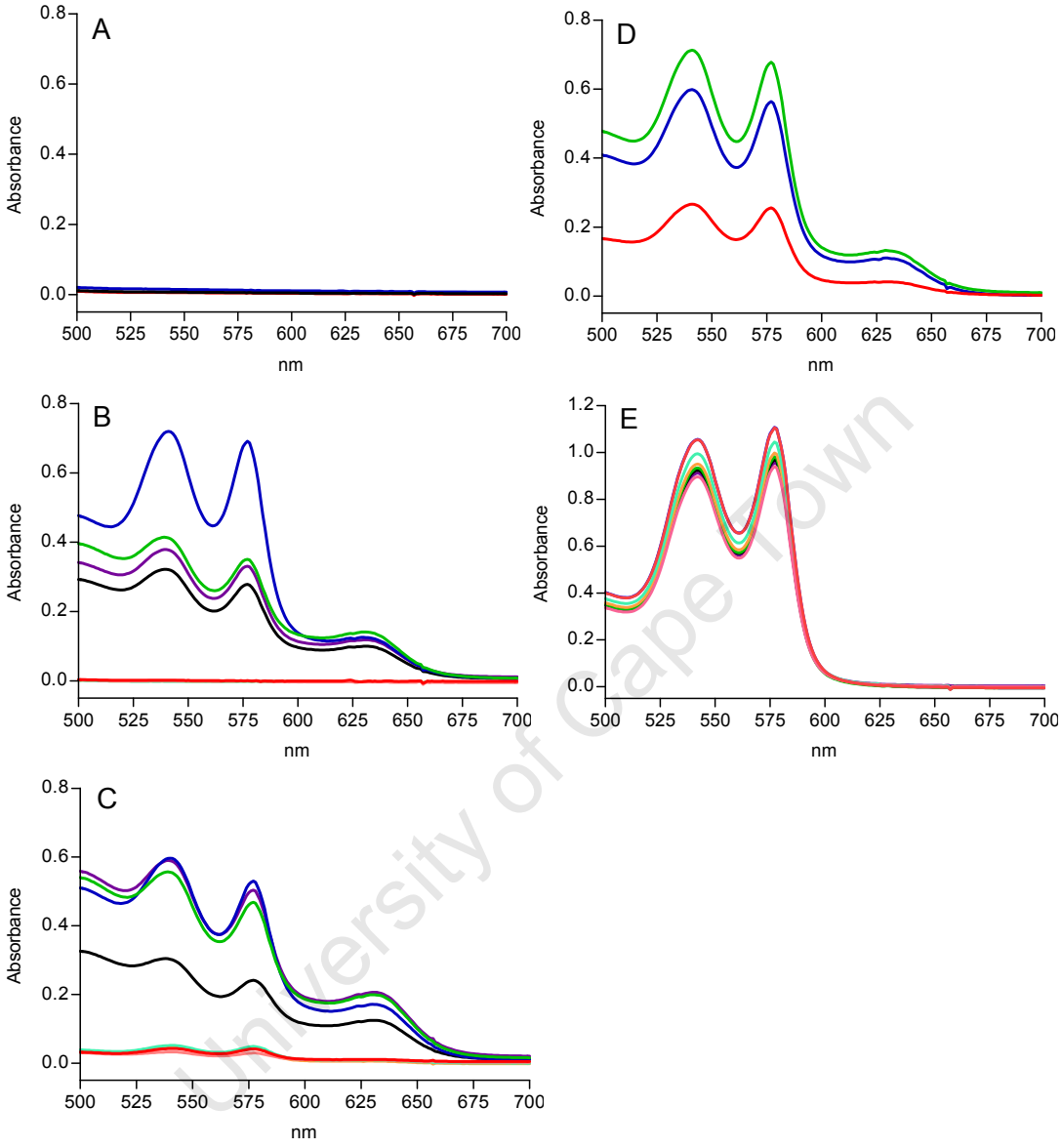


Figure 22. Red cell suspensions (1% Hct) from four Thoroughbred (black, purple, green, dark blue) and four Arabian-Crossbred horses (red, pink, orange, light blue) were exposed to 50mM H_2O_2 (in PBS, at 37°C), and the supernatants evaluated for haemolysis after (A) 1 minute, (B) 1 hour and (C) 20 hours. (D) Suspensions of the red cells from two of these Throroughbred horses and one of the Arabian-Crossbred horses were also incubated with 20mM AAPH, and the supernatants evaluated after 1 hour. (E) Total triton-X-induced haemolysis of a suspension of red cells from each of the eight horses.

Next, a literature search was performed, and two reports found to support the idea that human red cells must have significant levels of XO/XD protein. Kaynar et. al (2005) investigated red cell XO/XD activity (!!), in addition to the activities of various

known red cell antioxidant enzymes, in healthy human subjects versus lung cancer patients. They reported that, in addition to having elevated red cell catalase activity, the red cells of the cancer patients had significantly increased XO/XD activity compared with the red cells of the healthy subjects, which had very low but nevertheless measurable XO/XD activity [173]. Since red cells cannot synthesize new proteins, the increased activity of red cell XO/XD seen in these patients implies that much inactive XO/XD protein is present in healthy human red cells - surprisingly, these investigators made no reference to Al-Khalidi's conclusions, and to the fact that their results challenged the dogma.

The other report supporting the presence of XO/XD in human red cells is by Tavazzi et. al (2000) [174]. These investigators exposed a suspension of washed, catalase-inhibited (1mM NaN_3) human red cells (5% Hct) to increasing concentrations of H_2O_2 (0-10mM, in phosphate buffer with 10mM glucose). They found an H_2O_2-concentration-dependent decrease in ATP, accompanied by an increase in IMP (see below), inosine, hypoxanthine, *as well as xanthine and urate*!! Again, surprisingly, these investigators made no mention of the fact that red cells do not supposedly have the machinery to make xanthine and urate from hypoxanthine, but their results indicate that horses are *not* unique amongst mammals in having the potential to produce urate within their red cells.

The above-mentioned report by Tavazzi et. al. highlights the possibility that red cell XO/XD may also be activated by a single exposure to *in vitro* or *in vivo* oxidative stress (such as with an exhaustive bout of exercise). However, on attempting to replicate the Tavazzi experiment (for this thesis), despite finding an H_2O_2-concentration-dependent increase in hypoxanthine and decrease in ATP level, no urate or xanthine became detectable, even at the highest H_2O_2 concentration. An opportunity then arose to examine the red blood cells of rats being forced to undergo exhaustive physical exertion. None of the rats however showed any red cell urate production at any of the various time points evaluated after the stressor (data not shown). These findings point rather that XO/XD activity in red cells may only increase detectably on repeated or chronic exposure to oxidative stress (an adaptive-type response). It is hypothesized that red cell basal XO/XD activity in the blood donor in Tavazzi's study might have been greater than in the red cells from the donors used in the current study (fitter individuals, or smokers possibly used in the Tavazzi study?). Tavazzi et. al. subsequently found that, whereas AMP deaminase (see Fig 2, Literature Review and Background Work) from other mammalian cells is paradoxically always inactivated by exposure to H_2O_2, that the AMP deaminase from

red cells is activated by exposure to H_2O_2 [175], explaining how xanthine and hypoxanthine were formed in their earlier study. Therefore, under conditions of oxidative stress, the benefit of providing substrate to produce urate in the red cell under conditions of oxidative stress may be considerably greater than in other cell types (see also Chapter 3).

(v) Free tryptophan: inter- and intraspecies variation

On comparison of the reverse-phase HPLC profiles of the red cells from the various mammalian species, as illustrated in figure 11 of section iii, further species-specific differences in the size of three other peaks were observed. The identities of the LMW substances responsible for these peaks were initially determined by their shared characteristics with known substances present in the standards mix. Support for a suspected identity was always provided by co-elution with a standard solution of that substance.

Species differences in tryptophan levels were evident for both red cells and plasma. A visibly higher level of free tryptophan was present in the red cells of some species compared with humans. Tryptophan, as for tyrosine, is a phenolic, UV-absorbing amino acid. As for tyrosine, although it has numerous well-described physiological roles in nucleated cells, none of these roles are apparently present in the red cell. Significantly however, although little mentioned in the literature, is that tryptophan and its oxidative metabolites have also been shown to have *in vitro* antioxidant activity [132, 176] (see Chapter 2); however, a physiological role in antioxidant defense has not been suggested before.

In human red cells, the mean free tryptophan level was 7.5 $\pm$ 1.6μM (Fig 23A). Beutler's 2001 textbook value is given as 24 $\pm$ 4μM [3], but this value is referenced from publications dating from the 1950's and 1960's. Another report found that tryptophan was observed only irregularly in whole blood and washed red cells [83]. Such discrepancy may be because different washing protocols for the red cells were used, and as for tyrosine, tryptophan may be easily lost from the red cells in the wash steps [83].

As for tyrosine, tryptophan levels in the red cells from many of the other species evaluated were significantly higher compared with human levels, with horses and buffalo having a 5-fold, and cows having a 7-fold higher red cell tryptophan level. Notably, although tryptophan levels were in a much lower range than tyrosine levels, levels of these two amino acids in the red cells seemed to follow a similar species

distribution (compare with Fig 12A, section iii). In other words, the same animal species that had high red cell tyrosine levels relative to humans also had elevated red cell tryptophan relative to humans. Since tryptophan and tyrosine transport into human red cells, occurs via both the L-system and the aromatic amino acid-specific T-system [92, 177], heterogenous expression or activity level of these transport systems amongst different species may be one explanation for the similar trend of intra-erythrocytic levels of these two aromatic amino acids.

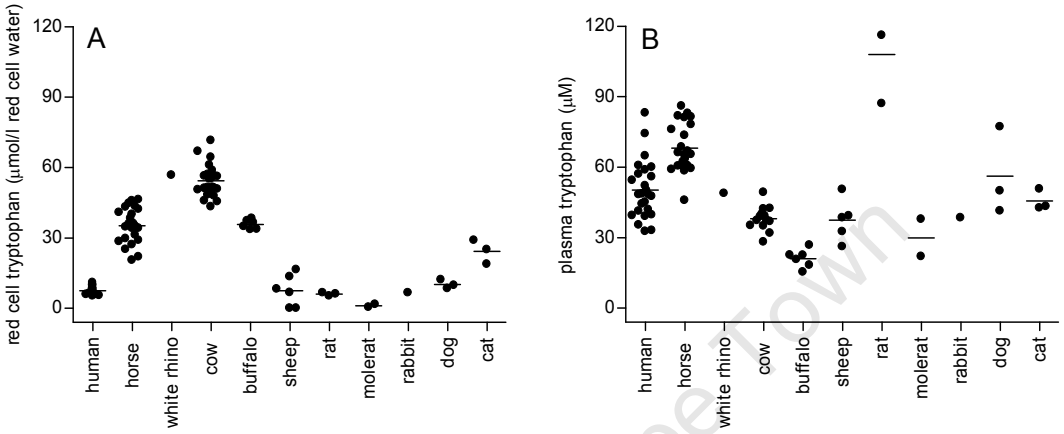


Figure 23. Tryptophan concentrations in the (A) red cells and (B) respective plasmas from various mammalian species, including humans. Horizontal bars indicate means. The Unpaired t test was used to determine *P* values to compare tryptophan levels between species. The Paired t test was used to determine *P* values when comparing plasma and red cell tryptophan levels within species.

However, in contrast with what was found for tyrosine, the mean plasma tryptophan level (Fig 23B) was at least equivalent or higher than the respective mean red cell level for each species, (except for cows and buffalo, see below). The mean plasma tryptophan level in humans was 56.2 $\pm$ 18.7μM (which compares well with a previous report of 45 $\pm$ 9μM [83]). Horses and particularly rats had the highest plasma tryptophan levels. The elevated plasma levels in these animals may be due to dietary factors - for example, horse diets are often supplemented with tryptophan because of its calmative properties [178], and this has been shown to increase plasma tryptophan levels [179]. However, despite the high plasma tryptophan levels, rats had red cell tryptophan levels similar to human levels. Therefore mean E:P tryptophan ratios (as for tyrosine) varied significantly between species. Most interestingly, whereas levels in the red of this essential amino acid cell were generally lower than the respective plasma level, this was not the case for cows and buffalo. In these two related species, red cell tryptophan levels were significantly greater than the plasma levels ($P < 0.0001$).

Surprisingly, red cell tryptophan levels in cows (Fig 24A) and buffalo (not shown), as for humans (Fig 24B), correlated positively with plasma levels, with an E:P ratio of 1.4, 1.7 and 0.15 respectively. In contrast, red cell levels in horses (Fig 24C) and sheep (Fig 24D) showed no correlation with plasma levels.

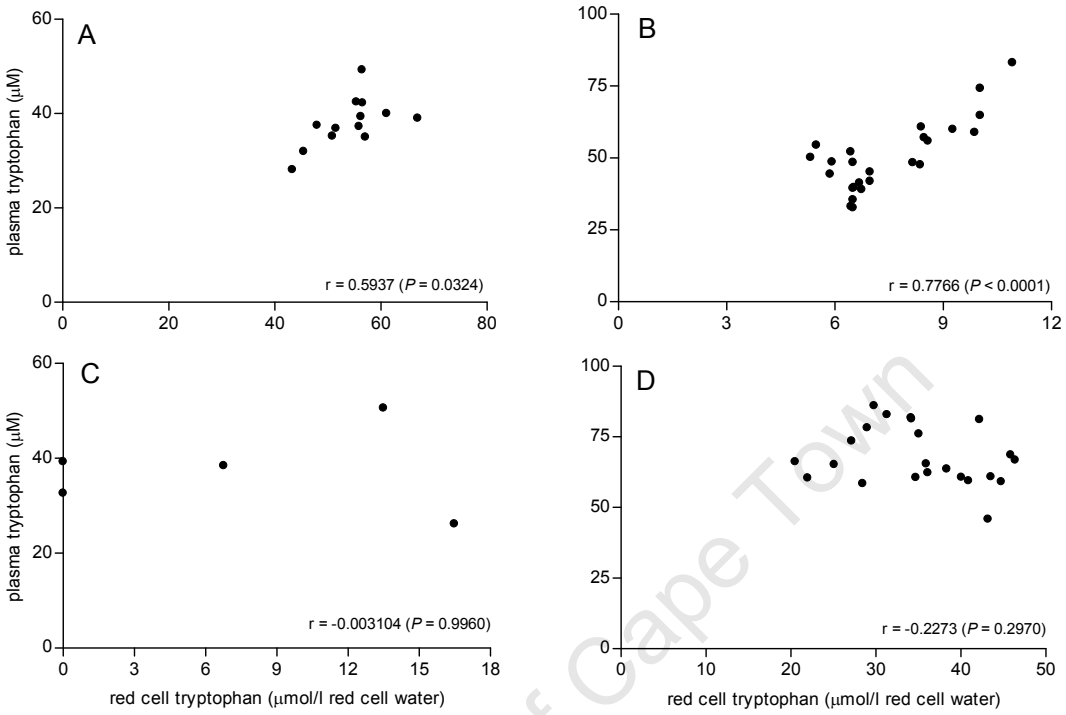


Figure 24. Comparison of the tryptophan concentration within red cells and of plasma for (A) cows, (B) humans, C) sheep and (D) horses. The Pearson test was used to determine statistical significance of correlation, except in (D) where the Spearman r was determined.

Therefore, whereas in humans (and all the other non-bovid species evaluated, particularly rats) there appears to be a relatively restricted entry of plasma tryptophan into the red cells, the red cells from cows and buffalo seem to accumulate tryptophan, analogous to the accumulation of tyrosine in the red cells of some other species. This accumulation is plasma-level dependent. Such active uptake is likely to utilize a significant amount of the red cell's energy, implying an active role for the elevated tryptophan (and tyrosine) levels in the red cell.

(vi) NAD(H) and NADP(H): inter- and intraspecies variation

In addition to the pronounced interspecies variability in red cell levels of urate riboside, urate, tyrosine and tryptophan, levels of the major electron-transfer substances NAD(H) (Fig 25A) and NADP(H) (Fig 25B) were also highly variable between the different species although to a lesser extent, with the red cells from

many of the species evaluated appearing to have much higher levels than seen in human red cells.

It is necessary to note that, although these co-enzymes exist *in vivo* both in an oxidized and reduced form, acid extraction of the red cell contents leads to complete oxidation of all the NADH and NADPH. Therefore, although only NAD^+ and $NADP^+$ (oxidized forms) are present in the extracts, as represented by figures 25A and B, these represent the total NAD(H) and NADP(H) present in the red cells of the various species respectively.

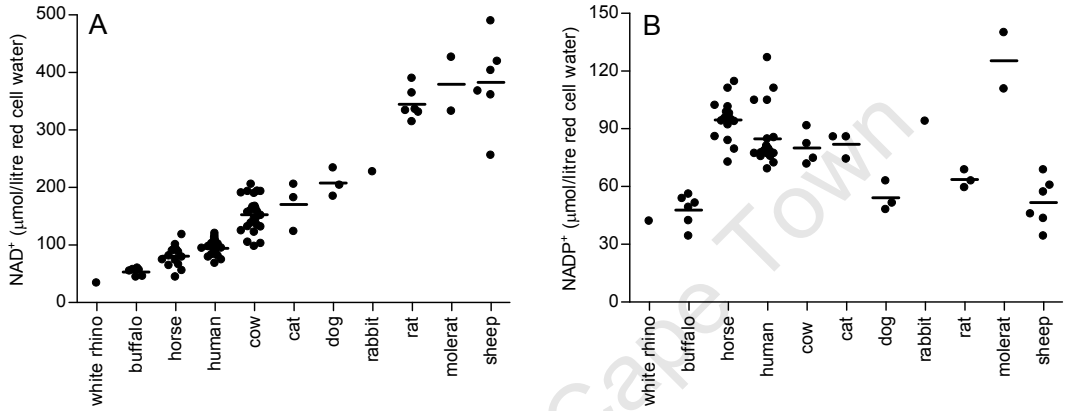


Figure 25. (A) NAD^+ and (B) $NADP^+$ concentrations in the red cells from various mammalian species, including humans. Horizontal bars indicate means.

Surprisingly little comparative data is available on levels of NAD(H), and even less on NADP(H) within red cells. In this study, the mean level of NAD^+ in human red cells was 94.2 $\pm$ 2.6µM - this compares with a previous report by Harkness et. al. (1969) of 70µM NAD in human red cells [27], and another relatively more recent report by Micheli et. al. (1993) of 50.8 $\pm$ 7µM for combined reduced and oxidized NAD(H) in human red cells [180]. The mean level of NAD^+ in sheep red cells was almost 4-fold greater than in human red cells, with similarly high levels in rats and naked molerats. Our finding that both rats and sheep have higher mean levels of NAD(H) than the other species, being 345µM and 383µM respectively, is in accordance with another published report comparing levels in the red cells of rats and sheep with those from horses, rabbits and guinea pigs using a spectrophotometric assay [181]. The only other report found to compare NAD levels amongst various mammals was the study by Harkness et. al. in 1969 using low resolution chromatography [27]. Although these investigators did not measure levels in sheep or rats, they found similarly high levels in dolphins as found for sheep in the current study. Whereas the red cells from rats

clearly had higher concentrations of NAD(H) than was present in the human red cells, rat and human fibroblasts did not show such a difference in NAD(H) levels (HPLC profiles not shown).

Although red cell extract levels of $NADP^+$ also varied between species, the difference in mean level of $NADP^+$ between the species with the lowest (buffalo) and the highest (naked molerat) level was only 2.5-fold, this being much less than the variation for NAD^+ which was almost 8-fold. No other reports comparing NADP(H) levels in different animals were found. The $NADP^+$ and NAD^+ levels clearly did not correlate. As expected, no NAD^+ or $NADP^+$ was present in the plasma extracts (see Fig 11, section iii).

New molecules of both NAD^+ and $NADP^+$ can be synthesized through a salvage pathway in the red cell, whereby NAD^+ is enzymatically synthesized from nicotinic acid and PPRP. After attachment of AMP, glutamine provides an amino group for completion of the synthesis of NAD^+. Enzymatic phosphorylation of NAD^+ by ATP then forms $NADP^+$ [1]. Large oral doses of nicotinic acid have been shown to promote an increase in NAD^+ (but not $NADP^+$) concentration in human red cells [182], so it is at least theoretically possible that differences in NAD(H) levels within the red cells of different species may reflect differences in dietary nicotinic acid intake; this would however not explain the large intraspecies difference found in NAD(H) level in sheep red cells since they all came from the same herd. Notably, tryptophan is a precursor molecule for nicotinic acid, and rats had particularly high plasma tryptophan levels (presumably diet-related) (see Fig 23B, section v). Rats may thus have large amounts of nicotinic acid formed in their plasma. On the other hand, plasma tryptophan levels were relatively low in sheep, and thus are unlikely to explain the elevated NAD(H) in this species. Alternatively the differences in interspecies differences in NADH level may be genetic, having been induced by different species requirements of this important antioxidant within the red cell. The much more consistent levels of NADP(H) amongst mammalian species, and the fact that changes in NAD^+ production do not influence $NADP^+$ production may reflect that maintenance of NADP(H) levels within a narrower range is more crucial to survival.

(vii) *Inter- as well as intraspecies variability in red cell LMW substances at a spectrophotometric glance*

A simple and rapid spectrophotometric diode array scan (absorbance spectrum 220nm-350nm) of red cell acid extracts provides a concise visual illustration of the

more striking differences evident in the red cell extracts of some mammalian species (Fig 26).

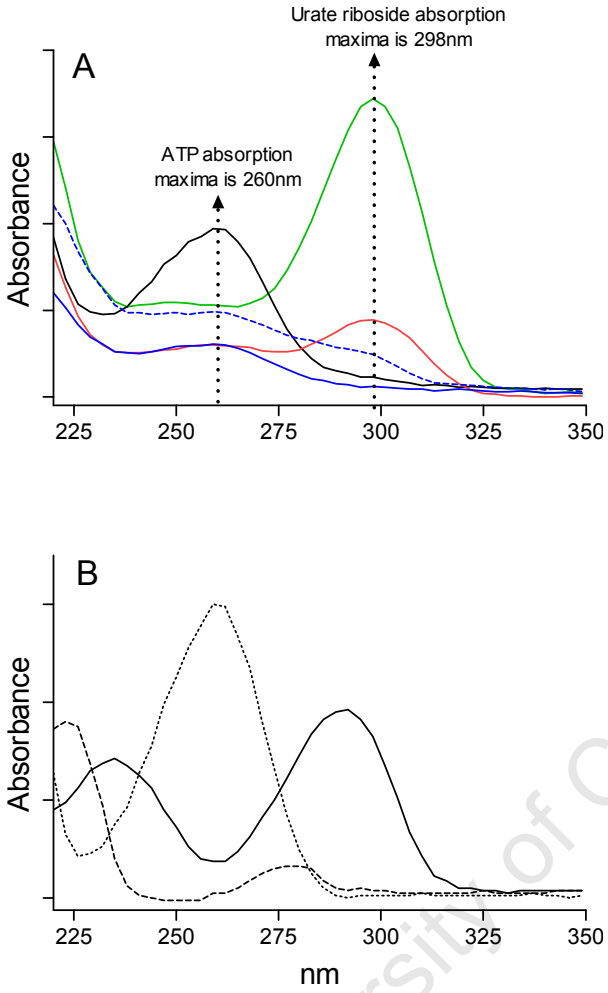


Figure 26. (A) Characteristic absorption profile of diode array scans of the red cell acid extracts of human (black), cow (green) buffalo (red) and horse (blue). The dashed blue line is the extract from a horse that was shown on HPLC to have a large concentration of both urate and tyrosine (horse C in Fig 9). Maximum absorption for urate riboside is 297-298nm, whilst that for urate is 293nm. A nanodrop (Qiagen) was used, pathlength = 1mm.

(B) Diode array scans of standard solutions of tyrosine (dashed), urate (solid) and ATP (dotted) (100μM each in phosphate buffer, pH 7.4). Note that due to the significantly lower extinction coefficient of tyrosine compared with urate, ATP and many other LMW substances, that even large amounts of tyrosine in an extract may at first glance be significantly underestimated when observing HPLC profiles at 260/280nm and most other wavelengths.

The fact that the spectrophotometric diode array pattern of red cell extracts varies so significantly between some animals may enable this method to provide a forensic tool for an initial rapid determination of whether blood is of human origin or not.

Possibly even more useful is that a single absorbance reading of cow and buffalo red cell extracts measured at 298nm correlates significantly with the respective urate riboside HPLC peak area (measured at 280nm) (Fig 27A), since urate riboside completely dominates the HPLC profile at this wavelength. Smith et. al. (1981) quantified urate riboside in cow red cells by extrapolation from a spectrophotometric reading [84], and this same method was used in the current study.

Similarly, in the current study it was further shown that single absorbance readings of horse red cell extracts at 293nm correlate significantly with the respective urate

HPLC peak areas (measured at 280nm) (Fig 27B). Tyrosine level could not however be determined using a single spectrophotometric reading.

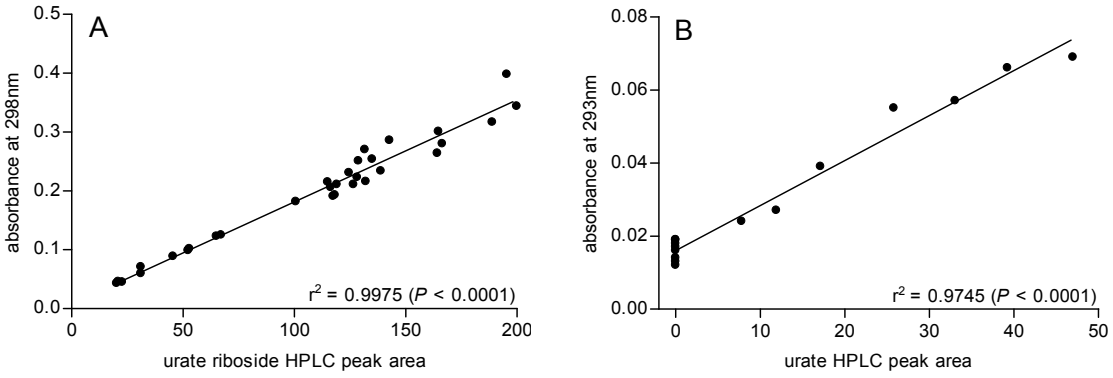


Figure 27. Comparison of a single absorbance reading versus HPLC peak area (measured at 280nm) for (A) urate riboside (single reading at 298nm), and (B) urate (single reading at 293nm). A nanodrop (Qiagen) was used, pathlength = 1mm. The Goodness of Fit test was used to determine linear regression.

Thus a single spectrophotometric reading, as opposed to HPLC, may be used as a simpler, more rapid and cost-effective method to determine levels of some of these substances in acid extracts. This may be particularly useful if levels of these substances are found to be useful biomarkers of oxidative stress.

(E) SUMMARY

Using ion-exchange HPLC, interspecies differences in the variety and levels of UV-absorbing LMW substances within red cells have previously been reported to exist. For example, many mammalian species have significantly lower levels of red cell ATP compared with humans. However the identities of some unretained substances responsible for large early-eluting peaks seen on ion-exchange HPLC of red cell extracts from some species were never determined. In order to begin to clarify some of these evolutionary differences, a combination of anion-exchange and reverse-phase HPLC was used to determine the identity of the LMW substances accounting for some of the species-specific variability in red cell profiles. Levels of these substances in the red cells and plasma of the various mammalian species were determined, and are summarized in table 2 below.

Compared with levels in human red cells, high levels of tyrosine were found in the red cells not just of the *Perissodactyla* Order, as was anticipated, but also in various other mammalian Orders (*Carnivora* and some *Artiodactyla*). In the red cells of the same species, levels of tryptophan were also significantly higher than for humans. Urate riboside was the prominent peak in the red cells from cows and buffalo and seems to be specific to the *Bovinae* Subfamily. Its absence in both the plasma and fibroblasts from these animals indicates that its formation is likely to be limited to their red cells. The red cells from sheep, rats and naked molerats had particularly high levels of NAD(H) (which is not present in plasma) relative to the red cells from the other species, whereas NADP(H) showed less interspecies variation. The red cells from horses were particularly interesting; in addition to having high levels of tyrosine, horses were the only mammalian species (other than humans) to have urate in their red cells, with extraordinarily high levels (800μM) found in the red cells of some horses. Unlike for humans where red cell urate may be explained by the high urate levels, plasma levels of urate in horses were negligible (as expected), and could not therefore account for the high levels of red cell urate in this species. Similarly, plasma levels of tyrosine were very similar and low across all mammalian species, and could not account for the very high levels of tyrosine found in the red cells of some species. In cows and buffalo, red cell levels of tryptophan were also higher than plasma levels. Therefore, it may be concluded that the red cells of these mammalian species must be actively accumulating such LMW substances.

Table 2. The mean levels of various soluble antioxidants found in the red cells/plasma (where applicable) from various mammalian species relative to levels in human red cells (set as the 100% control), as determined from the reverse-phase HPLC profiles. In brackets are the actual concentrations (μM) of these substances. Where levels are greater than that present in human red cells/plasma, these are highlighted in blue. Urate riboside is not included because it is undetectable in all species other than *Bovinae*.

	Tyrosine	Tryptophan	Urate	NAD(H)	NADP(H)
Human (25)	100/100 (68/60)	100/100 (8/50)	100/100 (118/238)	100 (94)	100 (85)
Horse (20)	410[$]/95 (279[$]/57)	470/136 (38/68)	153[$]/1 (181/2)	86 (81)	111 (94)
White rhinoceros (1)	1485[*]/112 (1010[*]/67)	757/98 (61/49)	0/17 (0/40)	36 (34)	49 (42)
Sheep (6)	150/143 (102/86)	100/75 (8/38)	0/1 (0/2)	407 (383)	61 (52)
Cow (24)	[#]/83 ([#]/50)	720/76 (58/38)	1/9 (1/21)	163 (153)	94 (80)
Buffalo (6)	[#]/48 ([#]/29)	477/42 (38/21)	1/9 (1/21)	56 (53)	56 (48)
Rat (3)	56/189 (38/113)	80/216 (6/108)	6/15 (7/36)	367 (345)	75 (64)
Naked molerat (2)	0/118 (0/71)	13/60 (1/30)	2/13 (2/31)	404 (380)	147 (125)
Rabbit (1)	63/115 (43/69)	88/77 (7/39)	0/2 (0/5)	241 (227)	110 (94)
Dog (3)	153/102 (104/61)	135/112 (11/56)	0/8 (0/19)	221 (208)	64 (54)
Cat (3)	537/75 (365/45)	324/91 (26/46)	0/5 (0/12)	181 (170)	96 (82)

Note: [$] Because of the skewed data, these values significantly underestimate the very high levels present in some horses.

[*] This value in 8 black rhinoceros was 778.8μM, and 1145% that for humans [2].

[#] Unable to determine this value from the HPLC profiles (see Results and Discussion, section iii).

Whereas evolutionary adaptations are likely to explain most of these interspecies differences in red cell LMW substances, the accumulation of large amounts of urate in the red cells of horses is thought to reflect an inducible biochemical pathway of urate production which may be common to the red cells of many, or even possibly all mammals - it is proposed that mammalian red cells have inactive XO/XD enzyme, which may become activated, and even upregulated under certain conditions, including regular physical exertion as in the case of the athlete horses sampled for this study.

The unexpectedly large degree of intraspecies variation in red cell levels of some of these substances, particularly of tyrosine and urate in horses, and urate riboside in cow/buffalo reflects significant intraspecies genetic heterogeneity for the ability to accumulate these substances in the red cells from these species.

CHAPTER 2

Investigating a role for free tyrosine and tryptophan in endogenous antioxidant defense

(A) INTRODUCTION

In Chapter 1, some of the differences in the variety and level of red cell UV-absorbant, low molecular weight (LMW) substances amongst mammalian species were clarified and quantified. High levels of free tyrosine and tryptophan, as well as NAD(H), NADP(H), urate and urate riboside were shown to be accumulating in the red cells of some species. It is logical that red cells would only make use of their limited energy supplies to synthesize/accumulate such substances if this served a useful physiological role. Urate and NADH both have well-established antioxidant functions, although an antioxidant role for urate within red cells has never been suggested. Urate riboside, a LMW substance unique to the red cells of some bovids (see Chapter 1, Background Work and Literature Review), has been shown to have similar *in vitro* antioxidant properties to urate [36-38].

Specifically within the mature, anucleate mammalian red cell, elevated levels of tyrosine and tryptophan would not be required for protein synthesis. Although these amino acids also serve as precursor molecules for acetyl-CoA and various other specialized biomolecules such as melanin, epinephrine, thyroxin, and serotonin [183], neither the Krebs cycle nor any of the other specialized biochemical pathways are represented in the mature red cell. Could the significant accumulation of free tyrosine and tryptophan in the red cells of some mammalian species be revealing/illuminating an unsuspected role for these LMW substances in antioxidant defense?

(B) LITERATURE REVIEW

Reduction-oxidation (redox) reactions occur continuously as part of an O_2 -rich (21%) environment for both biotic and abiotic processes, for example the (abiotic) rusting process. Although O_2 is poorly reactive with most biological molecules, oxidation reactions do nevertheless occur in biological organisms, thought probably to involve the presence of transition metal ions. The more reactive superoxide ($O_2^{\cdot -}$) free radical is produced, which will then further accelerate the oxidation process [8]. The simplest defense to limit such non-specific oxidation reactions is to reduce O_2 concentration within the organism. In complex organisms such as mammals, the circulating red blood cells safely deliver a minimum intracorporeal amount of O_2 to the peripheral tissues by regulating its release from haemoglobin (Hb) as required.

Many reactive species (RS), including reactive oxygen species (ROS) such as $O_2^{\cdot -}$, reactive nitrogen species (RNS) and reactive chlorine species (RCS) are however generated 'deliberately' under physiological conditions within cells. One of the largest sources of RS in most nucleated animal cells is the respiratory chain in the mitochondria that converts molecular O_2 to water. A few percent of O_2 molecules continuously leak from the electron transport chain as $O_2^{\cdot -}$, which will then form hydrogen peroxide (H_2O_2) enzymatically, or by spontaneous dismutation. This process alone results in a substantial basal level of oxidants *in vivo*. Although the mammalian red cell is spared from producing mitochondrial-derived $O_2^{\cdot -}$, a low continuous flux of $O_2^{\cdot -}$ and hence H_2O_2 is produced within the red cell via the auto-oxidation of oxyhaemoglobin (oxyHb) to methaemoglobin (metHb) - approximately 3-4% of the oxyHb is estimated to undergo auto-oxidation every day [184, 185].

Investigation of the physiological role played by H_2O_2 and other RS in intracellular signaling via redox reactions is a rapidly developing field [8, 186, 187]. The RNS, nitric oxide ($NO^{\cdot}$), is synthesized within various cells by the $NO^{\cdot}$ synthase enzymes and has many important physiological roles [8]. The enzyme xanthine oxidase/xanthine dehydrogenase (XO/XD) (see Chapter 1, Results and Discussion, section iv) can produce $O_2^{\cdot -}$, thought to play a role in defending the gastrointestinal tract against pathogens [188]; XO/XD also produces $NO^{\cdot}$ [189]. Phagocytic cells produce large amounts of intracellular $O_2^{\cdot -}$ via NADPH oxidase during the respiratory burst, and this $O_2^{\cdot -}$ has been shown to be crucial in the eradication of certain bacteria. Hypochlorous acid (HOCl) is produced in activated neutrophils by the heme enzyme myeloperoxidase (MPO) through chlorination of H_2O_2 [109]. Although HOCl is microbicidal and cytotoxic *in vitro*, its actual role in bacterial killing has been

questioned [190]. RS such as H_2O_2 and NO· produced within cells readily move across membranes into the extracellular fluid and plasma [8, 191], and HOCl is released into surrounding tissues during an inflammatory reaction. The $O_2^{\cdot -}$ radical however does not readily cross biological membranes unless specific channels exist [8], such as the anion channel of the red cell [192].

Although $O_2^{\cdot -}$ and H_2O_2 themselves react relatively slowly with most biological molecules, reaction of $O_2^{\cdot -}$ and H_2O_2 with other molecules to form more highly reactive RS leads to significant cellular damage. Such oxidative damage has been implicated as a primary or at least contributing pathophysiological factor in a wide range of disease processes [8]. For example, H_2O_2 will react with unbound transition metal ions, especially iron, manganese and copper ions via Fenton chemistry to form the highly oxidising hydroxyl radical (OH·). The OH· radical reacts with a wide range of molecules and will damage DNA, proteins and lipids. Carbon-centered radicals may be formed (eg. in lipid membranes) on reaction with OH·. These carbon radicals can then combine with O_2 to form peroxyl radicals, which themselves oxidize adjacent lipids, thus initiating the propagation of lipid peroxidation [8]. Peroxyl radical formation and peroxidation of proteins also occurs *in vivo* [193-195]. Similarly, the reactivity of the physiologically important NO· molecule is greatly enhanced by its rapid reaction with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$), which can lead to both the oxidation and nitration of various susceptible biological molecules [196]. HOCl is a highly reactive oxidizing and chlorinating species, and has been shown to be involved in causing inflammation-associated cellular damage [109, 197]. Singlet oxygen, formed on photosensitisation of some molecules which then transfer the excitation energy to an adjacent O_2 molecule, can be highly damaging and plays a large part in the aging process (especially in the skin and eye). It may also be produced in activated neutrophils [8]. In addition to being formed endogenously, various damaging RS may be also be derived exogenously (eg. oxidant drugs).

In order to survive an oxidizing environment, aerobic organisms have necessarily evolved an array of antioxidant defense mechanisms. A “disturbance in the prooxidant-antioxidant balance in favour of the former”, is the classic definition of ‘oxidative stress’ by Sies (1991) [198], and is most usually initiated by an increased level of RS. Oxidative stress can eventually lead to oxidative damage, including cell death, if adaptation by upregulation of the antioxidant defenses does not re-establish the prooxidant-antioxidant balance. The new simplified definition of an antioxidant according to Halliwell and Gutteridge (2007) [8], two names well-known in the field of free radical research, is “any substance that delays, prevents or removes oxidative

damage to a target molecule". The term antioxidant defense thus encompasses many different substances that all ultimately serve to limit oxidative damage.

Antioxidants may be classified in various ways [8], including whether they are proteins or LMW antioxidants. The antioxidant proteins may perform their role enzymatically or non-enzymatically. Proteins such as transferrin and ferritin act by decreasing the formation of damaging RS; they sequester and thus minimise the availability of pro-oxidant iron ions within plasma and nucleated cells respectively. Other proteins such as albumin can act as antioxidants by scavenging various RS.

Three enzyme processes, namely superoxide dismutase (SOD), catalase and the glutathione peroxidase (GPx)/reduced glutathione (GSH) system are responsible for the catalytic removal of $O_2^{\bullet -}$ and H_2O_2. The SOD family of enzymes is responsible for removing $O_2^{\bullet -}$ by accelerating its dismutation to H_2O_2.

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

The SOD enzymes are present in almost all eukaryotic cells [8]. Copper and zinc-containing SOD is located mainly in the cytosol and is composed of two protein subunits, each of which has one copper and one zinc ion at the active site. Manganese-containing SOD (from higher organisms) is composed of four protein subunits, and contains manganese at its active site. It is located largely in the mitochondria. Both SODs are also present in the peroxisomes of cells [199]. The selenium and manganese ions at the active sites of these SODs catalyse the dismutation reaction by undergoing alternate oxidation and reduction [8].

Catalase and GPx then both function to remove the H_2O_2 (GPx can also remove organic peroxides [200]). Catalase is present in the cytoplasm, peroxisomes and nucleus of eukaryotic cells [199]. Especially high levels of this enzyme are present in liver and red blood cells [8]. Catalase contains four subunits, each of which has a Fe(III)-haem at its active site [201]. As shown in the reaction below, catalase donates 2 electrons to reduce one H_2O_2 molecule and forming compound I, where the iron is an Fe(IV) species with an additional electron having been removed from the porphyrin ring to form a porphyrin-cation radical (haem$^{\bullet +}$), and sometimes referred to as $^+$heme-Fe(V)=O [9]. Compound I then receives 2 electrons from another H_2O_2 molecule to return to its ferric state (the H_2O_2 is oxidized to water and oxygen in this step) [8].

catalase Fe(III) + H_2O_2 → compound I + H_2O

compound I + H_2O_2 → catalase Fe(III) + H_2O + O_2

Net : $2H_2O_2 \rightarrow 2H_2O + O_2$

The catalytic rate of mammalian catalase activity is amongst the highest of the known enzymatic rates [202]. Catalase activity is proportional to the H_2O_2 concentration over a wide range of the latter i.e. it is efficient at high H_2O_2 concentrations, but slower at destroying low concentrations of H_2O_2. Certainly catalase appears to be essential for protection against higher levels of H_2O_2 - acatalasemic red cells are very sensitive to exogenous H_2O_2 [203]. The finding that metHb levels in acatalasemic red cells from both humans [203] and mice [204] are normal supports the proposal that catalase has a limited role to play in unperturbed red cells. During exposure to lower concentrations of H_2O_2, slower peroxidatic reactions of catalase are noticeable, and the one-electron reduction of compound I can also form inactive catalase (compound II). In this way, exposure to H_2O_2 that is generated at a constant rate, catalase can reach a steady state in which much of it is inactive. One role of bound NADPH may be to help maintain enzyme activity by acting as a preferred electron donor to compound I under conditions where H_2O_2 supply is limited. NADPH may also reconvert compound II to active catalase [202].

The GPx family of enzymes is widely distributed in animal tissues, and is present in all cellular compartments [199], with especially high levels present in liver and kidney tissue. At least four types exist - cytosolic GPx (GPx1), is the classical enzyme, while another type of GPx (GPx2) is present in the cells lining the gastrointestinal tract as well as liver cells. GPx3 is present in plasma. GPx1-3 are each composed of four protein subunits, each of which bear an atom of selenium (present as selenocysteine) at the active site [8]. The GPx enzymes, unlike catalase, follow Michaelis-Menten relationships and efficiently remove low concentrations of H_2O_2, as well as organic peroxides, by coupling reduction of these RS with the oxidation of the cofactor GSH to form GSSG [207]. It has been proposed that the primary physiological role of GPx lies in its ability to detoxify organic peroxides [205, 208].

$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$

$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$

The GSH cofactor, a tripeptide, is synthesized in the cytoplasm of all animal cells, the liver being the most active organ [8]. High levels of GSH are present intracellularly eg. 2mM in the red cells [1]. Catalase activity is also dependent on the reducing potential of its attached reduced NADPH cofactor [209], which can provide the required 2 electrons to compound I when H_2O_2 levels are low, so as to limit formation of the inactive compound II [8]. Hence, maintenance of an adequate baseline level of the reduced forms of the LMW cofactors GSH and NADPH via the hexose monophosphate (HMP) shunt (see below) is crucial to the adequate functioning of the 2 main H_2O_2-removal systems in cells. In the red cell (Fig 28), the continuous removal of H_2O_2 is particularly necessary because the auto-oxidation of OxyHb is accelerated by H_2O_2, as well as by various other oxidants. Excessive MetHb formation leads to reduced oxygen-carrying capacity, and haemoglobin denaturation, precipitation and cell lysis may occur if red cells are exposed to sufficient oxidative stress [210, 211].

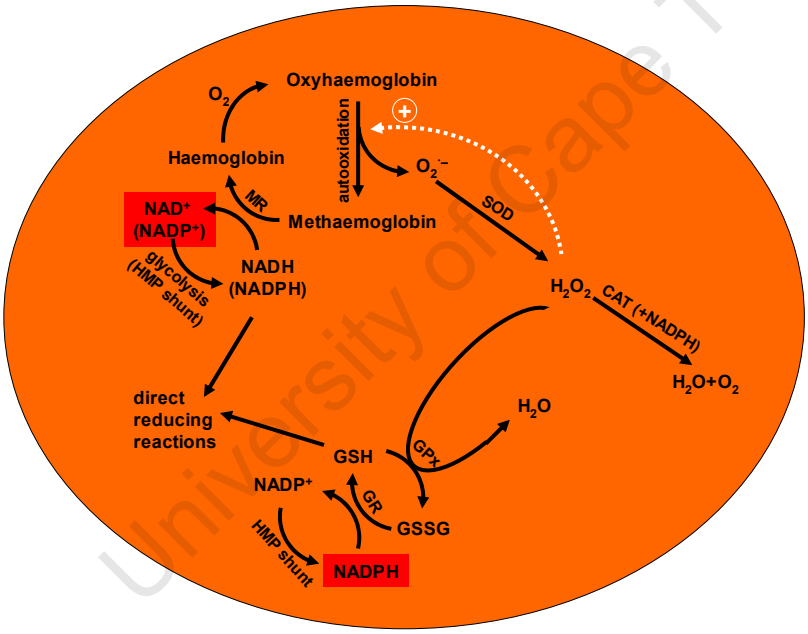


Figure 28. Schematic representation of $O_2^{\cdot-}$- and H_2O_2 - neutralising pathways within the red cell. All reactions, other than oxyhaemoglobin auto-oxidation and reduction, also occur in other cell types. The source of $O_2^{\cdot-}$- and H_2O_2 in nucleated cells is mainly from the respiratory chain in the mitochondria. (SOD=superoxide dismutase; CAT=catalase; GPx=glutathione peroxidase; GR=glutathione reductase; MR=methaemoglobin reductase)

Oxidised $NADP^+$ is reduced back to NADPH via the HMP shunt (see also Fig 1, Chapter 1). In turn, NADPH serves to provide the electrons for the reduction of GSSG back to GSH by the enzyme glutathione reductase (GR). These cofactors are

therefore continuously cycled between the oxidized and reduced forms without being consumed. The balance (ratio) between the oxidised and reduced forms of these substances is an important component of what is termed the redox state of cells [212]. Under physiological conditions, most (up to 99%) of the cellular glutathione exists in the reduced GSH form [8]. Similarly, the $NADP^+$/NADPH ratio is approximately 0.04 in human red cells [180] (and about 0.005 in other mammalian tissues [213]). Regulation of glucose flux through the HMP shunt is dependent on the availability of $NADP^+$ [1, 15], but is also inhibited by NADPH [214]. Any oxidant substance/drug that oxidizes GSH, and hence increases the $NADP^+$/NADPH ratio, will stimulate the shunt. In the normal red cell, the rate of the HMP shunt can be increased significantly by shunt activators [40], and by as much as forty-fold [215].

As shown in figure 28, NADH plays a vital role in the red cell as the main electron-donating cofactor for the reduction of metHb back to Hb via methaemoglobin reductase (MR), although NADPH can function as an alternative cofactor [216]. The oxidised form, NAD^+, is reduced back to NADH via glycolysis. In contrast with the $NADP^+$/NADPH, the NAD^+/NADH ratio in healthy mammalian tissue has been reported to be around 1 [212]. However within red cells (human), the ratio is approximately 27 [180]. Therefore in the red cell specifically, low levels of the reduced form relative to the oxidized form are maintained, this however obviously being sufficient to provide the electrons for the critical reduction of metHb, which is maintained so as to account for only 1% of total Hb [217]. In fact, any surplus NADH in the red cell is oxidised back to NAD^+ by the reduction of pyruvate to lactate, this being the major product of glycolysis in red cells under normal circumstances [1].

Extracellular fluids including plasma contain little or no catalase activity. Only low activities of SOD and GPx (the GPx3 form only) are present in plasma [218] and in some extracellular tissues such as in seminal plasma [219, 220], the extracellular tissues of the eye [8, 221] and the mucus linings of the respiratory and intestinal tracts [8]. Therefore unlike within the intracellular environment, extracellular fluids including plasma do not have significant $O_2^{\bullet -}$- or H_2O_2- removing capacity. Levels of GSH in plasma are also much lower than within cells, being only 2μM in human plasma [222]. Higher levels (40-200μM) are however present in alveolar lining fluid [8]. GSH, as for NADH and NADPH, can also donate electrons directly to various RS [8, 223], for example HOCl [224] and $ONOO^-$ [225] *in vitro*. Therefore, in tissues where GSH is present at high concentrations, direct scavenging of these RS is feasible *in vivo* [8].

Cells and extracellular fluids including plasma are also equipped with an array of LMW antioxidant substances, many of these acting as 'sacrificial agents' by being preferentially oxidised by RS to prevent damage to essential enzyme systems" biomolecules. These LMW antioxidant substances are either synthesised *in vivo* (eg. urate), or are derived from the diet (eg. vitamin C/ascorbate, which cannot be synthesized by some vertebrates, including humans [226]). Besides its important role as cofactor for numerous enzymes, and various other metabolic functions, *in vitro* data suggests that ascorbate is likely to be providing significant *in vivo* LMW antioxidant protection against various RS. Mammalian cells accumulate ascorbate from the surrounding body fluids against a concentration gradient, with intracellular levels reaching millimolar concentrations. Levels in plasma are relatively low (30-90μM), but are higher in cerebrospinal fluid, aqueous humour of the eye, seminal fluid, gastric and respiratory tissue fluids. Ascorbate is a powerful reducing substance, reacting with most RS including the radicals formed from oxidation of other antioxidants such as urate and α-tocopherol radicals. The one-electron oxidation of ascorbate yields the semidehydroascorbate (SDA) radical (also known as ascorbyl). This product is very non-reactive, this being the essence of ascorbate's antioxidant activity. Left to themselves, 2 ascorbyl molecules will undergo slow disproportionation, regenerating some ascorbate and forming dehydroascorbate (DHA).

2SDA → ascorbate + DHA

DHA is unstable and breaks down into a mixture of products. In healthy mammalian tissues, the ascorbate/DHA ratio is high, and most cells possess enzymes that convert DHA or SDA back to ascorbate at the expense of GSH or NADH [8]. In addition, red cells (as well as neutrophils) take up plasma DHA and convert it back to ascorbate [227](see also Chapter 3).

Vitamin E is believed to be one of the most important endogenous, lipophilic LMW antioxidants. Yet, as for vitamin C, although much *in vitro* evidence of its antioxidant activity exists, evidence for its antioxidant effect in healthy humans is limited [8]. Vitamin E is not a specific substance, but rather the term Vitamin E is a nutritional term first used to refer to a fat-soluble 'factor', a dietary lack of which caused a variety of specific symptoms in various animals. Vitamin E is now known to refer to eight naturally-occurring substances that have such lipophylic anti-oxidant activity, α-tocopherol being one of these substances. Blood tocopherol levels in healthy humans are 20-35μM. Because α-tocopherol is fat soluble, it concentrates in the

interior of cell membranes and in lipoproteins. The significant antioxidant activity of α-tocopherol is by virtue of its ability to inhibit lipid peroxidation - it is able to scavenge lipid peroxyl radicals before they have a chance to react with the adjacent fatty acid side chain. Alpha-tocopherol is capable of reacting with a second peroxyl radical, producing a non-radical product. Various LMW antioxidants (including GSH) are known to be able to reduce the α-tocopherol radical back to α-tocopherol *in vitro*, with ascorbate-dependent recycling thought to be the most important *in vivo*. Singlet oxygen is also quenched by α-tocopherol [8].

Urate, traditionally considered to be a metabolically inert end-product of purine metabolism, is believed to provide potent antioxidant protection, particularly at the very high levels present in human plasma (mean of 350μM) (see also Chapter 1, Results and Discussion, section iv). The concentration of urate in human plasma is several-fold higher than that of the other recognized LMW antioxidant substances present in plasma, namely ascorbate and α-tocopherol [39, 228]. In humans, urate is also present intracellularly [8], including within red cells (approximately 120μM), and in various other body fluids such as saliva (100-200μM) [229], and respiratory tract lining fluids (90-250μM) [230].

Urate can act as an antioxidant by binding iron and copper ions [231], and also by directly scavenging many different RS *in vitro*. It reacts with ozone [232], $ONOO^-$ [233, 234], HOCl [235], singlet oxygen and peroxyl radicals [39]. Urate has also been found to stabilise ascorbate in biological fluids, without itself being consumed. This stabilizing effect appears to be due to an inhibition of iron-catalyzed oxidation of ascorbate due to iron chelation by urate [236]. Urate is able to protect red cell membrane ghosts from lipid peroxidation [39], and cell culture experiments have been used to show urate's ability to protect other cells against various RS such as ozone [237, 238]. In isolated organ preparations, urate protects against reperfusion damage induced by activated granulocytes, cells known to produce a variety of radicals and oxidants. There is now evidence for such processes not only *in vitro* and in isolated organs, but also in the human lung in vivo [239-241], a site of direct exposure to many exogenous RS, and which has relatively high urate levels. Infusion of urate was also found to have neuroprotective effects in some animal models [142, 143], and infusion of urate into humans during severe physical exertion leads to a smaller rise in markers of oxidative damage [242]. In the process, urate radicals may be formed that are themselves capable of oxidizing other molecules [8]. Ascorbate is able to reduce these radicals [243]. Otherwise urate is converted to innocuous products such as allantoin. Allantoin, as well as oxonic/oxaluric and parabanic acids

and other unidentified products, are also formed when urate reacts with H_2O_2 plus Hb (or hemin) [235] (see also Chapter 3, Literature Review). An increased concentration of allantoin has been observed in the body fluids from patients with various chronic conditions known to be associated with oxidative stress, such as rheumatoid arthritis [244], chronic renal failure [245] and diabetes [246], as well as after acute exhaustive exertion [104, 105] and after short-term whole body exposure to extreme cold [160], this taken as evidence that urate does indeed react with RS *in vivo*.

Many other LMW substances have been shown *in vitro* to have antioxidant activity against various RS, but their contribution to antioxidant defense *in vivo* is still unconfirmed (eg. bilirubin, ergothioneine [8])

Measures of the antioxidant capacity of biological samples can either focus on determining levels of individual antioxidants (eg Vitamin C content), or alternatively can act to provide an estimation of the total antioxidant capacity (TAC) of the sample. The latter approach attempts to 'summarise' the activity of all antioxidants present in the sample into a single value. Various 'TAC assays' exist, for example the 'oxygen radical absorbance capacity' (ORAC) and the 'total radical trapping antioxidant parameter' (TRAP) assays. Such TAC assays and have been used to evaluate and compare the antioxidant capacity of a range of pharmacological compounds, foods, beverages [247, 248] and also biological samples such as plasma and seminal plasma. A change (usually a decrease) in plasma TAC in individuals over time, or between healthy controls and diseased individuals, is often used in clinical studies as an indirect measure of the association of oxidative stress with the event or disease process, or of response to antioxidant therapy [8, 249].

The contribution of a putative antioxidant to the sample's TAC can also be determined, this being calculated from its concentration in the sample and its TAC value (determined using a standard solution of the substance). In this way, urate has been found to be a major contributor to the TAC of human plasma with several of the TAC assays [8]. Important to note, however, is that by virtue of the different chemistry behind these assays (in particular different oxidants), and because the time-course over which they are conducted is different (see Results and Discussion), the various assays to measure TAC contributions often do not always give the same results [249]. Using the TRAP assay, for example, the major contributors to the TAC of human plasma were found to be protein (10-50%), urate (35-65%), ascorbate (up to 24%) and vitamin E (5-10%) [250]. Using the ORAC assay on total/whole ($ORAC_T$) and protein-precipitated ($ORAC_{LMW}$) human plasma samples, it has been shown that

the precipitated fraction (which would include proteins as well as lipoproteins and their associated lipophilic antioxidants) account for 85-90% of the $ORAC_T$ value of human plasma against peroxyl radicals [116, 251]. Another study showed that the lipophilic components of plasma account for 28-33% of this value [252]). Hence, certainly if using the ORAC assay and the interest is in LMW antioxidant molecules, proteins in plasma samples should first be precipitated, allowing determination of the TAC of just the LMW components of the sample (TAC_{LMW}) in order to prevent 'masking' of the LMW substance's antioxidant value by the proteins.

In human plasma the sum of the TAC contributions from the known plasma antioxidants is less than the TAC of plasma i.e. there are as yet 'unidentified' antioxidant components. This could reflect synergy between antioxidants such that they work better in combination than individually. This could also reflect a contribution from unsuspected LMW antioxidant substances to the TAC_{LMW} of plasma [8]. Various pieces of scientific evidence exist that together seem to provide sufficient grounds for the proposal that endogenous levels of free tyrosine and/or tryptophan may be playing a yet insufficiently appreciated antioxidant role. This evidence is presented next.

Most tyrosine molecules in biological tissues are present as residues within proteins. These tyrosine residues are targets for redox reactions. Much attention has therefore focused on the reactions of *protein-bound* tyrosine residues with various RS. Although reaction with these protein residues often eliminates the initiating RS to form more stable, non-reactive products (see below), these reactions are generally unfavourable because the protein is usually damaged and marked for degradation [253-255].

In vitro experiments show that free tyrosine is a target for most of the same RS that attack protein-bound tyrosine including singlet oxygen [256], $ONOO^-$ [257] and HOCl [109]. As for protein-bound tyrosine residues, the oxidation of free tyrosine leads to various end-products, depending on the RS. For example, exposure of free or protein-bound tyrosine to singlet O_2 forms tyrosine peroxides [256] which can be removed by GSH and ascorbate and the GPx/GSH enzyme system (although the latter cannot remove protein-bound peroxides, consistent with substrate size being a key factor) [258]. Exposure to most other RS leads to the formation of free or protein-bound tyrosyl radicals. Free and protein-bound tyrosyl radicals may be reduced back to tyrosine by various reducing substances such as GSH, ascorbate, urate, and also free tyrosine [259-264]. Alternatively, tyrosyl radicals may dimerise to form dityrosine

via the ortho-ortho cross-linkage of two tyrosyl radicals [265] (Fig 29). Dityrosine is also formed when tyrosine reacts with H_2O_2 plus a non-specific peroxidase (for example horseradish peroxidase [265] or Hb [4, 254]) – see also Chapter 3. Dityrosine is a very stable end-product [6], and will therefore not take part in further oxidation reactions.

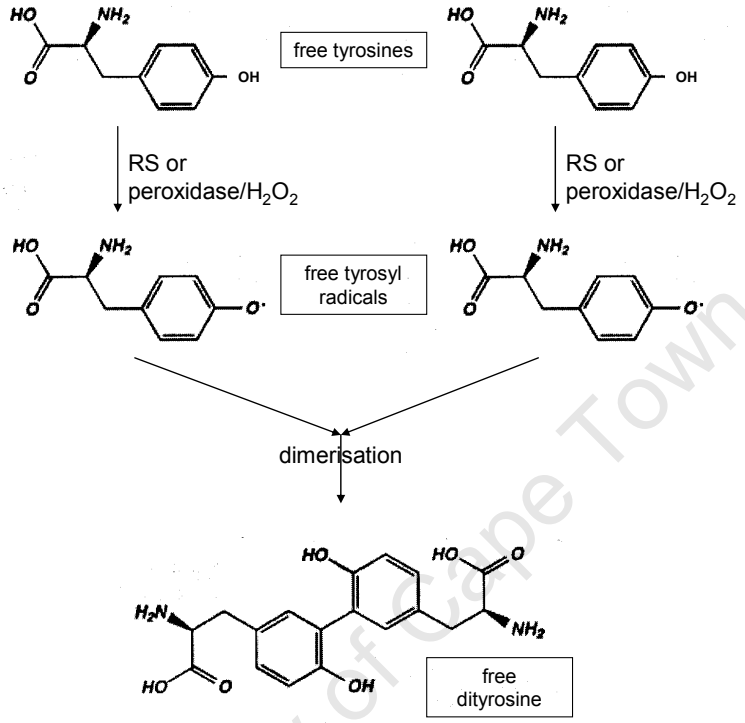


Figure 29. Free tyrosyl radical and dityrosine formation via the oxidation of free tyrosine by various RS, or the peroxidase/H_2O_2 antioxidant system (adapted from DiMarco et. al. (2007) [6]).

When dityrosine crosslinks form between protein-bound tyrosyl radicals, this can lead to protein unfolding and eventual degradation. However, Moosmann et. al. (2000) have suggested that tyrosine and tryptophan residue-enriched peptides representing stretches from the transmembrane domains of integral membrane proteins serve to protect cells from oxidative destruction, with these amino acids acting as 'sacrificial' residues [176]. The distributional pattern of such tyrosine and tryptophan-enriched peptides seems to constitute a general principle of membrane protein architecture, being found in many diverse classes of membrane proteins independent of their primary function, and they do not seem to be a requirement for membrane protein stability or correct insertion. Moosmann et. al. suggested that oxidized tyrosine and tryptophan residues could then be reduced by various electron-donating LMW substances. They also suggested that this may explain the high vulnerability of low-

protein, neuronal membranes to oxidative stress, as is seen in neurodegenerative disorders [176].

Free tyrosine may, as for these membrane bound tyrosine residues, play a role in antioxidant defense as a 'sacrificial' amino acid by being preferentially targeted by RS. Phenolic ring-containing substances are known to have antioxidant potential [266], and various phenolic compounds, including tyrosine, have more recently been investigated for their antioxidant properties against $ONOO^-$ and as potential therapeutic drugs to limit oxidative damage occurring in inflammatory conditions. Van Dyke et. al. (2007) showed that free tyrosine in micromolar amounts had significant *in vitro* antioxidant activity against this physiological RS [196].

The possibility that endogenous free tyrosine may be playing an important antioxidant role *in vivo* is supported by the study of van Overveld et al. (2000), which investigated the antioxidant activity of seminal plasma [66]. Reactive species are produced within this fluid which bathes spermatozoa. The presence of RS in seminal plasma is important for a number of spermatozoal functions [267]. However, spermatozoa are also particularly susceptible to oxidative stress because their constituent lipids are rich in polyunsaturated fatty-acid side-chains [219]. The large variety and high levels of antioxidant defenses present in this fluid are therefore crucial to maintaining a well-controlled prooxidant/antioxidant balance. The SOD and GPx enzymes are present, as are iron-binding proteins (lactoferrin and transferrin). High levels of urate (235μM) and ascorbate (568μM), and lower levels of hypotaurine (35μM) present in seminal plasma have each been suggested to contribute to the LMW antioxidant defense [8, 268, 269]. Van Overveld et al [66] found deproteinised human seminal plasma to have an unusual, slow but continuous antioxidant activity using the 'trolox equivalent antioxidant capacity' (TEAC) assay, this being one of the established TAC assays. Using the same assay, they also evaluated the various putative LMW antioxidant substances (urate, ascorbate, tocopherol and hypotaurine). They included tyrosine since it is present at such high levels in spermatic fluid (2116μM [65]). They found free tyrosine to have powerful antioxidant activity, and to have the same TEAC curve as that for seminal plasma, whereas urate for example showed immediate and complete radical-trapping ability. They concluded that, compared with all the other substances evaluated, tyrosine was the most important antioxidant contributor to seminal plasma TAC using the TEAC assay. They suggested for the first time that a high level of free tyrosine in seminal plasma is providing important antioxidant protection [66].

As compared with seminal plasma, levels of free tyrosine in other tissues are relatively low, being approximately 60μM in plasma. This level is however not very different to levels of the antioxidants ascorbate and α-tocopherol in blood. Yet free tyrosine has not previously been considered to be an important naturally occurring antioxidant. It is worth considering that even if levels of free tyrosine are insufficient to prevent oxidative damage to other biomolecules, the preferential cross-linking of a protein-bound tyrosyl radical with a free tyrosyl radical formed in close proximity, rather than with another tyrosyl residue in the same protein, could limit protein damage/unfolding. Finally, it is noteworthy that patients with phenylketonuria, an autosomal recessive disease causing reduced tyrosine formation, show signs of increased levels of oxidative stress, and a deficient capacity to handle an increased level of RS [270].

As in the case of tyrosine, free tryptophan is never included in the textbooks as being a physiologically relevant antioxidant. Many of the same RS that attack tyrosine are also reactive with tryptophan, both protein-bound and free amino acid forms [256, 258, 271, 272]. Oxidative modification of protein-bound tryptophan, as for tyrosine residues, can be very detrimental. For example, oxidation of tryptophan and tyrosine residues of the SOD enzyme protein results in impaired enzyme activity [271]. As for tyrosine there is a small body of scientific evidence to indicate that free tryptophan, and/or its oxidative metabolites (Fig 30) do have antioxidant activity. Cadenas et. al (1989) showed that 5-hydroxytryptophan (5-OH-Tryp), the precursor to serotonin, was able to protect microsomal lipids from peroxidation [273]. Christen et. al (1990) investigated the possibility of a physiological role for free tryptophan and its oxidative metabolites as inducible antioxidants in lung tissue. They showed that low micromolar concentrations of hydroxylated tryptophan metabolites (but not their corresponding nonhydroxylated precursors) were able to scavenge peroxyl radicals with higher efficiency than equimolar amounts of either ascorbate or Trolox. They suggested that the 100-fold induction of indoleamine 2,3-dioxygenase (IDO) seen in mice suffering from acute viral pneumonia (IDO being the first enzyme in the catalytic formation of kynurenine oxidative metabolites from tryptophan), represents a local antioxidant defense in such inflammatory diseases (Notably, XO was also significantly induced in the lungs of these mice) [132]. Such an upregulation of IDO occurs in various tissues in response to infections and other pathological conditions, and has also been suggested to contribute to scavenging of RS [8].

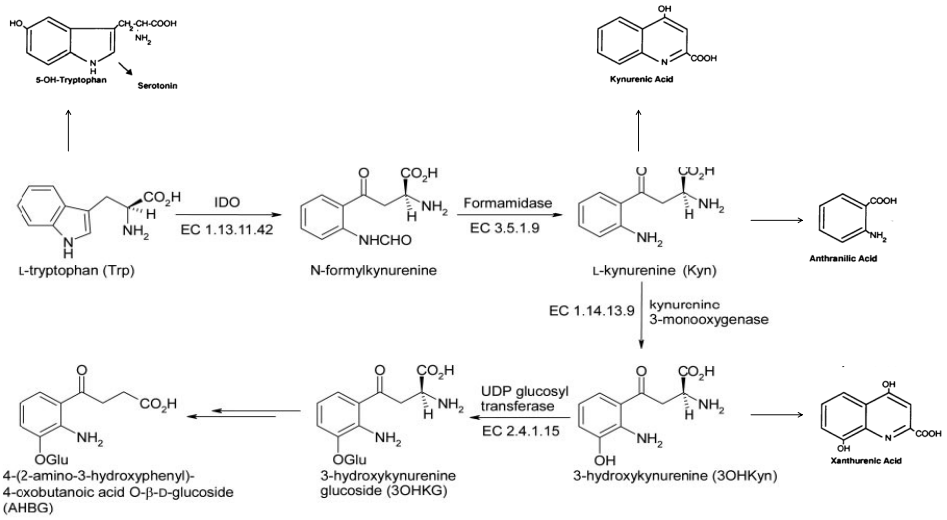


Figure 30. Part of the tryptophan oxidative metabolic pathway (adapted from Streete et. al. (2004) [7]).

A significant rate of synthesis of tryptophan oxidative metabolites (particularly 3-hydroxykynurenine glucoside) occurs in the human lens [274, 275]. These UV filter compounds are poor photosensitizers and protect the lens against light of wavelength 300-400 nm (UVA) [276]. The possibility that some of these tryptophan oxidative metabolites may also be providing antioxidant protection in the human lens has been suggested [277]. Notably, Truscott and co-workers reported a decreased level of free tryptophan metabolites in cataract lenses compared with normal lenses, despite a higher tryptophan and normal kynurenine concentration in the cataract lenses. This appears to be due to a defect in one of the steps that converts kynurenine to 3-hydroxykynurenine glucoside [7]. Such an impairment may be a predisposing factor to cataract formation since evidence of oxidative stress and damage are detectable in cataract lenses [7, 278-280].

Although the enzyme IDO, which can be induced in many cell types by interferon-gamma [281], is not likely to be present in plasma or in red cells [1], tryptophan can also undergo non-enzymatic oxidative modification, as indicated by Halliwell et. al (2007) (Fig 31). Reaction of tryptophan with various RS forms *N*-formylkynurenine. Ring-hydroxylated products such as 5-OH-tryptophan may also be formed [8]. This implies that tryptophan oxidative metabolites may be formed non-enzymatically in plasma and red cells in the presence of oxidative stress.

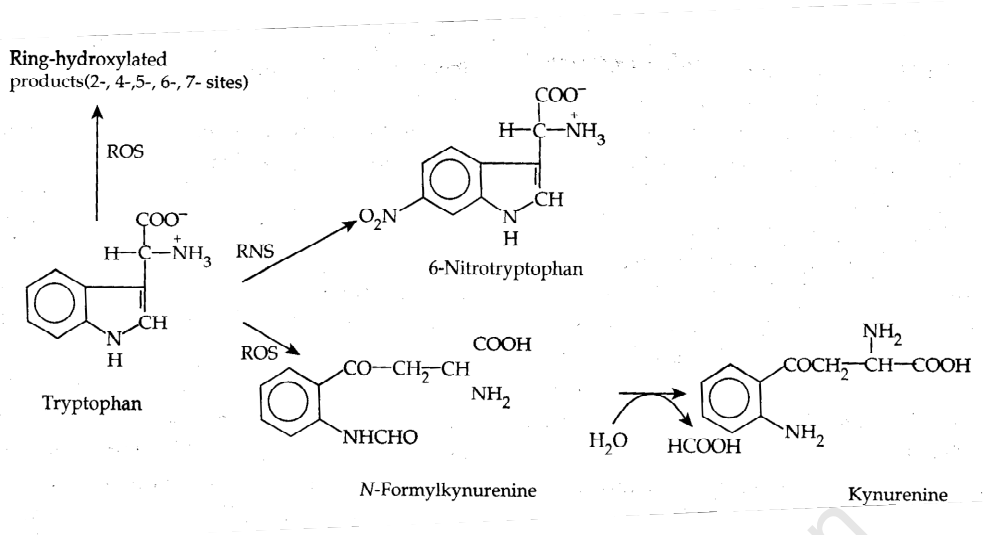


Figure 31. Oxidative modifications of tryptophan [8].

In conclusion, it is therefore possible that free tyrosine and tryptophan (and/or its metabolites) may have antioxidant activity by serving as 'sacrificial' LMW substances, and may be contributing to the residual TAC_{LMW} of plasma. The TAC_{LMW} of red cell contents has not previously been reported, and the potential LMW antioxidant contributors have not been determined. The high levels of tyrosine in the red cells of some mammalian species, the high levels of urate found in the red cells of some horses, and the accumulation of tryptophan and urate riboside in bovid red cells as reported in Chapter 1, may each be contributing significantly to the LMW antioxidant capacity of these red cells.

(C) AIMS

To (i) compare the oxygen-radical absorbance capacity (ORAC) value of tyrosine, tryptophan, and urate, (ii) to determine the ORAC value of acid-extracts of red cells and plasma from humans, horses and bovines, and (iii) to determine the antioxidant contribution of the above LMW substances to the ORAC values of these extracts .

(D) MATERIALS & METHODS

(i) Samples

Thirty micromolar solutions of commercially available LMW substances were made in 10mM potassium phosphate buffer (pH 7.4) (tyrosine, urate, xanthine, hypoxanthine, taurine and phenylalanine from Sigma, USA; NADH from Boehringer Mannheim, W. Germany; tryptophan from Hopkin & Williams Ltd, UK.). Diode array spectrophotometry was performed to confirm identity and concentration of solutions made. Aliquots of these solutions were frozen at -80^{o}C, and thawed immediately prior to use in the assay. Some of the aliquots of frozen red cell and plasma acid-extracts from humans, horses, cows and buffalo that were made for HPLC analysis (as described in Chapter 1) were thawed and used for the ORAC assay in order to determine the ORAC value of the LMW substances ($ORAC_{LMW}$) for the red cells and plasma of each species. Prior et. al. (2003) showed that use of perchloric acid was best of the options tested to remove proteins for determination of $ORAC_{LMW}$ [252].

(ii) The oxygen-radical absorbance capacity (ORAC) assay

This fluorometric, kinetic assay quantitates the ORAC value of antioxidant substances over time and to completion. The ORAC assay was first developed by Cao et. al. (1993) [251]. They used β-phycoerythrin as the target - this was subsequently shown to be very unstable, and the improved ORAC developed by Ou et. al. (2001) used fluorescein (FL) as the target/probe [282]. Blackhurst et. al. (2007) further modified the assay [283]. In the current study the latter method was used, with some further minor modifications.

The ORAC assay was performed on a top-reading fluorescence spectrophotometer (Varian, Australia), in white, flat-bottomed, 96-well microplates (Greiner Bio-One, USA) at ambient temperature. The excitation and emission wavelengths were 485 and 520 nm respectively. Each 300μl reaction was performed in duplicate (in consecutive wells) and allowed to proceed at ambient temperature. The final reactions contained:

50μl of phosphate buffer, pH 7.4 (12.5 mM)
100μl of fluorescein (31.9nM)[(a)]
50μl of the sample[(b)] or trolox standard[(c)]
100μl of AAPH (110μM)[(d)]

The following control reactions were also included in each assay:

300μl buffer alone (background fluorescence)

200μl buffer plus 100μl fluorescein (no free radicals)

(a) Fluorescein (FL) is a stable molecule which is susceptible to oxidative degradation by peroxyl radicals. A stock-1 solution (1.196mM) of FL (Sigma-Aldrich, USA) was made in buffer (stable indefinitely when stored at 4°C). A stock-2 solution (5.98μM) of FL in buffer was made from this every 2 months (stored at 4°C). A working solution (95.7nM) of FL in buffer was made from stock-2 on the day of the assay.

(b) This consisted of either thawed 30μM solutions of the commercially-available LMW substances, or thawed red cell (or plasma) acid-extracts which were 1 in 17 (or 1 in 12) dilutions of the actual red cell water (or plasma) (see Chapter 1).

(c) Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) represents a water-soluble form of tocopherol and serves as the antioxidant standard in the ORAC assay. A stock-1 solution (100mM) of Trolox (Sigma-Aldrich, USA) was made in absolute ethanol. A stock-2 solution (200μM) of Trolox in cold buffer was made, and aliquots frozen at -20°C. An aliquot was thawed on the day of the assay, serial dilutions (200μM -1.37μM) were made in cold buffer and used immediately. A control (0μM Trolox) reaction was always performed by adding 50μl buffer instead of Trolox to the reaction.

(d) AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) decomposes spontaneously at 37°C in aqueous solution to release a constant flux of peroxyl free radicals [8]. A working solution (330μM) of AAPH (Sigma-Aldrich, USA) was always made in pre-warmed buffer (37°C) immediately before addition to each well, as this has previously been reported to improve reproducibility of the assay [252]. AAPH was always added last to the well to initiate the reaction. Some investigators use a temperature-controllable fluorescence plate reader, and maintain the reaction temperature at 37°C.

All reaction constituents were added to each well, except for AAPH, and the plate was shaken for 10 seconds. Before reconstituting the AAPH, 'time 0' fluorescence readings of the two control reactions were made. The AAPH was added to the appropriate wells, the plate shaken for 10 seconds, and fluorescence measurements made and recorded (Cary Eclipse software, Australia) at 2 min, 5 min and then every

5 min thereafter until 20 min, then every 10 min until 60min, and then every 15 min until all reactions had reached baseline (zero) fluorescence. The decrease in fluorescence over time reflects the oxidative degradation of FL.

Graphpad Prism Statistical Software Version 4.01 (2004) was used to convert the data into an average fluorescence/time plot/curve for each duplicated reaction (Fig 32), determine the area-under-curve (AUC) for each reaction, and perform the subsequent analyses.

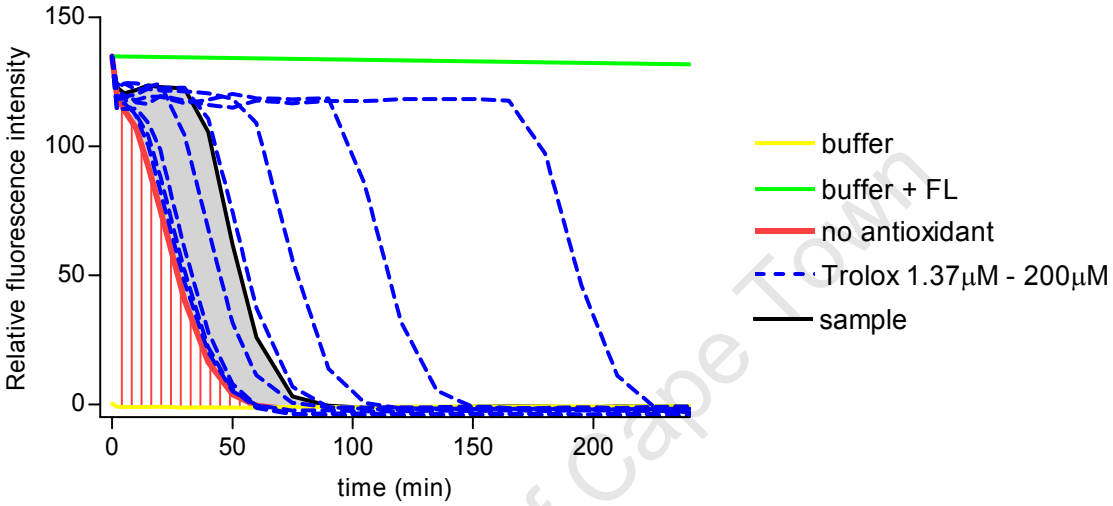


Figure 32. Time-dependent fluorescence decay of fluorescein after the addition of AAPH in the presence of serial dilutions of Trolox antioxidant, and control reactions and a typical sample (black line). The red striped area represents the background AUC; the grey area would be the samples corrected AUC.

A Trolox standard curve (AUC versus Trolox concentration) was produced for each assay (Fig 33), and the AUC for each sample extrapolated to a Trolox concentration. The ORAC value of each sample is thus measured as μmol Trolox equivalents (TE)/litre sample. The definition of one ORAC unit is the protection provided by a 1μM Trolox solution [251].

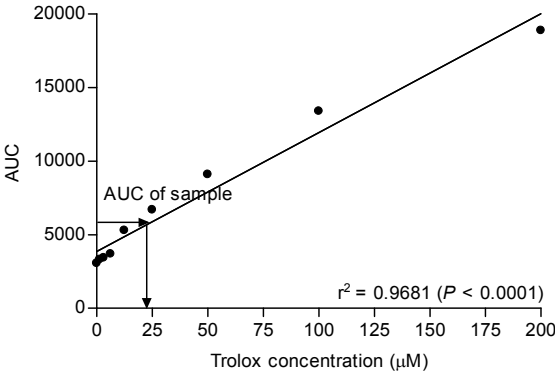


Figure 33. Trolox standard curve. The Goodness of Fit test was used to determine the linear regression. The ORAC value (as μmoles Trolox Equivalent per liter) of a sample is determined by extrapolation from the standard curve.

Where the samples were acid extracts of red cells or plasma, the ORAC value of the extract was multiplied by a further factor of 17 or 12 respectively, these being the dilution factors of the biological samples (see Chapter 1, Materials and Methods).

(iii) Statistical considerations

Unless otherwise indicated, data are presented as mean ± SD. Linear regression was determined using the Goodness of Fit test. When comparing means, the Unpaired t test was used to determine statistical significance, unless the data was skewed, in which case the Mann Whitney test was used. The Paired t test was used when comparing plasma and red cell levels within the same species, except where the data was skewed, in which case the Wilcoxon signed rank test was used. The Pearson test was used to determine statistical significance of correlation, unless the data was skewed in which case the Spearman r was determined. A P value < 0.05 was considered statistically significant. The intra-assay and inter-assay coefficients of variation (CV) for the ORAC assay were 17% and 4.5% respectively, using urate as the quality control solution. It was noted that well position in the 96-well plate significantly and consistently contributed to the large intra-assay CV. Ou et. al. (2001) also noted this effect and performed their duplicate reactions in a "forward-then-reverse" order to correct possible errors due to signal drift associated with different positions of the same sample [282]. A large intra-assay variation was also shown by Prior et. al. - they determined this to be caused by a number of factors, and a lower CV could be obtained using 48-well plates, and when excitation/emission could be set from the bottom of the plate [252].

(E) RESULTS AND DISCUSSION

Many different assays exist to measure TAC [249]. These assays vary in terms of the pro-oxidant and target/probe used, as well as how the measurement is made (fixed time point versus assays that allow reactions to reach completion). The ORAC assay is one such well-established assay to measure TAC. It quantifies antioxidant scavenging activity of a substance/sample against the peroxyl radicals released by thermal decomposition of water-soluble AAPH, by measuring the protection it gives to the target or probe (namely FL) from oxidation. The protective effect of an antioxidant substance or sample is determined relative to the antioxidant activity of the known antioxidant Trolox, a water-soluble from of tocopherol [282], and given as μmoles TE/litre.

When Cao et. al. first reported developing the ORAC assay, they measured the ORAC values of urate and vitamin C, two well established endogenous antioxidants present at high levels in human plasma [251]. However since then, investigations have focused more on using TAC assays such as the ORAC assay to evaluate the antioxidant capacity of food, beverages and potential therapeutic drugs, rather than on evaluating the antioxidant activities of baseline levels of LMW substances, and their contributions to the antioxidant capacity of biological tissues. In Chapter 1 the detection of unusually high levels of urate and tyrosine in the red cells of horses relative to man, and the accumulation of tryptophan in the red cells of cows and buffalo was reported. In order to determine if these LMW substances may have antioxidant ability, the ORAC values of equimolar solutions (30μM) of tyrosine and tryptophan, as well as urate and various other LMW substances known to be present in red cells and/or plasma were determined.

Examination of the fluorescence curves for each LMW substance revealed not only differences in AUC, but also very different kinetic profiles for the various substances (Fig 34). TAC assays that are designed to determine antioxidant capacity at a fixed time point (eg. the TEAC assay) would give very different results for the same substance, depending on the time point at which the measurement is taken. One of the advantages of assays like the ORAC assay that measure an AUC for reactions which are allowed to run to completion (i.e. back to baseline), is that this solves many of the problems associated with kinetics and lag-time effects [251].

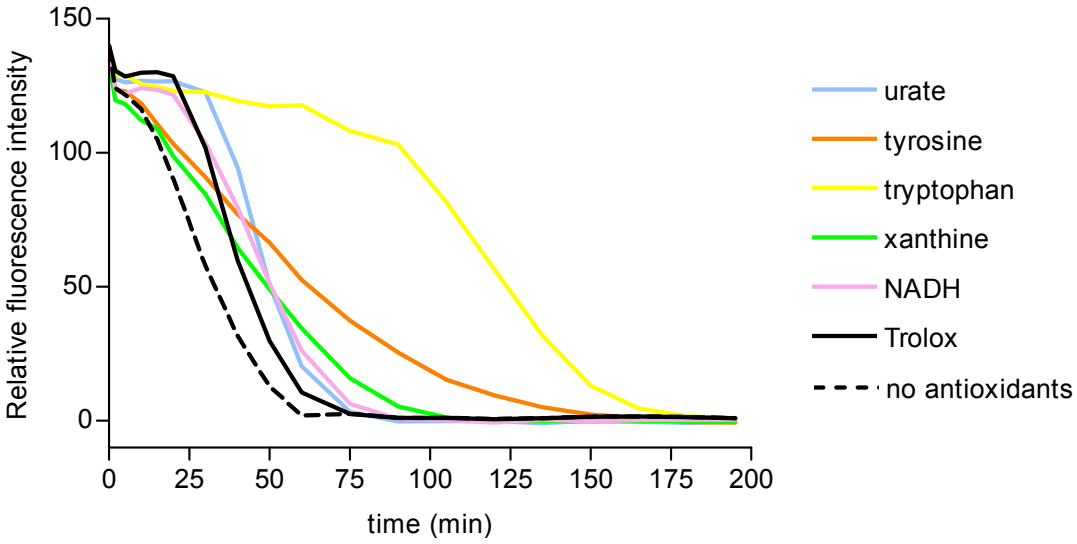


Figure 34. Time-dependent fluorescence decay of fluorescein after the addition of AAPH in the presence of 30μM solutions of urate, tyrosine, tryptophan, xanthine, NADH, or 12.5μM Trolox as standard, or no antioxidant.

Figure 34 shows that reactions containing Trolox or urate have very similar FL degradation profiles. Urate and Trolox have the same high rate of reaction with the water-soluble peroxyl radicals formed from AAPH - after an initial plateau region, indicating complete FL protection, the fluorescence decreased rapidly and at a rate similar to that found in the control (no antioxidant), indicating complete oxidation of the urate/Trolox. These profiles are identical to that illustrated by Cao et. al. (1993) for urate and Trolox [251]. The extent of the plateau for a substance is related to its concentration in the reaction (see Fig 32). Although an equimolar solution of NADH showed a shorter plateau than for urate, the rate of FL-degradation after the plateau phase was slightly slower than for urate and Trolox, being identical to that in the control.

Tyrosine showed a significantly lower affinity for the peroxyl radicals than Trolox and urate. Instead of a plateau region, tyrosine seems rather to be providing partial, continuous protection to FL. This same slow radical trapping curve was shown by van Overveld for tyrosine (and seminal plasma) using the TEAC assay, and suggested to be important in maintaining a careful balance between pro-oxidants and antioxidants for adequate spermatozoal function [66] (see also Literature Review). A similarly slow, fluorescence-degradation profile was evident for reactions containing an equimolar amount of xanthine, although the protection was less than that provided by tyrosine.

Reactions containing tryptophan showed a strikingly different protective profile. There was an extended plateau-type phase, well beyond that for equimolar concentrations of urate. This plateau tended to drop off very slowly, up till a point where no further antioxidant protection was evident. Therefore tryptophan was more efficient to limit oxidation of FL (see below).

The ORAC value for each of the LMW substances was then determined from the respective AUC (Fig 35).

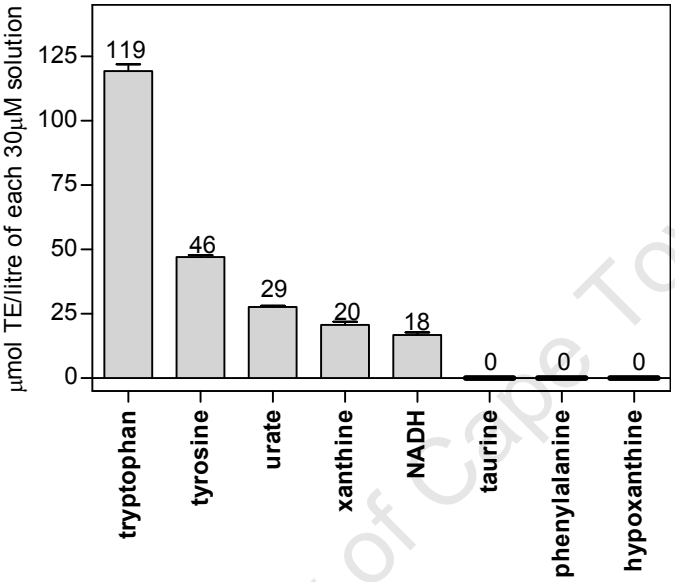


Figure 35. The mean (and SD) ORAC values of equimolar (30µM) solutions of some LMW substances. Results represent 4-7 replicates (performed on different days) for each substance.

A 30µM solution of urate was determined to have an ORAC value of 29µmoles TE/litre, indicating that the antioxidant capacity of urate to protect FL against peroxyl radicals is just slightly less than an equimolar amount of Trolox. Extrapolated, one ORAC unit (which by definition equals 1µM Trolox) equals 1.05µM urate. This result is in agreement with the findings of Cao et. al. (1993) who found that one ORAC unit equals 1.08µM urate [251]. Such extrapolations are possible since linear correlations between ORAC value and concentration of antioxidants tested have been shown [251, 284]. Quantitatively then, urate and Trolox must donate almost the same number of electrons, and at similar rates, to peroxyl radicals.

Although tyrosine clearly reacts at a slower rate than urate or Trolox with peroxyl radicals (Fig 34 above), its AUC shows it to have a greater (almost 2-fold) ORAC value than either of these antioxidants. A possible explanation is that whereas each

urate molecule may be donating 1 electron, forming stable and mildly oxidizing urate radicals, that 2 tyrosine molecules may donate 3 electrons via a disproportionation reaction between two tyrosine radicals (see Literature Review). Despite tyrosine's greater ORAC value compared with urate, the very different kinetics between tyrosine and urate should nevertheless be kept in mind – levels of antioxidants are likely to be continuously renewed *in vivo*, and urate would be a more powerful/complete antioxidant than tyrosine against peroxyl radicals because of its greater rate of reaction (or higher affinity for) with these radicals.

Tryptophan had an extraordinarily high ORAC value, this being almost 5-fold greater than the ORAC of the known antioxidant urate, the 30μM tryptophan solution having an ORAC value of 119μmol TE/litre. This reveals a complex antioxidant capacity for tryptophan, probably via the non-enzymatic, peroxyl-mediated formation of tryptophan oxidative metabolites (see Literature Review). Halliwell et. al. (2007) indicate that tryptophan can undergo non-enzymatic oxidative modification by RS to form these products, which have significant antioxidant activity as shown by Christen et al. (1990) and Cadenas et. al (1989), [132, 273]. An equimolar solution of tryptophan is therefore clearly able to donate many more electrons to peroxyl radicals than either urate or tyrosine.

As expected, the hypoxanthine and phenylalanine (included as 'negative controls') solutions both showed no ORAC activity. The ORAC value for the 30μM NADH solution was 18μmol TE/litre, NADH being an important intracellular reducing agent and able to donate electrons directly to peroxyl radicals [8]. Although suggested by some to have antioxidant activity [70-73, 285], taurine showed no antioxidant activity against peroxyl radicals in the ORAC assay - this is in accordance with Auroma et. al. (1988) who showed that unlike hypotaurine, taurine does not react rapidly with H_2O_2, $O_2^{\cdot -}$, $OH^{\cdot}$, or HOCl [74]. Unexpectedly, xanthine, which has not previously been evaluated as an antioxidant, had an ORAC value a little greater than that of NADH. Based on this data, both tyrosine and tryptophan, neither previously considered to have significant antioxidant activity, have greater antioxidant activity against peroxyl radicals than urate, with the relative peroxyl radical absorbing capacity of 1μM Trolox, urate, tryptophan and tyrosine being 1, 0.97, 4 and 1.5 respectively. The relative peroxyl radical absorbing capacity of 1μM NADH and xanthine were 0.67 and 0.6 respectively.

In order to determine the contribution of tyrosine, tryptophan and urate to the LMW component of red cell and plasma TAC, the ORAC assay was performed on

perchloric acid-extracts of red cell and plasma samples from humans (n=5 and n=4 respectively), horses (n=12), cows (n=4) and buffalo (n=6). Only deproteinised samples were evaluated firstly because it has been shown that proteins account for 85-90% of the $ORAC_T$ value of whole/total human plasma against peroxyl radicals [116, 251]. Hence, certainly if using the ORAC assay and the interest is in LMW antioxidant molecules, proteins in plasma samples should first be precipitated, allowing determination of the $ORAC_{LMW}$ of the sample ($ORAC_{LMW}$) in order to prevent 'masking' by the proteins. In addition, an ORAC value for total red cell contents cannot be determined, since hemoglobin quenches fluorescence [286].

In comparison with the plasma extracts from the different mammalian species (Fig 36 A), which all followed very similar kinetics (albeit surprisingly, considering the high urate in human plasma), the fluorescence curves in reactions containing red cells extracts (Fig 36 B) were unique for each species.

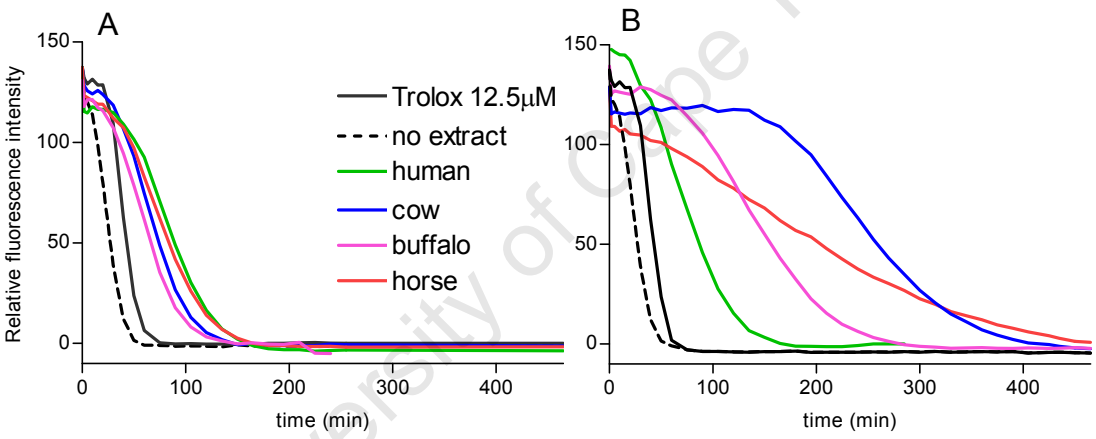


Figure 36. Time-dependent fluorescence decay of FL after the addition of AAPH in the presence of (A) plasma extracts, and (B) red cell extracts from humans, cows, buffalo and horses, illustrating the very different ORAC kinetics profiles for the red cell extracts from different mammalian species. Note that some profiles may start at different fluorescence intensities because the reactions were not all performed in the same run – this would however be taken into account when extrapolating the TE value from the respective Trolox standard curve performed with every run.

The curve for horse red cell extracts was reminiscent of the slow continuous radical-trapping ability of a tyrosine solution. The curves for human, cow and buffalo red cells each showed similar kinetics, although the AUC for buffalo and cows was clearly much larger than that for humans. The red cell LMW contents from cows in particular provided prolonged, complete protection to FL (as does urate) until a point where the FL degradation followed a more 'tryptophan-type' pattern.

The $ORAC_{LMW}$ value for each acid-extract was then determined (Fig 37 and Table 3).

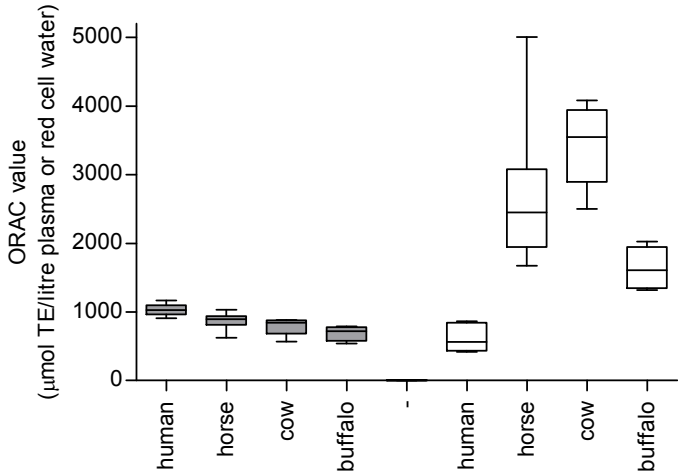


Figure 37. Box and whisker plots of the ORAC values of deproteinised red cell (empty boxes) and plasma (grey boxes) extracts from four mammalian species: humans (n=5), horses (n=12), cows (n=4) and buffalo (n=6). The Mann Whitney test for non-parametric data was used to determine *P* values between horses and humans where data was skewed, whilst the Unpaired t test was used otherwise. The Paired t test was used to determine *P* values when comparing plasma and red cell tyrosine levels, except for horses where the Wilcoxon signed rank test was used.

Table 3. The mean (± SD) ORAC values for red cell and plasma extracts from four mammalian species.

	Plasma $ORAC_{LMW}$ (μmol TE/litre plasma)	Red blood cell $ORAC_{LMW}$ (μmol TE/litre red cell water)
Human (n=5)	1032 ± 106	623 ± 207
Horse (n=12)	873 ± 115	2631 ± 948
Cow (n=4)	782 ± 148	3419 ± 694
Buffalo (n=6)	693 ± 95	1633 ± 305

The $ORAC_{LMW}$ of human plasma was 1032 ± 106μmol TE/litre plasma. This is similar to that previously reported by Prior et. al. (2003) [252]. Human plasma $ORAC_{LMW}$ was significantly higher ($P \leq 0.023$) than that from all three other species evaluated - this is in agreement with Ninfali et. al. (1998), the only other report comparing plasma TAC from different species, including cows and horses with that found in humans [116]. These investigators suggested this to be due to the presence of significant levels of urate in human plasma.

The ORAC value for red cells has not previously been reported. Whereas in humans, red cell $ORAC_{LMW}$ values were significantly lower than plasma $ORAC_{LMW}$ values (P = 0.0197), for each of the other three species the relationship was reversed, with red cell $ORAC_{LMW}$ values being significantly higher than the respective plasma $ORAC_{LMW}$ values ($P \leq 0.0029$). The $ORAC_{LMW}$ values of human red cells were also significantly lower than that of red cells from the other three species ($P \leq 0.0012$) – it is interesting to speculate why the red cell's LMW antioxidant capacity would have evolved to be so much higher than the respective plasma LMW antioxidant capacity (although not for humans) (see also Chapter 3).

The large intra-species variation in red cell $ORAC_{LMW}$ for horses and bovids was reminiscent of the large intra-species variation in tyrosine and urate levels amongst horses, and urate riboside levels amongst bovids, as reported in Chapter 1, and is similarly intriguing as it implies significant differences in antioxidant defense amongst individual.

It should be noted that the original ORAC assay, and as used in this study, is performed in aqueous solution. Therefore it measures the hydrophilic antioxidant activity, whilst lipophilic antioxidants have been found not to contribute to this value [282]. This may be because most of these antioxidants tend to be precipitated out with the plasma lipoproteins during the acid extraction step. The ORAC assay has been adapted by Prior et. al. (2003) to measure lipophilic and hydrophilic components separately (with hexane extraction of the lipophilic antioxidants), and using the same peroxyl radical generator for both components [252]. The total $ORAC_{LMW}$ values indicated in table 3 above may be predicted to be about 30% greater if the lipophilic LMW antioxidant component was included [249].

The antioxidant contributions provided by tyrosine, tryptophan, urate and urate riboside to the LMW component of red cell and plasma TAC were then determined (Fig 38). The antioxidant activities of 0.97, 4 and 1.5 μmoles TE/litre as determined above for every 1μmole of urate, tryptophan and tyrosine respectively present in red cell water or plasma was used to determine this contribution.

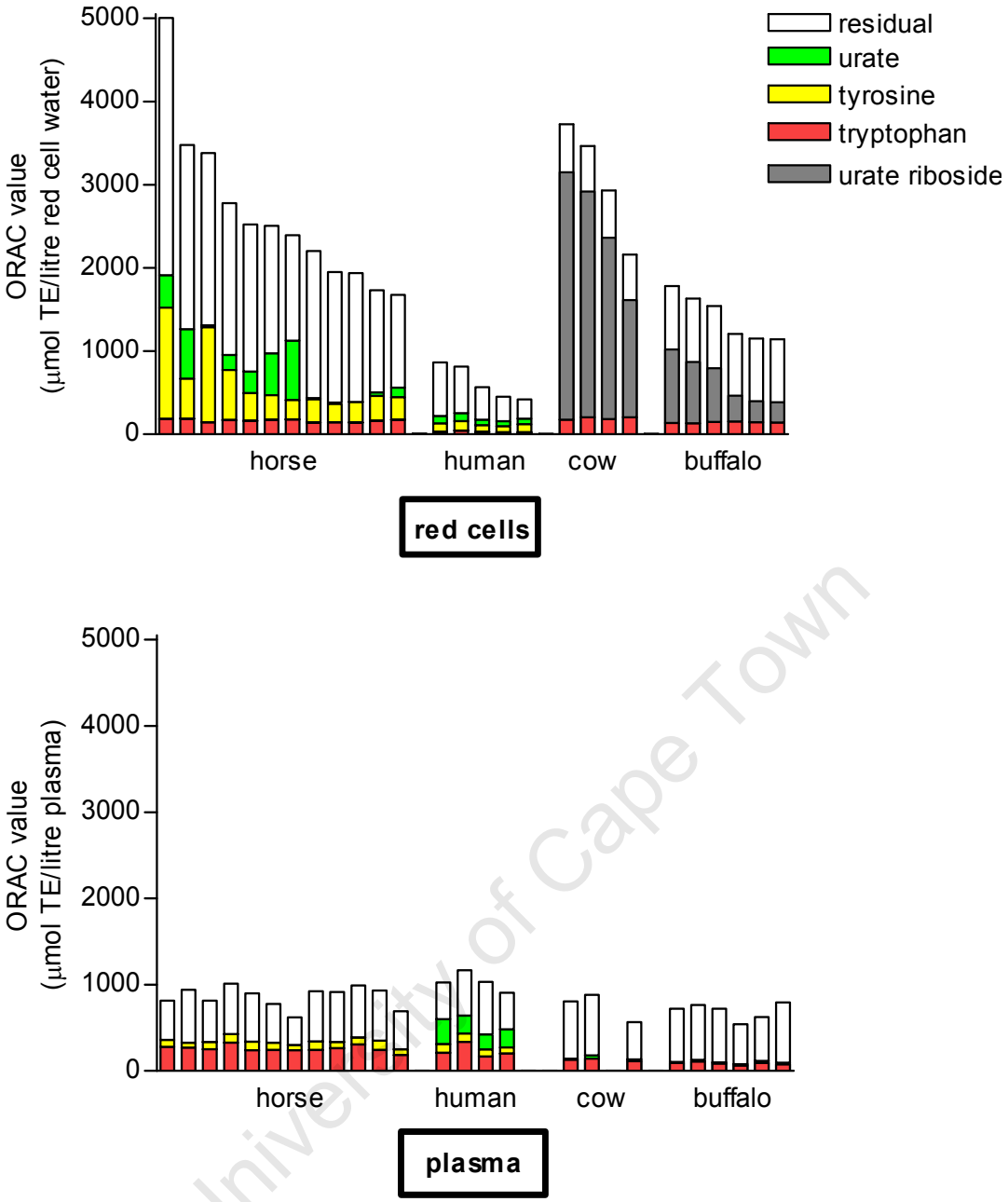


Figure 38. Stacked bar graphs of the contribution of tryptophan, tyrosine, urate and urate riboside to the $ORAC_{LMW}$ of red cells and plasma from a number of individual humans, horses, cows and buffalo. The red cell and plasma samples from the same individual are always aligned.

From figure 38, it is clear that there was no correlation between red cell and plasma $ORAC_{LMW}$ within each species.

Although urate did contribute significantly (17-29%) to human plasma $ORAC_{LMW}$, urate levels did not correlate with the plasma $ORAC_{LMW}$. The only other study found to report on urate contribution using the ORAC assay (n=4) showed a 16% contribution to, and also no significant correlation with, plasma $ORAC_{LMW}$ [116]. The

contribution from tyrosine to plasma $ORAC_{LMW}$ in humans was 8-10%. The contribution from tryptophan was as much as that from urate, being 16-29%.

Red cell urate concentration on the other hand was found to correlate significantly with the respective red cell $ORAC_{LMW}$ value in humans (P = 0.0125), and contributed between 11-17% of the LMW antioxidant activity against peroxyl radicals. Tyrosine contributed between 12-23%, and tryptophan between 3-5%. Unlike urate levels, levels of these two amino acids in the human red cells did not correlate with the $ORAC_{LMW}$ values.

In horses, the high levels of tryptophan present in horse plasma samples, and most-likely diet-induced (see Chapter 1, Results and Discussion, section v), were found to be a large component of the plasma $ORAC_{LMW}$, contributing 26-38% of the LMW antioxidant capacity against peroxyl radicals. Tryptophan concentration also correlated significantly with plasma $ORAC_{LMW}$ (P = 0.0112).

In horse red cells, it was the tyrosine concentration, rather, which correlated significantly with the highly variable red cell $ORAC_{LMW}$ values (P = 0.0002) (Fig 39 A). Tyrosine contributed between 10-24% of the $ORAC_{LMW}$ value. Although urate contributed a large component of the $ORAC_{LMW}$ in some horses (up to 30%), other horses had no red cell urate, and urate levels did not correlate with red cell $ORAC_{LMW}$. However, when the contribution from urate was added to the contribution from tyrosine for each horse, the correlation with red cell $ORAC_{LMW}$ value was improved over that with the correlation with tyrosine contribution alone (P < 0.0001) (Fig 39 B).

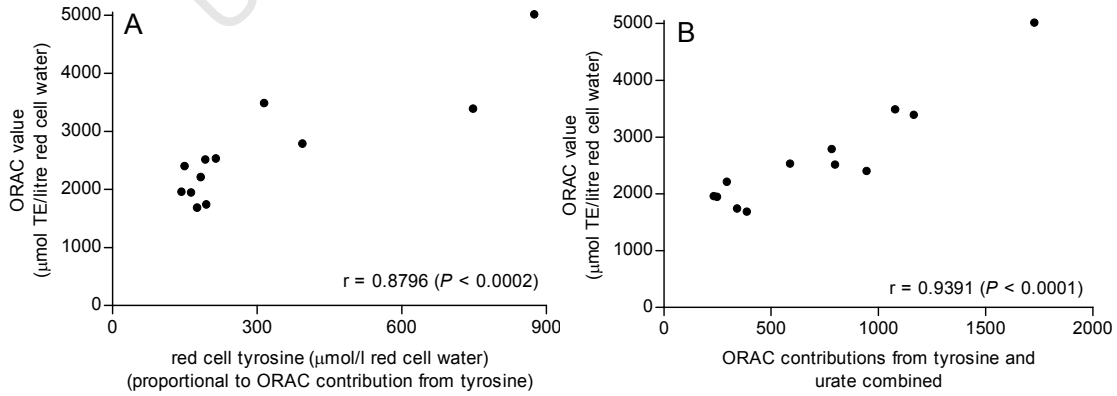


Figure 39. Comparison of the horse red cell $ORAC_{LMW}$ value and (A) horse red cell tyrosine concentration, and (B) combined $ORAC_{LMW}$ contribution from red cell tyrosine and urate in horse red cells. The Spearman test was used to determine statistical significance of correlation.

In horse red cells, tryptophan contributed only 4-10% of the $ORAC_{LMW}$. The contribution from tyrosine, tryptophan and urate in the horse red cells combined accounted for 19-47% of the red cell $ORAC_{LMW}$.

Despite the finding in Chapter 1 of an accumulation of tryptophan in the red cells from cows and buffalo, this being higher than in any of the other species evaluated, tryptophan was found to contribute only about 4-7% of the $ORAC_{LMW}$ in cow red cells, and in buffalo the contribution from tryptophan was only slightly more, being 7-10%. This may be attributed to the fact that urate riboside must be contributing an overwhelmingly large amount to the $ORAC_{LMW}$ of the red cells from these two species. Although the antioxidant contribution of urate riboside to the red cell $ORAC_{LMW}$ could not be determined directly, urate riboside concentration correlated with red cell $ORAC_{LMW}$ (Fig 40 A). Based on the linear regressions for cow and buffalo separately, as depicted in figures 40 B and C, a residual red cell $ORAC_{LMW}$ value of 750 and 900 μmoles TE/litre was determined for cows and buffalo respectively (at x-axis = 0). In this way the contribution of urate riboside to red cell $ORAC_{LMW}$ was thus determined to range from 57-73% in cows, and 18-45% in buffalo. The unlikely but small possibility that levels of another one, or more antioxidants are also correlated with these widely ranging red cell $ORAC_{LMW}$ values, cannot however be excluded.

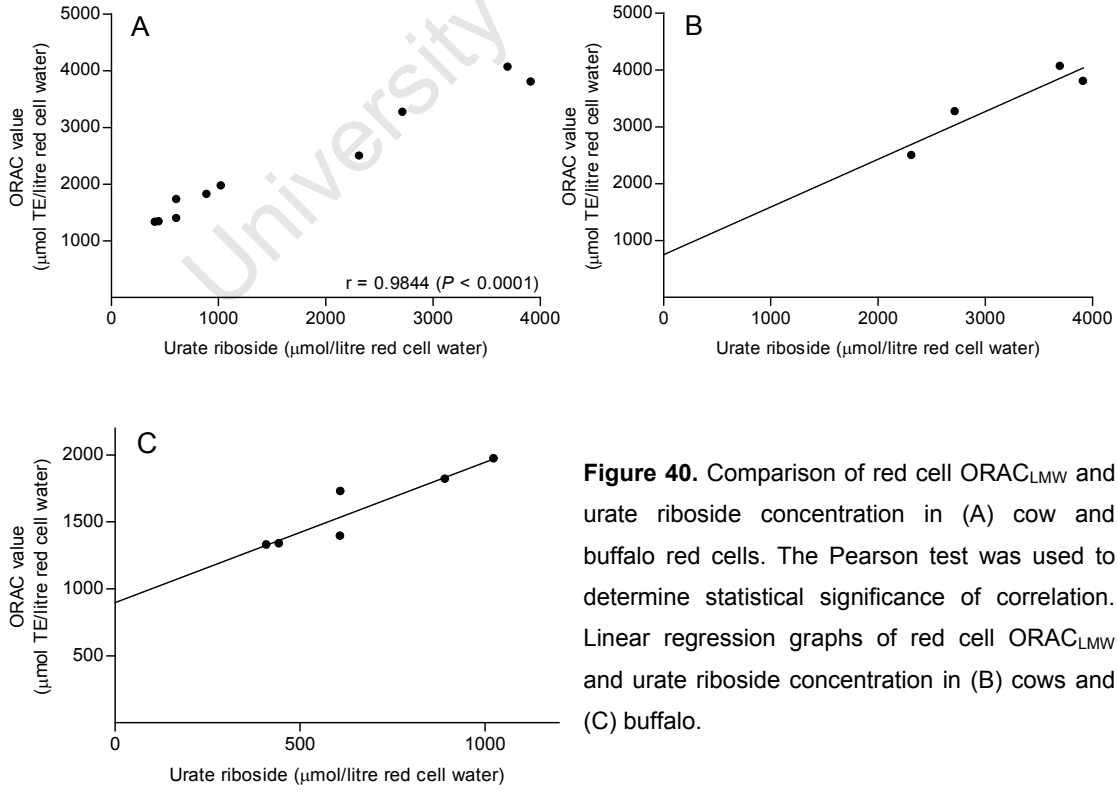


Figure 40. Comparison of red cell $ORAC_{LMW}$ and urate riboside concentration in (A) cow and buffalo red cells. The Pearson test was used to determine statistical significance of correlation. Linear regression graphs of red cell $ORAC_{LMW}$ and urate riboside concentration in (B) cows and (C) buffalo.

In red cells, high levels of reduced glutathione (GSH) are present (>2mM), and this antioxidant is likely to be contributing significantly to the residual $ORAC_{LMW}$ in the red cells. Vitamin C has an ORAC of about 0.52 μmol TE/litre according to Cao et. al. (1993) [251]. Therefore in humans, the known levels of Vitamin C [3, 218] would add only about 10μmol and 25-100 μmol TE/litre to the determined $ORAC_{LMW}$ of red cell water and plasma respectively. Although reduced NADH does have ORAC activity (Fig 35 above), all the NADH in the red cells would have been converted to NAD^+ during the extraction process, and hence would not contribute to the red cell extract ORAC values. If kept in the reduced form, its contribution would be about 30, 50, 60 and 90μmol TE/litre red cell water in buffalo, horses, humans and cows respectively. Other unsuspected antioxidants may also be contributing to the residual $ORAC_{LMW}$, as well as possible synergy between antioxidants (for example, the ability of ascorbate to reduce urate radicals, see Literature Review).

What is the biological significance of the ORAC value in assessing antioxidant ability? No single TAC assay or other measure of antioxidant capacity can be considered as a "total antioxidant capacity assay". In a biological system, whereas an antioxidant may be particularly reactive with a specific antioxidant, it may be less efficient in scavenging a different RS. The ORAC assay assesses antioxidant capacity against peroxyl radicals; further assessment of the antioxidant capacity of tyrosine and tryptophan against other oxidants, such as the $OH^{\cdot}$ or $O_2^{\cdot -}$ radicals, and ideally in a more complete biological system (for example cell-based *in vitro* assays) is required to generate an "antioxidant profile" and establish their antioxidant roles. The ORAC assay is however considered to be one of the most representative of the TAC assays of what may be occurring in the biological setting. The ORAC reaction proceeds as a classic hydrogen atom transfer (HAT) mechanism, rather than as a single electron transfer (SET) mechanism, as occurs in the TEAC and FRAP assays. Many scientists feel that the HAT reactions are most relevant to human biology. The AAPH provides a controllable source of organic peroxyl radicals, which is the predominant radical occurring in lipid oxidation in biological systems. Oxidation of proteins by the $OH^{\cdot}$ radical and other reactive species is also proposed to occur *in vivo* to form protein peroxyl radicals [194, 195]. Therefore, the ORAC assay, unlike many of the other TAC assays, uses an oxidant that is clinically relevant to measure antioxidant activity [247].

(F) SUMMARY

Both tyrosine and tryptophan have significant antioxidant activity against water soluble peroxyl radicals, as measured using the ORAC assay. The radical scavenging capacity of 1μM Trolox, urate, tryptophan and tyrosine is 1, 0.97, 4 and 1.5 respectively. The antioxidant contribution of physiological levels of tyrosine and tryptophan and urate ribosdie to the $ORAC_{LMW}$ of plasma and red cells of some mammalian species is similar or greater to the contribution by urate, and in some instances correlations of these with red cell $ORAC_{LMW}$ activity were demonstrable, e.g. for urate riboside in cows, and for combined urate plus tyrosine in horses. In conclusion, these results provide evidence to suggest that these free intra-erythrocytic amino acids have effective *in vivo* antioxidant activity, and that their contribution to endogenous LMW antioxidant defense mechanisms is underestimated, particularly in mammalian species found to accumulate high levels of these substances in their red cells.

CHAPTER 3

Unsuspected antioxidant strategies of mammalian red cells

(A) INTRODUCTION

Most of the enzymatic and non-enzymatic antioxidant capacity of whole blood is located within the red cells, both because it has high levels of these established antioxidants, and because it is the most abundant cell type in blood. In Chapter 2 it was demonstrated that the red cells from cows, buffalo and horses (although not humans) have significantly greater LMW antioxidant activity against peroxyl radicals (analogous with hydroxyl radicals) than the respective plasma. It was also shown that the high levels of tyrosine, tryptophan, urate and urate riboside present in the red cells of some mammalian species (as reported in Chapter 1) are likely to be playing a physiological part in such antioxidant activity, as well as in neutralising a variety of other biologically-relevant reactive species. But why do red cells require such an extensive antioxidant capacity, and what might the advantages be for the accumulation of these other putative LMW antioxidants specifically within the red cells?

(B) LITERATURE REVIEW

Compared with other tissues, a relatively low rate of superoxide ($O_2^{\cdot -}$) and hence hydrogen peroxide (H_2O_2) production occurs within the mature mammalian red cell [287]. Having no nucleus or subcellular organelles, $O_2^{\cdot -}$ production in the red cell is thought to arise exclusively from the auto-oxidation of ferrous Fe(II) oxyhaemoglobin (oxyHb) i.e. every so often a molecule of oxyHb releases $O_2^{\cdot -}$, leaving behind ferric Fe(III) methaemoglobin (metHb) [8] (Fig. 41). It has been estimated that about 3% of the oxyHb present in human red cells undergoes such oxidation every day [288] (4% in mice red cells [289]). MetHb, which is unable to bind oxygen, is continuously converted back to ferrous haemoglobin by the NADH(NADPH)-dependent methemoglobin reductase enzyme (ascorbate can also reduce metHb back to ferrous Hb, but only at millimolar levels [290, 291]). Normally, the amount of metHb present in human red cells is approximately 1% of total Hb [289, 292]. The high level of copper-zinc superoxide dismutase (CuZnSOD) present in red cells [8] rapidly catalyses the dismutation of two molecules of $O_2^{\cdot -}$ to form one H_2O_2 molecule. This maintains very low intra-erythrocytic levels of $O_2^{\cdot -}$, calculated to be 1.9 x 10^{-13} M in human red cells [287], and 5 x 10^{-13} M in mice red cells [289]. This value is 300 times smaller than that calculated for liver [287].

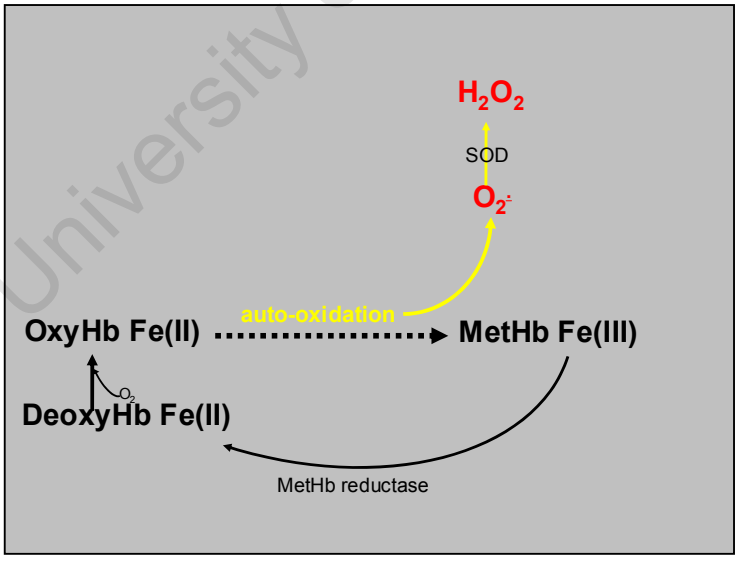


Figure 41. Auto-oxidation of oxyhaemoglobin.

H_2O_2 itself can accelerate the two-electron oxidation of oxyHb to form the Fe(IV) Hb species, ferrylhaemoglobin (ferrylHb) (Fig 42). FerrylHb is only an intermediate species.

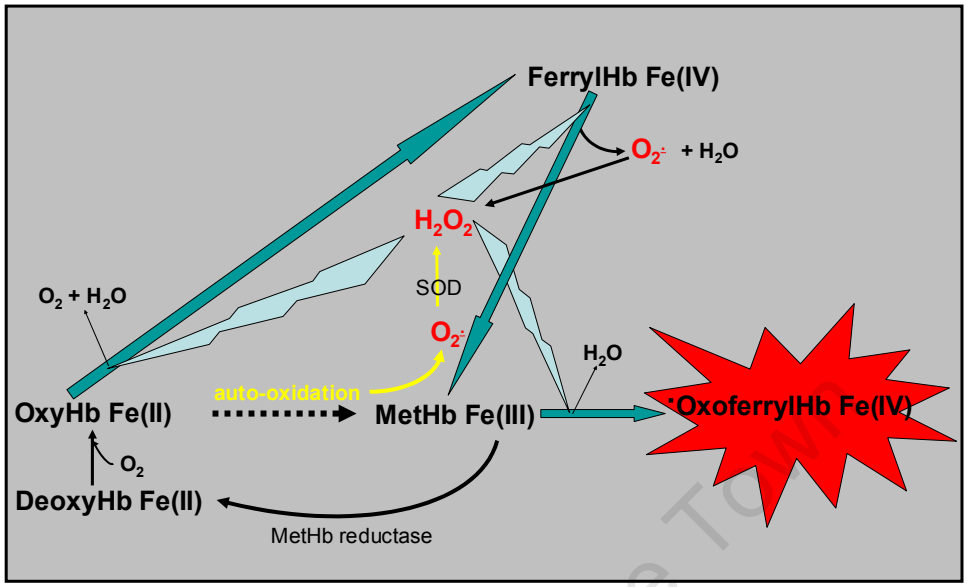


Figure 42. Reactions of oxyhaemoglobin, ferrylhaemoglobin and methaemoglobin with H_2O_2 (as reviewed and proposed by Nagababu et. al. (2004) [9]).

Nagababu et. al. reported that the subsequent reaction of H_2O_2 with ferrylHb to form Fe(III) metHb occurs via the one-electron oxidation of H_2O_2. A $O_2^{\cdot -}$ radical is concurrently produced within the haem pocket, and leads to the formation of fluorescent heme degradation products, as well as reforming an H_2O_2 molecule [293-295].

H_2O_2 also rapidly oxidizes metHb to yield oxoferrylhaemoglobin (oxoferrylHb). OxoferrylHb is two oxidizing equivalents above metHb - it is an Fe(IV) Hb species that has an additional electron removed from globin to produce a protein-bound radical, and referred to as ·HbFe(IV)=O [9]. FerrylHb and oxoferrylHb, both being Fe(IV) Hb species, have identical absorbance profiles on spectrophotometry [293]. As for the reaction of H_2O_2 with other haem-containing proteins (eg. myoglobin, catalase, peroxidase enzymes), formation and dissipation of the free radical in oxoferrylHb has been found to proceed via a complex series of transfer and transformation steps [296, 297]. Many similarities exist particularly between Hb and myoglobin oxidation reactions - with both, the formation of protein-bound tyrosyl radicals seems to be involved [261, 288, 296, 298, 299].

In the red cell, oxidation of Hb by H_2O_2 leads not only to loss of oxygen carrying capacity, but both the ferryl iron and the protein-bound radical of oxoferrylHb are able to initiate a range of oxidative chemical reactions with similar reactivity to the hydroxyl (OH·) radical [300-302]. The Hb itself also becomes modified/damaged, for example dityrosine formation has been shown to occur *in vitro* [254], whilst products reflecting the covalent modification of haem can be detected even in normal blood, and levels of these products increase significantly during acute oxidative stress (exercise) [300]. Therefore, although H_2O_2 itself is not a very reactive oxidizing species, the oxidative toxicity of H_2O_2 is greatly enhanced through its interaction with Hb within the red cell (the toxicity of oxidized Hb is hazardous not only within the red cell, but also systemically, for example with the use of Hb derivatives as a blood substitute [303]). H_2O_2 within red cells can also react rapidly with cytoplasmic LMW 'catalytic' iron (presumably released from Hb, particularly in older red cells) [8] to form the highly reactive hydroxyl radical (OH·). The formation of these highly reactive oxidizing species within the red cell in particular could be detrimental because this long-lived, circulating cell, with a polyunsaturated fatty acid (PUFA)-rich membrane, no longer has a nucleus and protein synthesizing machinery, and therefore generally cannot replace oxidised biomolecules.

In Chapter 2, using the Oxygen radical absorbance capacity (ORAC) assay it was reported that tyrosine, tryptophan, urate and urate riboside were each able to neutralize peroxyl radicals. The action of peroxyl radicals is believed to be similar to that proposed for the OH· radical [194]. Therefore, it is probable that accumulation of high levels of these LMW substances within the red cell cytoplasm may provide a physiological antioxidant role by neutralizing OH· radicals formed in the red cell. These LMW substances may also be involved in removal of the highly oxidizing oxoferrylHb species (see later).

Despite these putative antioxidant strategies for the removal of such highly oxidizing molecules, removal of H_2O_2 before it induces significant Hb oxidation or forms more oxidizing species is clearly preferable. High levels of catalase and glutathione peroxidase (GPx, specifically GPx1 in the red cell) activity within the red cell [8, 206] imply that, in a closed system, a very low steady-state concentration of H_2O_2 would be maintained within the red cells. This has been calculated to be 2×10^{-10} M [287]. In mice, this value was determined to be even lower (5×10^{-11} M [289]). These red cell H_2O_2 concentrations are to be compared with levels of 4-5 $\times 10^{-6}$ M reported for human plasma [304] and steady state levels of 10^{-7} to 10^{-8} for perfused rat liver tissue [8].

Since H_2O_2 is freely able to permeate most biological membranes, including that of the red cell [8], the red cells with their low production of H_2O_2, and high H_2O_2-removing capacity (and reserve), may act as 'sinks' for extracellular H_2O_2 [218]. Under conditions of increased systemic oxidative stress, red cell catalase and GPx activities can be further increased [108, 155, 158]. The red cell hexose monophosphate (HMP) shunt (see also Chapter 2, Literature Review) can also be activated several fold on exposure of red cells to oxidant substances that deplete reduced glutathione (GSH) or NADPH levels [40, 215]. As a consequence, healthy red cells do not easily undergo lipid peroxidation on *in vitro* exposure to H_2O_2 [8]. Red cell catalase, more than GPx, has been shown *in vitro* to be responsible for the removal of significant amounts of extracellular H_2O_2 [206, 305], and catalase activity has been shown to correlate with the ability of red cells to withstand H_2O_2-induced lysis [306, 307].

The proposed ability of the red cells to act as scavengers for plasma H_2O_2 should, theoretically, not only protect themselves but also the tissues they perfuse (as well as other blood cells) – as the red cells decrease plasma, and hence extracellular H_2O_2, the H_2O_2 produced inside nucleated cells should exit these cells into the extracellular fluid. Therefore, despite its highly pro-oxidant intracellular environment, it has been suggested that red cells may be playing an important physiological role in limiting oxidative damage to other cells/tissues [8, 217, 308, 309]. Various investigators have shown that red cells are able to reduce the H_2O_2 -induced damaging effects when added to various cell cultures (leukemic cell line [206], endothelial cells [310], neutrophils [309] and T-lymphocytes [311, 312]) to isolated rat lung tissue [310] and also to mouse embryos [313]. By removing extracellular H_2O_2, red cells also limit the formation of extracellular hydroxyl radicals and hypochlorous acid (HOCl) by stimulated neutrophils [305].

In contrast to H_2O_2, $O_2^{\bullet -}$ does not readily cross biological membranes except where specific channels occur [8]. Superoxide has however been shown by Lynch et. al. (1978) to be able to exit the red cell via the red cell anion exchange channel (band 3 protein) [192]. Although it may be presumed that $O_2^{\bullet -}$ would theoretically be able to enter the red cells via the same channel, Winterbourn et. al. (1987) found that $O_2^{\bullet -}$ (unlike H_2O_2) was not scavenged by red cells [305]. Therefore the red cell may not be involved in removing $O_2^{\bullet -}$ from plasma.

The red cell may be particularly permeable to various other RS because it possesses a larger number of band-3 membrane channels than most other cell types [314].

Peroxynitrite ($ONOO^-$), a strong oxidant formed by reaction between $O_2^{\bullet -}$ and nitric oxide ($NO^\bullet$) (see also Chapter 2, Literature Review), is one such RS that is easily able to cross the red cell membrane. Inside the red cell, $ONOO^-$ is rapidly inactivated by reacting with the large amount of oxyHb present, which itself is oxidized to metHb. It has been calculated that approximately 50% of peroxynitrite generated in the blood enters the red cell [315, 316]. The red cell also limits extracellular $ONOO^-$ formation by rapidly removing its precursor, namely $NO^\bullet$, from plasma. This small, relatively unreactive free radical diffuses freely across all membranes, but *in vivo* is taken up and removed within seconds by red cells because it is rapidly destroyed by oxyHb [317, 318]. (The high level of CuZnSOD present within the red cell [8, 319] reacts rapidly with the $O_2^{\bullet -}$ formed within the red cell, which may otherwise react with the $NO^\bullet$ to form $ONOO^-$ within the red cell). The red cell may also serve to scavenge HOCl from extracellular fluids – HOCl can penetrate the red cell membrane and GSH appears to provide significant antioxidant protection to other cellular components from HOCl within the red cell [224]. By virtue of its membrane permeability to various RS, together with fact that most of the non-enzymatic, as for the enzymatic, antioxidant capacity of whole blood is localised in the red cells [217], the red cells may be playing an intricate role in maintaining circulating redox balance.

Considering the reactivity of tyrosine, tryptophan and urate with various RS including $ONOO^-$ and HOCl (see Chapter 2, Literature Review), high levels of these LMW substances present in the red cells of some mammals relative to plasma levels may be promoting the scavenging ability of the red cells for such RS by serving as additional sacrificial antioxidant targets within the red cell. But if these LMW substances are indeed serving as direct antioxidants for RS that are being taken up from the plasma, then why not accumulate these LMW substances in plasma, for example as humans accumulate urate in their plasma? One possible explanation is that the kind of concentrations that are accumulating within the red cell may be hazardous if present in plasma (for example, gout, urate kidney stones, and the symptoms associated with hereditary tyrosinaemia). Therefore, high levels of these substances may rather have evolved safely packaged within the circulating red cells, which are then able to take up these RS from the plasma. Another possibility is that there may be a redox advantage for the accumulation of these LMW substances *specifically within the Hb-rich milieu of* the red cells – these LMW substances may be serving to promote completion of the peroxidase turnover cycle of metHb, and thereby improve the H_2O_2 -neutralising ability of Hb.

Whereas much emphasis has been placed on the pro-oxidant potential of Hb, reactions of this abundant red cell constituent (approximately 300g/litre packed red cells [13]) with various RS make for a highly efficient antioxidant system which is perhaps not yet fully appreciated [308]. As mentioned above, oxyHb acts as an oxidant by rapidly destroying both $NO^{\bullet}$ and $ONOO^{-}$ that enter the red cells [315, 316]. Another way, therefore, of looking at the oxidation of oxyHb and metHb by H_2O_2 is that, in-so-doing, H_2O_2 is removed. The efficiency of this particular antioxidant action of Hb is greatly enhanced by the fact that the product of oxidation, namely oxoferrylHb, can be reduced back to metHb, thereby setting up a catalytic cycle. One way in which this is suggested to occur is via a catalase-like, or catalatic-type, activity of metHb (Fig 43).

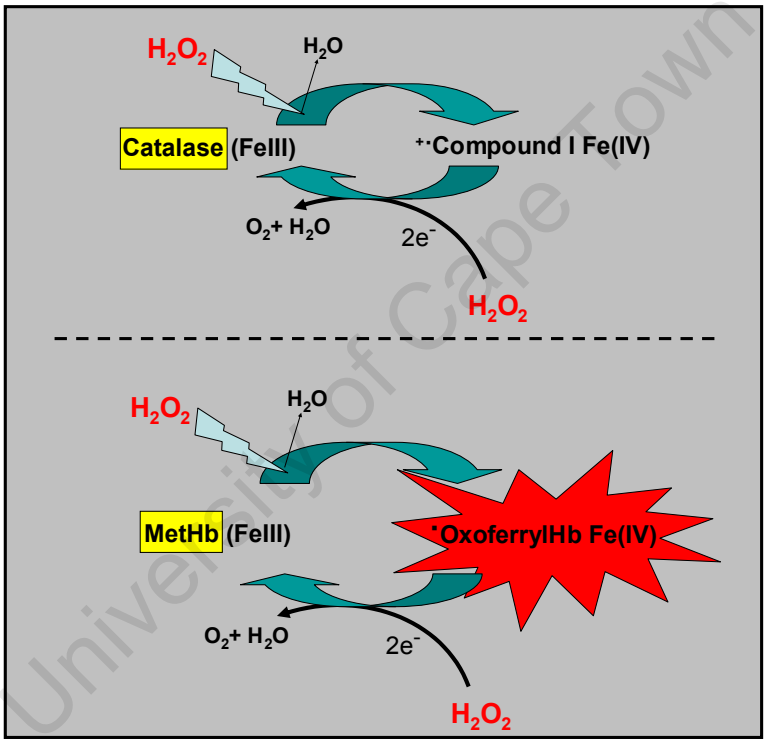


Figure 43. Catalase-dependent removal of H_2O_2 (above), and the catalatic activity of metHb, as proposed by Nagababu et al. (2004) [9] (below).

Both metHb and catalase (as was described in Chapter 2 Literature Review) are Fe(III) haem species. Both donate 2 electrons to reduce 1 molecule of H_2O_2 to H_2O. In the process, metHb is oxidized to oxoferrylHb, whilst catalase forms compound I. Each of these products are Fe(IV) species with an additional electron having been removed from the globin chain in the case of oxoferrylHb, and from the porphyrin ring to produce a porphyrin cation in the case of compound I. The oxoferrylHb species, like ferrylHb, can be reduced to metHb by H_2O_2. However, since the haem

degradation products observed when oxyHb reacts with H_2O_2 do not form when metHb reacts with H_2O_2 ([294], it has been suggested that this is because oxoferrylHb can abstract 2 electrons from one H_2O_2 molecule, reducing it to water and oxygen [293]. Therefore, unlike for the reaction of ferrylHb with H_2O_2, no $O_2^{\cdot -}$ is formed when H_2O_2 reacts with oxoferryl. Nagababu et. al. (2004) have therefore proposed that reaction of metHb with H_2O_2 is analogous with the reaction of catalase with H_2O_2, i.e. that metHb can remove H_2O_2 via catalase-like activity [9].

If metHb was able to function like catalase, then, as for catalase, 2 molecules of H_2O_2 should be removed with every cycle of one metHb molecule (reviewed by Nagababu et. al. (2004) [9]). However, an earlier study by Guilivi et. al. (1990) found that oxoferrylHb did NOT have catalatic-like activity - they showed that only 1 molecule of H_2O_2 is decomposed by each oxoferrylHb molecule per cycle [320]. Therefore, although metHb appears to be reformed on reduction of oxoferrylHb by H_2O_2 (in order to explain the continued cycling), no net removal of H_2O_2 occurs in this step. One possible explanation is that, as for ferrylHb (see Fig 42 above), only one electron is removed from 2 different H_2O_2 molecules during the reduction of oxoferrylHb. Each H_2O_2 would be oxidized to form $O_2^{\cdot -}$, and hence reform H_2O_2 (why this $O_2^{\cdot -}$ would not be causing haem degradation of oxoferrylHb is unclear). Then on spectrophotometry, Guilivi et. al. (1990) also showed that the end-product of the reaction between oxyHb and a continuous flux of H_2O_2 is an Fe(IV) Hb species [320] (presumably oxoferrylHb) – this suggests that, at least at these concentrations of H_2O_2, the rate at which H_2O_2 reduces oxoferrylHb must be slower than the rate at which metHb is oxidized by H_2O_2, which is again unlike the activity of catalase [8].

In the same study, Guilivi reported that, under more physiologically-correct *in vitro* conditions of using a greater oxyHb concentration, Fe(IV) Hb was only an intermediate product, with metHb being the final product [320]. They provided experimental evidence to support their explanation that, under such conditions, most of the H_2O_2 would be consumed by the oxidation of oxyHb, and that oxyHb (rather than H_2O_2) would donate an electron to the ferrylHb intermediate via a comproportionation reaction, whereby 2 molecules of metHb would be formed. Although they proposed this to be occurring between oxyHb and ferrylHb, they also showed in a separate experiment that oxyHb, as well as some LMW substances (see below), were able to donate the electrons to reduce oxoferrylHb back to metHb [320].

MetHb can therefore remove H_2O_2, by coupling its reduction to water using a suitable electron donor, for example oxyHb (and even H_2O_2 itself). This forms the basis for

the activity of the haem-containing, non-specific peroxidase enzymes (although these peroxidases are usually viewed rather as using H_2O_2 to actively oxidize substances for a specific purpose). As a result, Hb has been dubbed by some as a 'pseudoperoxidase' or peroxidase 'mimic', although it in fact acts as a true peroxidase [8, 321]. Figure 44 illustrates how compound I of horseradish peroxidase, a classic example of a non-specific peroxidase, receives two electrons in two one-electron steps, with each electron donor forming a radical (which may then dimerise). The exact steps whereby the oxoferryl iron and the protein radical of oxoferrylHb [322], as for the equivalent ferrylmyoglobin [323], are reduced are complex, but essentially as for the peroxidase enzymes, can involve the 2 electron reduction of oxoferrylHb by an electron donor.

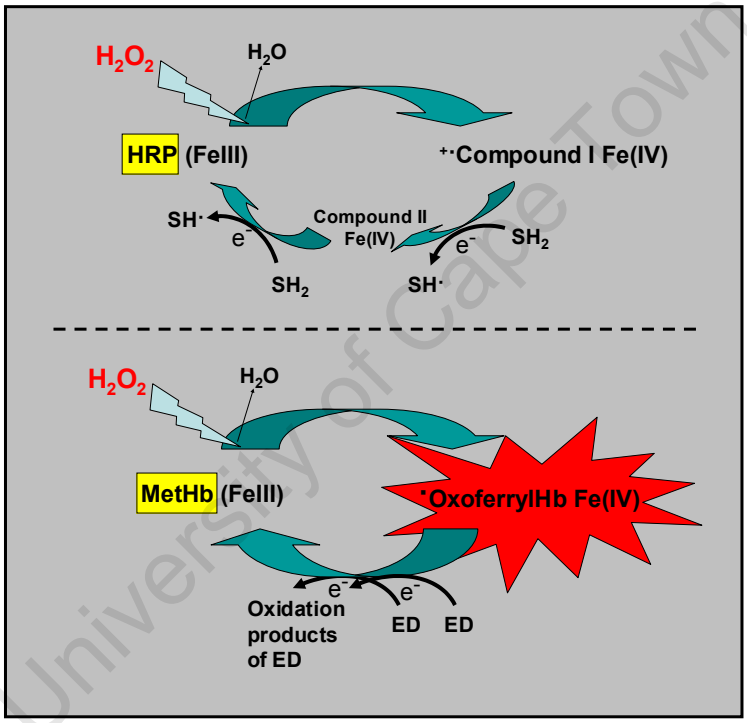


Figure 44. Non-specific peroxidase activity of horseradish peroxidase (HRP) (above), and the peroxidase-like activity of metHb (below). ED = electron donor.

Guilivi et. al. (1990) showed that urate could reduce oxoferrylHb back to metHb [320]. This is in keeping with the findings of Kaur et. al. (1990) that, although urate is not oxidized by H_2O_2, addition of Hb (or hemin) to the reaction leads to the formation of urate oxidation products [235] because the oxoferrylHb species formed is able to oxidize urate. These investigators showed that the rate constant for the reduction of oxoferrylHb was significantly greater for urate than for oxyHb (153 M^{-1} s^{-1} versus 23 M^{-1} s^{-1}). In their study, ascorbate also reduced oxoferrylHb, but with a much lower

rate constant, whilst GSH, NADH, and NADPH showed no detectable reducing activity. Another study also reported that urate reduced oxoferrylHb much more efficiently than did oxyHb (100 M^{-1} s^{-1} versus 20 M^{-1} s^{-1}) [321]. However, unlike in the Guilivi study, these investigators found that ascorbate had a 4-fold greater rate constant than urate to reduce oxoxferrylHb. Despite the significantly higher rate constants for urate and ascorbate for the reduction of oxoferrylHb, the concentration of urate (120μM, see Chapter 1, Fig 16) and ascorbate (40-70μM [292]) in human red cells compared with that of the oxyHb concentration (5mM [289]) means that oxyHb would by far be the most significant potential reductant for oxoferrylHb within the red cell [320].

However, if one now considers the extremely high levels of urate reported in Chapter 1 to be present in the red cells of elite-athlete horses (up to 800μM, see Results and Discussion, section iv, Fig 16), it may well be that under such conditions urate can play a physiological role in the elimination of oxoferrylHb radicals to reform metHb. At this concentration, the rate of removal of oxoferrylHb (and hence removal of H_2O_2) by urate should theoretically be at least in a similar range to that which intra-erythrocytic concentrations of oxyHb would be removing.

There are no reports on the reduction of oxoferrylHb back to metHb by tyrosine, tryptophan or urate riboside, these all being other LMW substances also reported in Chapter 1 to accumulate in the red cells of some mammalian species. However, it is known that the horseradish peroxidase enzyme can abstract electrons from either tyrosine (forming dityrosine) or tryptophan (unknown products formed) [324]. Similarly, myeloperoxidase (MPO) also catalyses the formation of tyrosyl radicals [197]. Harley et. al. (2004) showed that free tyrosine, like urate, although not oxidised by H_2O_2, is oxidized by H_2O_2 plus Hb (or hemin), and that dityrosine is formed [4], indicating at least that tyrosine (and possibly tryptophan also) can donate electrons to oxoferrylHb.

More recently, interest has grown on the subject of using the peroxidase activity of metHb plus a suitable electron donor to protect acellular and cellular-type haemoglobins (developed as “blood substitutes”) from auto-oxidation, as well as to limit the toxicity associated with these haemoglobin-based oxygen carriers (HBOC) [321, 325]. It is for such purposes that Atoji et. al. (2006) considered the possibility that, because horseradish peroxidase can use tyrosine as electron donor to eliminate H_2O_2, metHb plus tyrosine may similarly eliminate H_2O_2. They showed that the apparent rate constant for elimination of H_2O_2 was 10 times greater when tyrosine

(1mM) was added to solutions of metHb (5μM) and H_2O_2 (200μM), and that by coencapsulating a mixed solution of metHb plus tyrosine with oxyHb into vesicles (cellular-type HBOCs), the oxidation of oxyHb was significantly delayed, both *in vitro* and *in vivo*. This clearly indicates that tyrosine must be able to reduce oxoferrylHb back to the original metHb form.

It is proposed that a further evolutionary advantage for the novel finding of the accumulation of these LMW substances *specifically within the Hb-rich milieu of* the red cells may be to promote the peroxidase-like H_2O_2 -removing ability of metHb by being electron donors for the reduction of oxoferryl Hb, thereby providing antioxidant protection either specifically for the red cell, or more generally for the tissues perfused by red cells. The comparative effect of the presence of these and other putative electron donors on the metHb-dependent removal of H_2O_2 has not previously been reported.

(C) AIM

To compare the effect of urate, tyrosine, tryptophan and other putative electron donors present in red cells on the peroxidase-like ability of metHb to remove H_2O_2.

(D) MATERIALS AND METHODS

(i) Reagents

Tyrosine, urate, GSH, human Hb, BHT, methanol and xylenol orange were purchased from Sigma-Aldrich (USA); NADH from Boehringer Mannheim (W. Germany); tryptophan from Hopkin & Williams Ltd (UK).; ascorbate from Allied Drug Company (SA); Hydrogen peroxide and H_2SO_4 were purchased from Merck (USA), and Fe ammonium sulphate from Univar (USA).

(ii) Hb-peroxidase reactions

One milliliter reactions in 10mM potassium phosphate buffer (pH 7.4) were performed in eppindorfs at room temperature. All working solutions were made fresh. The H_2O_2 was always added last to the reaction.

The final reactions contained:

- 50μl Hb (10μM)[(a)]
- 900μl of one of the putative electron donors(250μM) [(b)]
- 50μl H_2O_2 (500μM)

Various control reactions were also performed:

No Hb catalyst
- 50μl phosphate buffer
- 900μl of one of the putative electron donors (250μM)
- 50μl H_2O_2 (500μM)

AND

No LMW reducing substance
- 50μl Hb (10μM)
- 900μl phosphate buffer
- 50μl H_2O_2 (500μM)

AND

H_2O_2 stability over time
- 950μl phosphate buffer
- 50μl H_2O_2 (500μM)

[(a)] Commercially-available human Hb (approximately 0.008g) was reconstituted in phosphate buffer (800μl). This was centrifuged at 10 000g for 10min to remove any precipitated Hb. The resulting concentration of this solution was determined spectrophotometrically according to Winterbourn et. al. (1990) [211]. This revealed that most (96%) of the soluble Hb was oxidized, being present as methaemoglobin as indicated by the manufacturer. A 200μM working solution was made in buffer.

[b] Urate, tyrosine, tryptophan, ascorbate, NADH and GSH solutions (278μM) were made in potassium phosphate buffer (pH 7.4). The concentrations of these solutions were confirmed using spectrophotometry. This provided a final concentration of 250μM of each LMW substance when adding 900μl of these solutions to the 1ml reactions.

(iii) Ferrous ion oxidation-xylenol orange (FOX) assay

The ferrous ion oxidation-xylenol orange (FOX) assay was used to determine how much H_2O_2 was still present in each of the reactions at various time points. The FOX assay was first developed (and later modified) by Wolff and coworkers (1990) as a sensitive peroxide-measuring technique [326-328]. It is a simple colorimetric method in which hydroperoxides (including H_2O_2) oxidise ferrous Fe(II) to ferric Fe(III) iron; this then forms a xylenol orange-ferric ion complex, which is a chromagen with maximal absorption at 560nm. The assay has been used to measure plasma lipid hydroperoxides [328], to determine H_2O_2 levels in human urine [329, 330], to compare the H_2O_2 scavenging ability (and by extrapolation the catalase activites) of red cell lysates from different mammalian species [307], and to determine the antioxidant capacity of various foods in preventing the formation of lipid hydroperoxides [331]. The method used in the current study is a modification of the original assay (protocol kindly obtained from Professor Dave Marais, University of Cape Town).

The FOX reagent mix[c] was prepared just prior to performing the assay. A volume of 180μl of the FOX reagent mix was added to the required wells of a clear 96-well plate (Greiner Bio-one, Germany), and evaporation over time was prevented by sealing the tops of the wells with cellotape. At 0, 10, 20, 40min, and every 20 min thereafter for 160min, 20μl of each peroxidase and control reactions was added to the FOX reagent mix in the appropriate wells. The plate was tapped to ensure adequate mixing of the reaction contents at each time point, and the wells sealed again. Thirty minutes after the last time point (sufficient to reach the plateau in the reaction between H_2O_2 and the FOX reagent mix [332]), the plate was shaken for 10 seconds and the absorbance at 560nm was determined on a spectrophotometer (Labotech, USA).

[c] The FOX reagent mix was made from 1 part FOX mix A and 4.5 parts FOX mix B. FOX mixes A and B were stored at 4°C for up to 2 weeks.

FOX mix A: 2.5mM Fe ammonium sulphate and 2mM xylenol orange in 0.25M H_2SO_4

FOX mix B: 0.88mM butylated hydroxytoluene (BHT) in analytical grade methanol (BHT is a potent antioxidant which, if analyzing a biological sample, will prevent the formation of any further lipid hydroperoxides).

A standard curve for H_2O_2 concentration (Fig 45), as well as measures of absorbance for the FOX mix A and the FOX reagent mix, were performed with each assay to control for consistency of the freshly made working solutions. The standard curve would also serve to determine actual H_2O_2 in reactions if required.

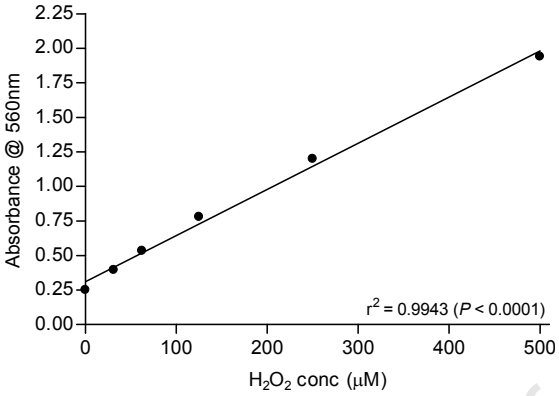


Figure 45. FOX assay standard curve for H_2O_2 concentration. The Goodness of Fit test was used to determine the linear regression.

(iv) *Statistical considerations*

The intra-assay coefficient of variation (CV) for the FOX assay was 0.8% (across the plate) and 2.2% (down the plate), using H_2O_2 concentration at 40min in a reaction containing Hb and urate as the quality control reaction.

(E) RESULTS AND DISCUSSION

Reaction of H_2O_2 with methaemoglobin (metHb), or oxyHb, leads to the formation of oxoferrylHb [294]. Both urate and ascorbate are able to efficiently reduce oxoferrylHb back to metHb, thereby providing the electrons for the peroxidase-like activity of metHb. Yet even in the absence of such reducing substrates, H_2O_2 is consumed, presumably because H_2O_2 itself can provide the electrons to reduce the highly reactive oxoferrylHb species. One possible evolutionary advantage for the high levels of urate, urate riboside, tyrosine and tryptophan within the red cells of some mammals may be that their presence may accelerate the metHb-dependent removal of H_2O_2, as has already previously been shown *in vitro* for tyrosine. The influence of equimolar amounts of urate, tyrosine and tryptophan on the peroxidase-like activity of catalytic amounts of metHb over time was therefore investigated, and compared with the control reaction of H_2O_2 plus metHb alone. Ascorbate, NADH and GSH (all established LMW antioxidants present in the red cell) were also compared. The FOX assay was used to determine the residual H_2O_2 present in the reactions at each time point.

Figure 46 below depicts the decrease, over time, in absorbance at 560nm (reflecting H_2O_2 concentration) in reactions starting with 10μM metHb plus 500μM H_2O_2 plus/minus one of various putative electron donors (each at 250μM). The ability of metHb on its own to remove H_2O_2 was observed, which is in agreement with the findings of Guilivi et. al (1990), that reaction of oxoferrylHb with H_2O_2 results in removal of H_2O_2 (by reforming metHb that then reduces 1 molecule of H_2O_2 per cycle) [320]. This supports the proposal that H_2O_2 itself can act as the electron donor to reduce oxoferryl. Significantly, however, all the LMW substances, except GSH, increased (to different degrees) the amount of H_2O_2 removed compared with the reaction of metHb plus H_2O_2 alone.

The overall rate of H_2O_2 removal in these reactions (if no catalase is present) is determined by the rates of the forward and reverse reactions of the peroxidase cycle. The forward reaction is dependent on the rate constant for metHb + H_2O_2, and the concentration of H_2O_2 and metHb. The reverse reaction is dependent on the rate constant(s) for oxoferryl + substrate(s), and the concentration of H_2O_2, metHb, and the LMW substrate if present. Initially, at least, since the rate of the forward reaction would be identical in each reaction, irrespective of the LMW substrate present, the difference in the initial rate of removal of H_2O_2 in each reaction with equimolar amounts of different LMW substance would be dependent solely on the rate

constants for these reactions. Therefore, although in each reaction the electron contribution from H_2O_2 towards the reverse peroxidase reaction cannot be separated from that of the contribution from the LMW substances, it may at least be said (as seen from Fig 46A) that the initial rate constants (within the first 10 min) for these LMW substances to reduce oxoferrylHb would be increasing in the following order:

NADH < GSH = tryptophan = tyrosine < urate < ascorbate

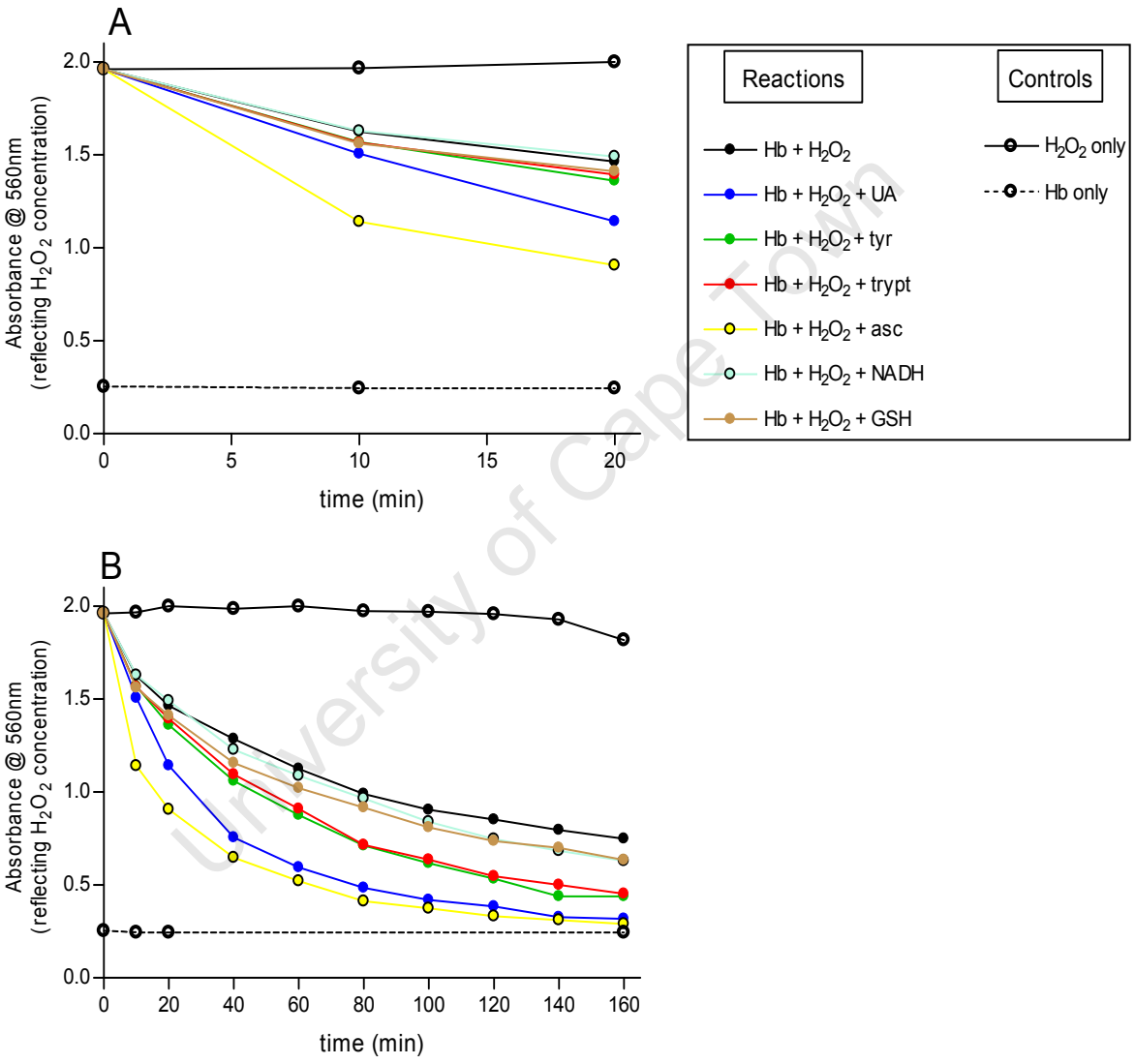


Figure 46. (A) The initial and then (B) sequential absorbance readings (at 560nm) of reactions in which the removal of H_2O_2 by the peroxidase-like activity of metHb in the presence of equimolar amounts of various LMW substances was evaluated. Absorbance readings directly reflect the H_2O_2 concentration left in each reaction. Each profile represents the average of two separate experiments, and each of the curves in the separate experiments showed the identical trend. Reactions (pH 7.4) contained 10μM metHb, 500μM H_2O_2 and 250μM LMW substance. (Asc=ascorbate; UA=urate; tyr=tyrosine; trypt=tryptophan).

Although the rate constants for urate and tyrosine are lower than that for ascorbate, their concentrations within the red cell need to be considered. The high levels of red cell urate in horse (150μM) and humans (120μM), and high levels of tyrosine in rhinoceros (1000μM), horses (280μM) and cats (360μM) may each be promoting the peroxidase activity of metHb (to be compared with ascorbate levels of 40-70μM in human red cells). In this way, these LMW may be contributing to formation of the H_2O_2 gradient between the red cell interior and the surrounding plasma.

Of note is that, although reactions with urate had a slower initial rate of H_2O_2 removal than reactions containing ascorbate, which is in keeping with the findings by Cooper et. al. (2008) who looked at oxidation rates of these substrates [321], a similar overall amount of H_2O_2 was eventually removed over time by both LMW substrates (Fig 46B). Reactions containing tyrosine and tryptophan showed a very similar pattern over time, with a larger residual H_2O_2 concentration remaining after 160min than for ascorbate and urate. Even with a prolonged reaction time, reactions with equimolar NADH and GSH eliminated only a little more H_2O_2 than occurred in reactions where only H_2O_2 could have provided electrons.

If H_2O_2 itself is able to provide the electrons for the reduction of oxoferrylHb, and hence for its own removal, then what would the advantage be of having additional electron-donating substances such as urate, tyrosine etc over the presence of H_2O_2 alone in the red cells together with metHb? Although H_2O_2 may appear to perform the same role as the LMW substances for the peroxidase activity of metHb, it should be noted that exposure of metHb to H_2O_2 leads to the formation of detectable Hb oxidation products, such as dityrosine [254]. Ramirez et. al. (2003) used an antibody to detect DMPO nitrone adducts formed on metHb protein radicals, and showed that ascorbate (at micromolar concentrations) and GSH (at millimolar concentrations) significantly reduced the level of nitrone adducts formed during oxidation of metHb by H_2O_2 [298]. Similarly, it has been found that, whereas the presence of ascorbate and urate during oxidation of metmyoglobin prevented dityrosine formation and reduced the oxoferryl form back to the original metmyoglobin form, addition of these substances *after* the reaction of metmyoglobin with H_2O_2 did not prevent dimer formation, and a modified form of metmyoglobin became apparent [260]. The presence of free tyrosine also has been shown to lead to decay of the protein radical formed during oxidation of metmyoglobin [262]. Therefore, it is possible that the reduction of oxoferrylHb by H_2O_2 does not lead to the complete recovery of metHb (although on spectrophotometry it appears very similar to metHb), and that this modified form of metHb becomes further damaged by the repeated

oxidation/reduction cycles. This may explain why the presumed peroxidase-like activity of metHb in the presence of H_2O_2 alone did not continue to remove all the H_2O_2. On the other hand, the presence of LMW substances such as urate and ascorbate with higher rate constants for reaction with oxoferrylHb may minimize the irreversible damage occurring to metHb on exposure to H_2O_2, thereby maintaining the integrity of the peroxidase enzyme system throughout a greater number of successive cycles, whilst sufficient concentrations of these substances are present.

It has previously been shown that the oxidation of both urate and ascorbate by metHb/H_2O_2 is linearly dependent on the concentration of metHb. The same concentration-dependent effect was found when the removal of H_2O_2 was evaluated in this study (in the absence of any LMW-reducing substance) (Fig 47A). Figure 47B shows the linear dependence of the removal of H_2O_2 (at 15mins) on the concentration of metHb - this reflects that metHb acts as a true enzyme [321]. In order to ensure a sufficient rate of metHb catalytic activity, 10μM metHb was used in the above reactions. This concentration has also been used by other investigators performing similar investigations.

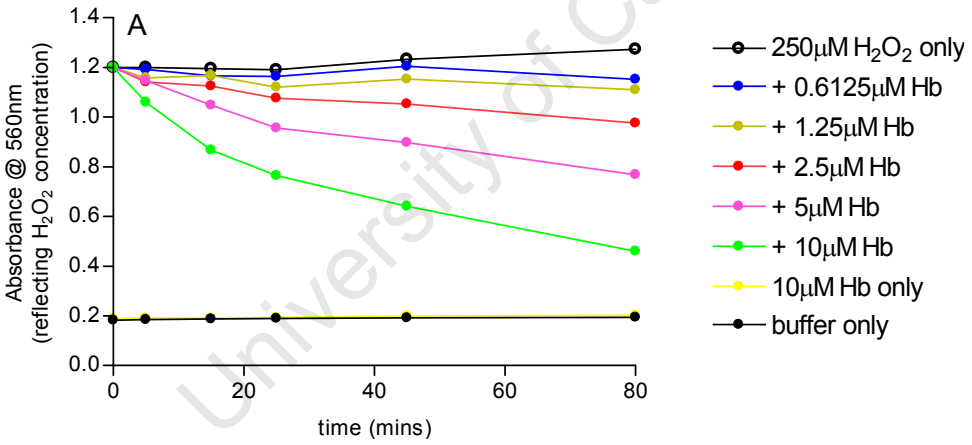
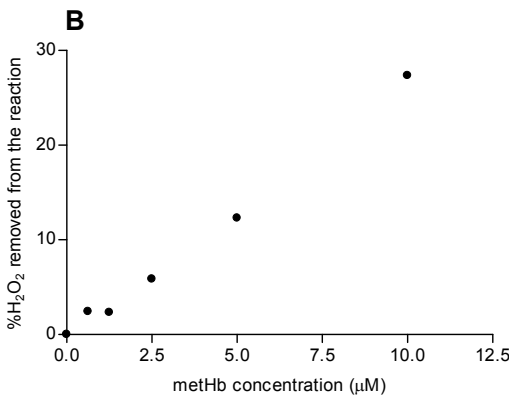


Figure 47. (A) Sequential absorbance readings (at 560nm) of control reactions (no metHb added) for the peroxidase-like activity of metHb in the presence of equimolar amounts of various LMW substances. Absorbance readings directly reflect the H_2O_2 concentration left in each reaction. Each result represents the average of two separate experiments, in the same trend was obtained. Reactions (pH 7.4) contained 500μM H_2O_2 and 250μM LMW substance. (B) As determined from figure A, metHb concentration dependence of the peroxidase-like activity of metHb at 15min.



An unexpected finding was obtained when performing the control reactions for the peroxidase-like activity of metHb. These reactions had the various LMW substrates plus H_2O_2, but no metHb. As anticipated, urate, tyrosine, tryptophan and NADH showed no direct scavenging of (and hence oxidation by) H_2O_2 (Fig 48). Ascorbate was able to remove significant amounts of H_2O_2 in the absence of metHb. To a much smaller extent, GSH also showed some ability to eliminate H_2O_2.

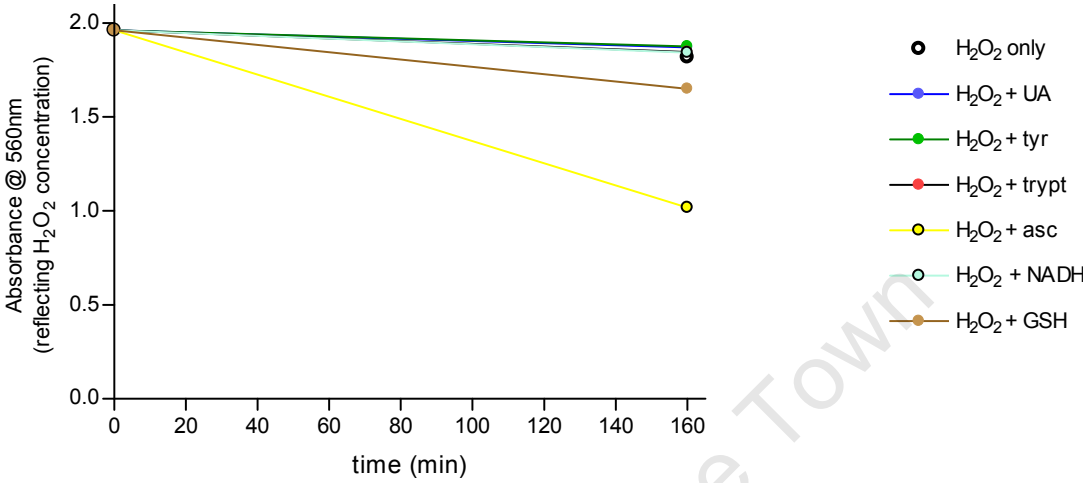


Figure 48. Absorbance readings (at 560nm), at time 0 and 160min, for control reactions (no metHb added). Absorbance readings directly reflect the H_2O_2 concentration left in each reaction. Each result represents the average of two separate experiments, in the same trend was obtained. Reactions (pH 7.4) contained 500μM H_2O_2 and 250μM LMW substance.

In vivo, catalase and the various peroxidase enzymes are responsible for removal of most of the H_2O_2. It is also possible that GSH and ascorbate may directly reduce H_2O_2, since although H_2O_2 is generally poorly reactive, it can however oxidise hyper-reactive –SH groups from some enzymes, as well as certain keto-acids such as pyruvate and 2-oxoglutarate. Certainly, ascorbate and GSH are believed to be among the most important antioxidants protecting oxyHb against auto-oxidation inside the red cells [292]. Although GSH showed a much lower rate of H_2O_2-removal than ascorbate, its intra-erythrocytic concentration is at least 30 times higher than that of ascorbate. It cannot however be excluded that a rapid interaction between ascorbate (and also GSH) and the subsequently-added Fe^{3+} in the FOX mix may have lead to the false impression that ascorbate in isolation reacts directly with H_2O_2.

Urate, although not oxidized by H_2O_2 alone, is known to be oxidised by the oxoferryl species produced on reaction of metHb (or hemin) with H_2O_2 [235]. Urate riboside, a LMW substance present in high concentrations in the red cells of cows and buffalo (see Chapter 1, Results and Discussion, section ii), has been shown by Smith et. al.

to have similar antioxidant properties to urate [36-38]. It was therefore investigated whether urate riboside would similarly be oxidized in the presence of H_2O_2 and hemin. The red cell acid extract from a cow (see Chapter 1, Materials and Methods) was exposed to H_2O_2, with and without the addition of 10μM hemin to the reaction (Fig 49).

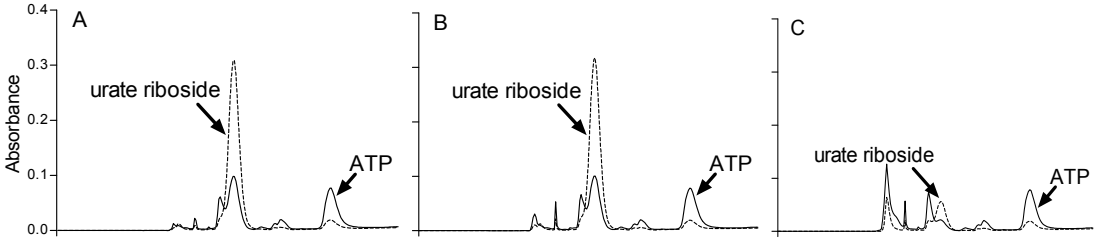


Figure 49. Anion-exchange HPLC of neutralized acid-soluble cow red cell extracts exposed, for 10min at room temperature, to (A) 0mM H_2O_2, (B) 2mM H_2O_2 and (C) 2mM H_2O_2 plus 10μM bovine hemin (Sigma).

Whilst exposure of a cow red cell extract to 2mM H_2O_2 showed no change in HPLC profile, the addition of 10 μM bovine hemin to the reaction caused significant oxidation of urate riboside, with no significant decrease evident in any of the other UV-absorbing substances present in the extract (Fig 4A). Although the rate of Hb-dependant H_2O_2 removal by urate ribsoside could not be compared with the other LMW substance present in red cells, the very high levels of urate riboside (millimolar range) present in the red cell of the cow may, as may be the case with high levels of urate in the red cells of some horses, provide some contribution to H_2O_2 removal within these red cells.

Therefore, it may be postulated that the presence of high levels of urate, urate riboside, tyrosine and even tryptophan may have evolved to improve the efficiency of the metHb peroxidase turnover cycle in removing H_2O_2. But how physiologically relevant is the peroxidase activity of metHb i.e. does it play a role in removing significant amounts of H_2O_2 *in vivo*?

The conventional view in more recent years has been that the two enzyme systems, GPx and catalase, together account for all the H_2O_2-disposal within the red cell, with GPx handling 'physiological' levels of H_2O_2, and catalase playing a greater role at higher concentrations of H_2O_2 [8]. However, other studies indicate that catalase and GPx alone, cannot account for all the H_2O_2 removed by red cells, and that other mechanisms are likely to be participating [205, 289]. The role of the peroxiredoxins

(Prx's), a family of peroxidase enzymes requiring thioredoxin and the NADPH-dependent thioredoxin reductase, in antioxidant defense in eukaryotic cells has only recently become appreciated [8, 289, 333]. They can also reduce organic peroxides and peroxynitrite [333]. They are widely distributed in animal, plant and bacteria cells [8]. In the red cell, the Prx enzymes (particularly Prx II) are some of the most abundant proteins (about 240μM Prx II) [289], Although Prx's can be inactivated by their substrate H_2O_2, and are slower at catalyzing H_2O_2 removal than GPx, their high cellular concentrations and low K_m values for H_2O_2 has led some to believe that they may be even more important peroxide-removing systems in animal cells than catalase or GPx, especially in disposing of low physiological levels of H_2O_2 [8, 334, 335]. Supporting this is the finding that knockout mice for Prx II gradually develop severe haemolytic anaemia and other signs of oxidative damage to their red cells [336].

The possibility that Hb, via its metHb peroxidase-like activity, may also be contributing to H_2O_2-removal within the red cell has been suggested, [205, 320], but never further investigated, or considered to be significant in human red cells. Although metHb is highly unlikely to compete with the high levels of highly active catalase in human red cells, it may play a role in removing low levels of H_2O_2 produced within red cells, as suggested for the peroxiredoxins. Given the extensively variable levels of catalase and GPx between different mammalian species (Table 1), it is also worth considering that the presence of high levels of LMW substances (eg. urate and tyrosine) in the red cells of some mammals may point to a greater contribution of the metHb-peroxidase system to H_2O_2 -neutralization in those red cells relative to human red cells.

Notably, black rhinoceros have extremely low levels of red cell catalase activity (<2% that present in human red cells [60]), and a diminished red cell rate of glycolysis through the HMP shunt [40. These features are likely to be underlying factors contributing to their inherent sensitivity to oxidant-induced injury {Paglia, 2000 #405, 337]. However, their red cells also have an unusually high level of free tyrosine, as reported by E.H. Harley and co-workers (2004) [2], and described in Chapter 1. It is conceivable that this may have evolved to promote the peroxidase-like activity of metHb as part of a compensatory H_2O_2-removal system. A similar concept was proposed by Galaris et. al. (1997) – they suggested that in myocytes, which have very low levels of catalase, the peroxidase-like activity of metmyoglobin (with ascorbate as electron donor) may be playing a significant role in the removal of H_2O_2 during oxidative stress [264]. Because lipid hydroperoxides can also initiate ferrylHb

chemistry, the presence of large amounts of dispensible LMW electron donors for oxoferrylHb may also have evolved in some mammalian species to assist GPx in the removal of organic peroxides.

Table 4. Comparative levels of various antioxidant enzyme activities and GSH content in the red cells of different mammalian species relative to levels in human red cells, which is set as the 100% control.

	SOD	Catalase	G6PD	GPx	GR	GSH
Human	100	100	100	100	100	100
Horse	126	68	160[5]	176		
Black Rhinoceros		≤2[6]	320[6]	1580[6]	80[6]	
Pig	101 117[1]	114 80[1]	109[5]	251 167[1]	78[1]	77[1]
Sheep	156 181[3] 133[4]	21 14[4]	13[3] 12[5]	506 357[3] 388[4]	38[3] 15[4]	138[3] 133[4] 148[5]
Cow	168, 272[3] 139[4]	55 64[3] 57[4]	125[3] 69[5]	200 450[3] 516[4]	25[3] 13[4]	115[4] 127[5]
Mouse	186 134[4]	43 36[4]		2687 981[4]	126[4]	104[4]
Rat	175[1] 200[3] 126[4]	40[1] 50[3] 79[4]	250[3]	333[1] 300[3] 441[4]	20[1] 19[3] 23[4]	115[1] 115[3] 119[4]
Rabbit	200 179[1] 264[3] 141[4]	115 80[1] 93[4]	138[3] 261[5]	274 200[1] 86[3] 122[4]	84[1] 88[3] 50[4]	132[1] 177[3] 107[4]
Dog	90[4]		116[5]	406[4]	41[4]	116[4] 90[5]
Cat	123[4]	171[4]	160[5]	488[4]	99[4]	119[4] 86[5]

Note. Because of the large variability in hemoglobin levels and size plus morphology of the red cells of the different species, the values obtained were all expressed per gram of Hb, and then extrapolated to a percentage of the human value (100%). All values that are equal to or greater than the respective human value are highlighted in blue.

SOD=superoxide dismutase; G6PD=glucose-6-phosphate dehydrogenase; GPx=glutathione peroxidase; GR=glutathione reductase; GSH= reduced glutathione.

Data referenced is from Maral et. al. (1977) [338], unless otherwise superscripted (1 = Godin et. al (1992)[339]; 2 = Avellini et. al. (1993)[340]; 3 = Suzuki et. al. (1984)[341]; 4 = Kurata et. al (1993)[342]; 5 = Kaneko et. al. (1974)[15]; 6 = Paglia et. al. (1992) [61].

An alternative way of looking at the evolutionary advantage of accumulating high levels of electron-donating LMW substances within the red cells of some species, for example tyrosine in the black rhinoceros, is that animals with lower levels of catalase or GPx may experience a higher level of oxidative modifications, including an increased rate of metHb and hence oxoferrylHb formation if their red cells. In such animals, electron-donating LMW substances could enhance the reduction of highly reactive oxoferrylHb radicals back to reusable unreactive metHb. Seen in this way, promoting the reverse reaction of the metHb-peroxidase cycle would be a defense mechanism, sacrificing tyrosine, for example, to prevent irreversible damage to haem or other nearby biological targets.

The finding of high tyrosine levels in cats was unexpected, considering what appears to be an adequate level of catalase and GPx. The Hb of cats, however, is known to be uniquely sensitive to oxidative damage resulting primarily from its high number of free sulfhydryl groups [343]. As a result, Heinz body formation is a common haematological abnormality in cats, and haemolytic anaemia ensues if the primary cause of the oxidative stress is not removed/treated (Heinz bodies are spheroidal red cell inclusions composed of denatured Hb, and are a late sign of oxidative damage). It is conceivable that cats specifically may have evolved the ability to accumulate high levels of tyrosine in their red cells in order to limit oxidative damage to Hb, by using tyrosine to reduce sulfhydryl radicals formed on their globin chains. Although cats are nevertheless highly susceptible to oxidative damage of their red cells, this susceptibility may be significantly worse without high levels of red cell tyrosine, and oxidative damage to peripheral tissues may be more evident.

Since certain physiological or pathological conditions may be associated with an increased rate of oxidation of oxyHb to metHb and then to oxoferrylHb, under such conditions it may be favourable to have an increased level of electron donors that can rapidly reduce oxoferrylHb radicals so as to limit further oxidative damage. In Chapter 1, it was suggested that the high stable levels of urate found in the red cells of some elite athlete horses may have been induced by their intense training schedule (exhaustive exercise is known to be associated with oxidative stress). It was further proposed that the ability to induce urate production within red cells (via the activation of inactive XO in these red cells by oxidative stress) may be a mechanism common to various/all mammalian species, rather than an evolutionary phenomenon limited to some horses. The increase in metHb concentration together with the increase in red cell urate level may lead to a greater rate of H_2O_2 -elimination within the red cell, and as a consequence from extracellular fluids.

(F) SUMMARY

In this Chapter, further evidence was provided to support the hypothesis that accumulation of high levels of urate, urate riboside, tyrosine and tryptophan within the red cells of some mammalian species may be species-specific evolutionary adaptations to increase the LMW antioxidant capacity of the red cell These LMW substances may not only serve as antioxidants to protect red cell contents against H_2O_2 and various other RS, but their presence at high levels within the red cell may promote the scavenging ability of the red cells to remove these RS from plasma. Accumulation of these substances may have evolved within the red cell in order to safely circulate high levels of these LMW antioxidants within a 'permeable package', in the same way that much of the antioxidant enzyme capacity of whole blood is conveniently packaged within the red cell. This data lends further support to the concept that the red cell is providing a convenient roaming 'package' of antioxidants, removing excess RS produced in blood and other tissues.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Although considerable detail is available concerning metabolic and structural features of the human red cell, it is well known that significant differences in various biochemical pathways exist when comparing the human red cell with the red cells from other mammalian species [15, 26, 94, 344-348]. This includes clear interspecies differences in the ion-exchange HPLC profiles of the red cells from different mammals [24, 25]; however many of these species-specific differences in red cell contents were never clarified.

In this thesis, a combination of reverse-phase and anion-exchange HPLC was used to further elucidate some of the differences in low molecular weight (LMW) content of the red cells from different mammalian species. By comparing the red cell and plasma profiles for each species, the unusual accumulation of substances such as tyrosine, tryptophan and urate within the red cells accounted for many of the interspecies differences. It was then proposed that the ability to accumulate such substances may play a role in red cell LMW antioxidant defense, and results of various *in vitro* experiments were consistent with such a hypothesis. These findings imply the existence of unsuspected antioxidant strategies in mammalian red cells, this being of interest to antioxidant biology in general, as well as having potential clinical application.

Oxidative stress, defined as a disturbance in the prooxidant-antioxidant balance in favour of the former [198], is not only a primary cause of disease, but is also now generally recognized as an important consequence of a large variety of chronic and acute conditions that affect not only humans, but also many different animal species. If re-establishment of the antioxidant/pro-oxidant balance does not occur, oxidative stress can then be a contributing/potentiating factor in the pathogenesis of the disease, by leading to oxidative damage of cellular components, including proteins, lipids and DNA. Therefore, although control of the initiating disease process is paramount, concurrent management of the oxidative stress may also be essential to limit progression of the disease, or prevent additional pathology.

Considering the likely role of the red cell as a roaming package of enzymatic and non-enzymatic antioxidants, permeable to and able to scavenge various reactive

species (RS), then changes in antioxidant levels of the red cells may provide an accurate and practical indicator of the extent of oxidative stress. Moreover, therapies that support the red cell in its ability to scavenge RS would not only protect the red cell, but may also protect the rest of the body against oxidative damage; however species-specific differences in antioxidant defenses may need to be considered. The fact that different mammalian species seem to have evolved different LMW red antioxidant strategies (probably as a consequence of differing environmental pressures) may have relevance to the management/prevention of veterinary conditions associated with oxidative stress.

For example, in mammalian species that accumulate high levels of red cell tyrosine, therapies aimed at increasing (or at least maintaining) red cell tyrosine levels may help to support endogenous antioxidant strategies, potentially useful for treatment in the acute situation of oxidative stress, or more generally as a preventative measure. Cats for example, which are particularly susceptible to oxidant drug-induced Heinz body haemolytic anaemia [343], may benefit from tyrosine supplementation to maintain their red cell free tyrosine levels under conditions of oxidative stress. Similarly, the black rhinoceros (*Diceros bicornis*) is a species particularly susceptible to developing a potentially lethal acute haemolytic anaemia when placed in captivity [30, 40, 61, 349], and dietary iron overload leading to oxidative stress is suspected to be a likely precipitating factor [350, 351] - the use of tyrosine supplementation for black rhinoceros in captivity may reduce this risk.

In addition, high levels of red cell tyrosine in various species may have the potential to be exploited as biomarkers of oxidative stress (although in some species such as horses, the large intraspecies variation in red cell tyrosine level is likely to be a limiting factor). Harley and co-workers (2004) have previously reported that black rhinoceros in captivity had significantly lower red cell tyrosine levels compared with their free-ranging counterparts [2]. Significant interest has also arisen more recently in the measurement of dityrosine as a biomarker of oxidative stress. This is a stable product of oxidative damage to proteins from various tissues (see Chapter 2) and is released into plasma. Elevated levels can be detected in urine samples from humans suffering from conditions associated with an elevated level of oxidative stress [6]. Therefore, in animals that have high levels of free tyrosine in their red cells, measures of red cell tyrosine, or of its oxidation product dityrosine, in red cell extracts may be useful biomarkers of oxidative stress. In preliminary investigations, it was found that a significant level of dityrosine was evident in the red cell extracts from healthy cats, compared with the extracts from horses with similar levels of red cell

tyrosine. This may be an indication that cats particularly rely on an increased level of baseline oxidative modification of red cell free tyrosine, which may be serving to maintain the reduced state of their oxidant-susceptible haemoglobin.

Red cell urate riboside levels may, in an analogous fashion, provide a biomarker of oxidative stress in bovids (although a large degree of intraspecies variability in urate riboside level may be a limiting factor). *In vitro* experiments indicate that urate riboside levels decrease in a manner proportional to the amount of hydrogen peroxide that cow red cells are exposed to. Various infectious diseases in cows and buffalo may give rise to oxidative stress; and since tuberculosis (TB) in humans has been shown to be associated with systemic oxidative stress [352, 353], bovine TB may be associated with changes in urate riboside levels. A small, preliminary study is currently underway to determine urate riboside levels in a TB-positive buffalo herd versus a TB-negative herd from the same area in South Africa. Bovine TB is endemic in African buffalo and a number of other wildlife species in South African game reserves [354]; such a biomarker may therefore prove to be useful as a rapid screening test for increased levels of oxidative stress in buffalo herds in such areas.

Attempts to increase the level of red cell urate riboside, by providing more substrate for the enzyme for example, may be another possible research area - improving the endogenous antioxidant defenses of TB-infected buffalo may limit the contribution of oxidative damage to the pulmonary tissue destruction associated with TB. The practicality of this may present a challenge, however, and more feasible may be a preventative approach - if the antioxidant defense system of healthy buffalo/cows can be increased, a better adaptive immune response may develop on exposure to the organism, either during infection or during vaccination. This should theoretically increase resistance to the development of active TB.

Perhaps most fascinating and with the most potential for further translational research, is the unprecedented finding of high levels of urate accumulating within the red cells of some horses sampled for this thesis. Unlike the ability to accumulate tyrosine or make urate riboside, which are most likely due to evolutionary red cell adaptations (probably involving gain mutation(s) for urate riboside, and parallel loss

mutation(s) for tyrosine), it was proposed in Chapter 1, that the ability to accumulate urate in horse red cells was reflective of the presence of XO/XD within red cells of some (or even perhaps all) mammalian species.

Although red cells have historically been presumed to have no XO/XD because no enzyme activity was detected, both active *and inactive* levels of this enzyme are now known to exist in various nucleated cells; red cells however were never re-evaluated for the presence of inactive XO/XD. Moreover, it has been shown that the activity of XO/XD can be further increased in these other tissues by various factors including oxidative stress [117]. The elite-athlete horses sampled for this thesis are believed to have inadvertently provided an *in vivo* model of repeated oxidative stress - their extreme fitness regime would have been associated with regular episodes of oxidative stress which may have lead to an adaptive ('hardening') antioxidant response, including the activiation of otherwise inactive red cell XO/XD, detected as an increase in red cell urate level.

This proposal, that red cells are able to produce urate under conditions of oxidative stress by virtue of the presence of red cell XO/XD, has never been investigated to date, and may be an unsuspected red cell strategy to 'upregulate' its antioxidant defenses when required. Further research to investigate these ideas is to be undertaken shortly as part of post-doctoral work. If these hypotheses are substantiated, this may have potential application to various fields of veterinary and human medicine, including sport's science.

Finally, significant variation in red cell urate and tyrosine in horse red cells (as for urate riboside in cows) seems to reflect a large degree of intraspecies genetic heterogentity for those substances in these two species - it is fascinating to speculate what such intraspecies variability implies for individual animals within each species, if indeed these substances are providing significant antioxidant protection; and under which pathophysiological conditions their role becomes valuable to the individual.

"The greatness of a nation and its moral progress can be judged by the way the animals are treated…"

Mahatma Gandhi

APPENDIX

[a]Most investigators wash the red cells twice or even three times [23, 125, 173, 181]. In this study, red cells were washed once only as it has been shown that washing red cells can cause mechanical disruption [307], and also more relevant to this study, several amino acids including tyrosine [83], as well as urate [5], are easily washed out of human red cells. Considering the paucity of UV-absorbant LMW substances detectable in the plasma of all animals evaluated (see Fig 11, Chapter 1, Results and Discussion, section iii), urate in human plasma is the only UV-absorbing substance with potential to be problematic with one wash of red cells. In order to determine if plasma urate could be contributing to the red cell urate concentration when using one wash step only, the following control experiment was done: A human blood sample was obtained and the red cells separated from the plasma. After one wash step, 200µl of the N/S supernatant above the red cell pellet was extracted as per the red cell protocol, and an HPLC profile created. The only substance detectable (as expected) was a very small urate peak. It was determined that even if as much as 10% of the 200µl of 'packed red cells' extracted was actually trapped N/S plus residual plasma, by extrapolation, this amount of contaminating plasma in the N/S would contribute no more than 5µM to the red cell's urate concentration of approximately 115 µM (i.e. 4%). One wash step is therefore optimal to obtain a representative red cell HPLC profile.

[b]The addition of 300µl N/S to the 200µl packed red cell prior to the addition of the PCA, was found to prevent clumping of the red cells that occurs otherwise on addition of PCA to packed red cells. The use of ice-cold PCA has been previously recommended in order to minimize acid hydrolysis of the nucleotides [17]. However, no difference in extract ATP concentration was evident whether the subsequent 10min centrifugation step was performed at room (22^oC) temperature or at 4^0C.

[c]The slow addition of K_2CO_3 is recommended because significant bubbling (release of CO_2) occurs on reaction of K_2CO_3 with PCA.

$$2HClO_4 + K_2CO_3 \rightarrow 2KClO_4 + H_2CO_3$$
$$\downarrow$$
$$H_2O + CO_2$$

[d]Either KOH or K_2CO_3 are usually used by others to neutralize PCA-extracts, both forming the insoluble $KClO_4$ with PCA. However, use of these alkalis is associated

with a number of problems: it is very easy to overshoot the neutralization step with the strong alkali KOH, and other investigators tend to use the alkaline base K_2CO_3. In this study it was found that, even with K_2CO_3, a constant final extract pH of 7 was difficult to obtain, and a constant amount of K_2CO_3 gave variable final pH. Careful and time-consuming titration of the K_2CO_3 was therefore required, particularly because it was easy to overshoot the correct pH with the high molarity K_2CO_3 required (so as not to dilute the extract too much further). Despite this, the final pH still seemed unpredictable. It was determined that a number of other factors also contributed to a variable final extract pH when using K_2CO_3: On addition of the K_2CO_3 to the acid extract, the time-dependent bubbling off of the CO_2 meant that pH would not be stable (rising slowly) until all the H_2CO_3 (which is a weak acid) had dissociated, and all the CO_2 had bubbled out of the sample. This often resulted in overshooting the final pH because too much K_2CO_3 had been added before the pH had stabilised. After addition of K_2CO_3, the pH of the extract was also affected by whether or not a lid was placed on the tubes whilst left on ice. Lastly, the pH of the thawed extracts was very often more alkaline than originally determined.

The significance of this altered pH of the extracts was that the elution position of certain substances, particularly urate, was effected, its peak often being more difficult to separate from other peaks. Urate also is known to degrade more rapidly in alkaline solutions [244]. Finally, equimolar (50µM) uric acid solutions at different pH (in buffer of pH 7, versus in water pH<7) vary quite significantly in their diode array scans, with more acidic solutions showing a left shift, as well as reduced peak absorbance. Since this work was performed using fixed UV-absorbance readings of 260nm and 280nm, quantification of pH-sensitive substances such as urate would be affected by final extract pH.

For these reasons, a modification of part of the extraction was developed towards the end of the thesis work, using 1M K_2HPO_4 which is a much weaker alkali.

$$HClO_4 + K_2HPO_4 \rightarrow KClO_4 + KH_2PO_4$$

Some of the red cell and plasma suspensions were extracted using this improved method, which ends up with the same final dilution of the red cell contents: One milliliter of ice-cold 0.9M PCA was added to the 500µl red cell or plasma suspensions whilst vortexing vigorously, giving a final PCA concentration of 0.67M. The acidified samples were immediately centrifuged at 4000rpm for 10 minutes at 22^{o}C to sediment the protein precipitate. Then 1300µl of this protein-free extract was

transferred to a fresh 5ml tube, and neutralized by the addition of 1200µl of a 1M K_2HPO_4 solution. Since no CO_2 is produced, no bubbling occurs when using K_2HPO_4, and the resultant pH is very consistently 7 on Whatman's pH paper, even after prolonged storage at -80°C. The only potential disadvantage of using K_2HPO_4 as the alkalinizing agent is that the resultant high phosphate concentration in the extract has significant UV absorbance throughout the spectrum used for nucleotide detection (i.e. 260nm and 280nm). However, with reverse-phase HPLC the potassium phosphates are not retained at all, and the peak does not interfere with other substances. With anion-exchange, the phosphates are retained, but the peak created does not co-elute with any other major UV-absorbant red cell LMW substance, and can easily be subtracted from the extract profile using computer editing. Diode array spectrophotometry cannot however be performed on K_2HPO_4-neutralised extracts because of this buffer's significant UV-absorption.

[e]For practical purposes, HPLC analysis of the animal extracts had to be batched. This meant that some extracts would need to be stored for up to one week prior to HPLC analysis. It was therefore decided to freeze all extracts at -80°C after the extraction step and prior to HPLC, as recommended by Bartlett (1986) [17]. Although the effect of a delay in the extraction of whole blood and washed red cells on LMW substances has been substantially reported [78, 81, 82]), the effect of delayed HPLC analysis on the extracts has not actually been described. The effect of freezing, and then storage at -80°C on levels of some of the LMW substances in a human red cell (Fig 50A) and a plasma extract (Fig 50B) was therefore investigated.

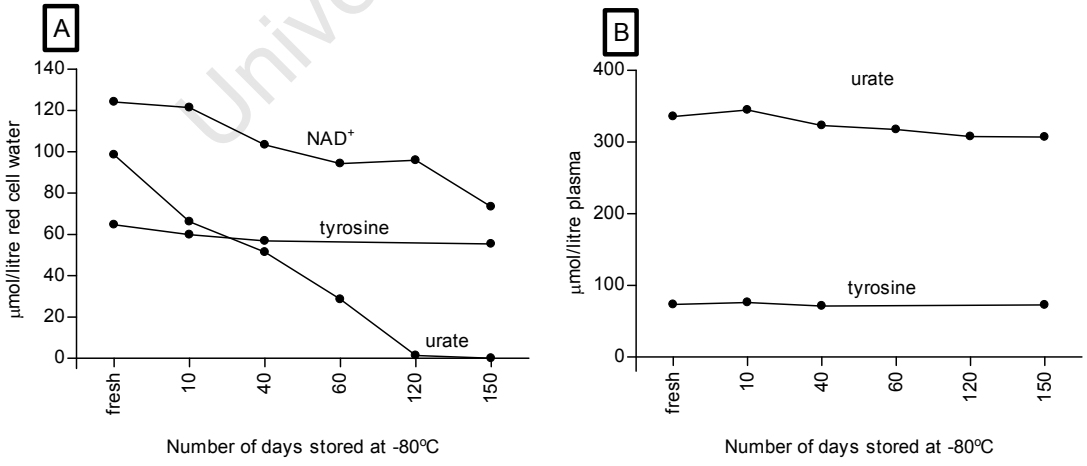


Figure 50. Stability, whilst in storage at -80° C, of (A) urate, tyrosine and NAD^+ in a human red cell extract, and (B) urate and tyrosine in a human plasma extract. Multiple aliquots of the extracts were made and then frozen simultaneously. An aliquot of the red cell and plasma extracts were thawed at each time point, and reverse-phase HPLC profiles produced.

It is therefore probable that in this study some measurements of urate in the red cell extracts, and NAD^+ to a lesser extent, may have been underestimated (possibly up to 30%); nevertheless, urate levels were in agreement with textbook values. Although reverse-phase HPLC is suboptimal for ATP determination, levels of this nucleotide appeared unaffected by long-term storage at -80^{o}C. The results indicate that extracts, particularly red cell extracts, should ideally be analysed fresh without prior freezing, especially if measuring urate. Interestingly, plasma urate was found to be a lot more stable to freezing than was red cell urate. Plasma urate levels were only minimally decreased, even after 5 months of storage at -80^{o}C. It was determined that the routine spectrophotometric assay used to determine plasma urate (based on the method of Praetorius using purified uricase [355]) could not be used to alternatively and rapidly measure red cell urate, as there seemed to be a large amount of a substance in the red cell extracts that interfered with the assay, giving a very large falsely-elevated level.

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