

Chapter 5

Methaemoglobin as an Indicator of PMOR Activity

5.1 Background

Here, the reduction of intracellular metHb by the provision of NADH in the extracellular medium was used as a probe of possible transmembrane electron transport from outside to inside RBCs. MetHb is confined to the intracellular compartment, thus changes in its reduction rate will reflect changes in the availability of intracellular reductants.

MetHb is often defined as oxidised Hb; that is the haem iron is oxidised from the ferrous (Fe^{2+}) state to the ferric (Fe^{3+}) state. In addition, in metHb the sixth coordination site of the haem iron is liganded to a water molecule; this shifts the absorbance maxima from that of Hb causing metHb to be chocolate brown in colour (acid form). At pH ~ 8 the water molecule is replaced by a bound hydroxyl group (alkaline form; Jaffé, 1981). This site is empty in deoxyHb and occupied by molecular oxygen in oxyHb (Jaffé, 1981). Due to this occupation of the sixth coordination site, metHb is unable to bind molecular oxygen. As the number of oxidised haem sites in the Hb molecule increases, unoxidised sites show an increase in oxygen affinity and a decrease in cooperativity, resulting in an inability to exchange molecular oxygen (Perrella, *et al.*, 1993). The α -chain of Hb is more readily oxidised than the β -chain (Mansouri and Winterhalter, 1974), thus intermediate oxidised Hbs exist mainly as $\alpha_2^{+3}\beta_2^{+2}$ rather than $\alpha_2^{+2}\beta_2^{+3}$ (Tomoda, *et al.*, 1978).

MetHb can be formed by the slow reaction of molecular oxygen with Hb; the transfer of an electron from the ferrous iron in the haem to molecular oxygen results in the

formation of metHb and superoxide (Jaffé, 1981). In human RBCs approximately 3% of total Hb is cycled to the metHb state every day; however, due to metHb reduction mechanisms the steady-state level of metHb is ~1% (Giardina, *et al.*, 1995). *In vivo*, metHb is predominately reduced by the NADH:cyt b_5 -MetHb reductase (NADH-MR) system (Figure 5.1A), which requires the cofactors cyt b_5 and NADH, the latter being continually regenerated via glycolysis (Jaffé, 1981). MetHb is also reduced through minor pathways such as NADPH-dependent metHb reductase (Figure 5.1B), and direct reduction by intracellular antioxidants such as ascorbate and GSH (Figure 5.1C). Like the NADH-MR system, the NADPH-dependent pathway requires an intermediate electron acceptor. The exogenous electron acceptor, methylene blue, quickly reduces metHb by this pathway (Jaffé, 1981). Free flavin and riboflavin have been suggested as physiological electron acceptors; however as flavin concentrations are low under normal conditions this pathway usually plays a minor role (Jaffé and Hultquist, 1995).

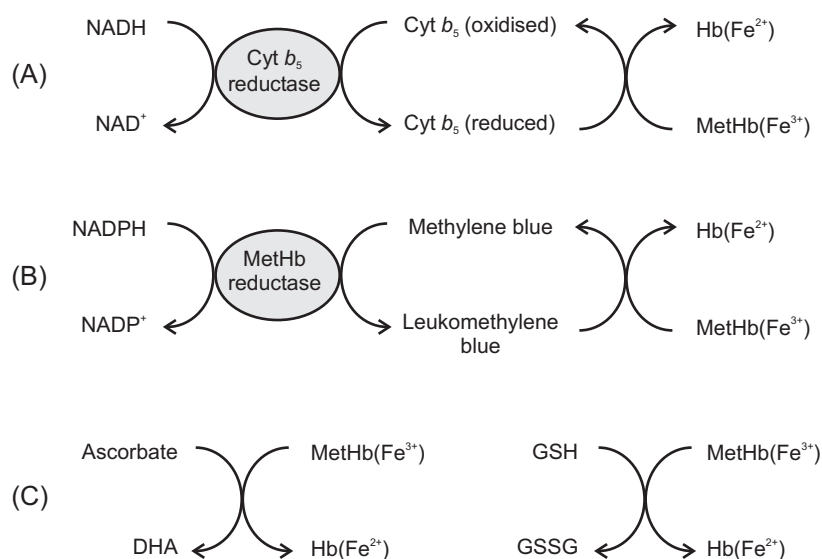


Figure 5.1: Metabolic pathways for the reduction of MetHb to Hb. (A) NADH-dependent cyt b_5 reductase pathway (NADH-MR). (B) NADPH-dependent reductase. (C) Direct reduction with endogenous reductants.

Methaemoglobinaemia, or the accumulation of metHb above normal steady state levels, can be caused by an increase in the rate of metHb formation (i.e., haem oxidation due to exposure to certain chemicals), a deficiency in the metHb reducing capacity (i.e. NADH-cyt b_5 reductase deficiency), or structural abnormalities of Hb

which stabilise its oxidised state (HbM; Lukens, 1993). Interestingly, cases of methaemoglobinaemia are rare in patients with NADH-cyt *b*₅ reductase deficiency as the minor pathways for metHb reduction can compensate for the loss of the main pathway and keep the steady-state metHb levels low (Jaffé and Hultquist, 1995). Methaemoglobinaemia can be induced by therapeutic agents (e.g., nitrites and quinones), domestic and industrial chemicals (e.g., nitrates in well water and food, nitrous gases and room odorizers), sulfonamides, aniline dyes, and a variety of aromatic amides and nitro-compounds (Hsieh and Jaffé, 1975; Lukens, 1993; Jaffé and Hultquist, 1995). Reducing agents may lead to the indirect oxidation of Hb through the production of ROS. Other chemicals such as nitrates and anilines are metabolised *in vivo* to compounds (e.g., nitrite) which subsequently oxidise Hb (Jaffé and Hultquist, 1995).

The symptoms of methaemoglobinaemia vary in intensity with the extent of metHb in the system. MetHb concentrations of 10–25% produce cyanosis, but no other apparent ill effects; at 35–45% metHb exertional dyspnea and fatigue are common symptoms; as the MetHb increases to 60%, extreme lethargy and stupor occur; and greater than 70% of the Hb in the oxidised form can be lethal (Lukens, 1993). Methaemoglobinaemia is treated by intravenous delivery of methylene blue. Methylene blue rapidly reduces metHb by the NADPH-dependent metHb reductase pathway (Figure 5.1B; Lukens, 1993), but requires a functioning oPPP to recycle NADPH; hence patients with G6PDH deficiency suffering from methaemoglobinaemia cannot be treated in this way. Methylene blue has also been shown to inhibit guanylate cyclase (Jin, *et al.*, 1995); hence alternatives to methylene blue with similar efficacy are desirable. Ascorbate, as mentioned above, can reduce metHb directly; however *in vitro* studies have shown that greater than 10 mM ascorbate is required to accelerate metHb reduction, making ascorbate an impractical alternative (Dotsch, *et al.*, 1998). *In vitro*, increasing the intracellular NADH concentration has been shown to increase rates of metHb reduction (Hsieh and Jaffé, 1975; Zerez, *et al.*, 1990), through increased availability of NADH for metHb reduction by the cyt *b*₅ reductase pathway. Thus if intracellular NADH can be increased by import of reducing equivalents through PMOR activity, metHb reduction should also be increased.

5.2 Motivation

In Chapter 4 the effect of extracellular NADH on intracellular metabolism was investigated by using NMR. The results did not provide conclusive evidence for electron transport from the extracellular NADH to intracellular oxidised metabolites and suggested the involvement of LDH in the effects of NADH. In this chapter, in an attempt to clarify the interpretation of these results, metHb was used as the endpoint intracellular electron acceptor. The reduction of intracellular metHb by extracellular NADH was studied in RBCs from a variety of mammalian species known to have different metHb reduction rates. The working hypothesis was that if metHb was reduced by extracellular NADH, in the absence of glucose, this would suggest an increase in intracellular NADH, possibly through transmembrane electron transport from extracellular NADH to intracellular NAD^+ , and that this effect might have species dependency. The rate of NADH oxidation and supernatant LDH activity were compared with MetHb reduction rates to clarify the involvement of LDH in the effects of NADH.

5.3 Results

5.3.1 *MetHb reduction in human and pig RBCs in the presence of extracellular NADH*

In human RBCs, extracellular NADH was shown to be capable of replacing glucose as the main source of reducing equivalents for metHb reduction. Over 8 h, human RBCs reduced 53% of the metHb when supplied with 5 mM extracellular NADH and 40% of metHb when supplied with 5 mM glucose (Figure 5.2A). Iodoacetamide (1 mM) inhibited the reduction of metHb by NADH or glucose almost completely, and no metHb was reduced in the absence of both NADH and glucose. The combination of extracellular NADH and glucose resulted in 72% of the metHb being reduced to Hb, while pyruvate and extracellular NADH were less effective than NADH alone, with only 22% of metHb reduced after 8h. The membrane-permeable redox dye

methylene blue rapidly reduced the metHb, with full reduction occurring in less than 2 h.

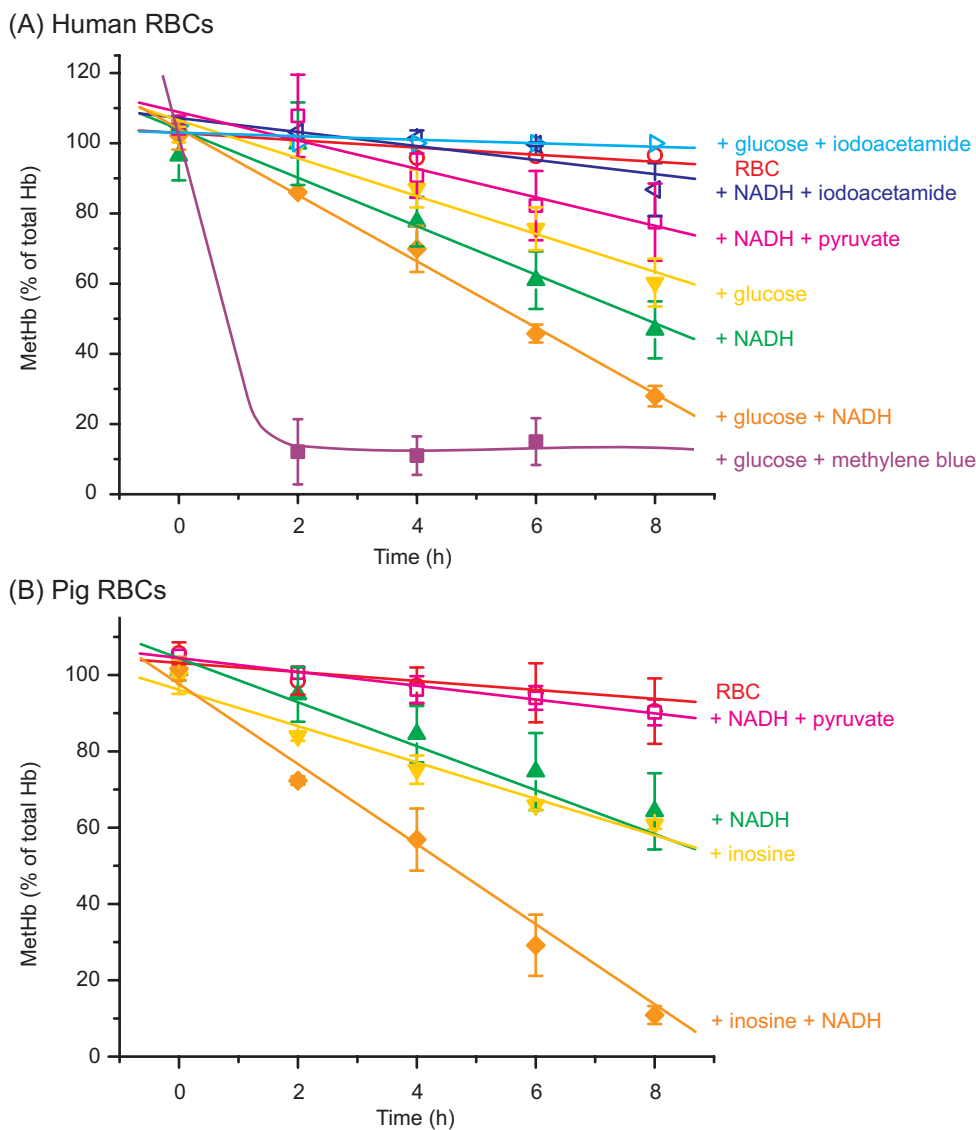


Figure 5.2: MetHb reduction in human (A) and pig (B) RBCs. RBCs were incubated with NaNO_2 to convert all Hb to metHb, then incubated at 37 °C for 8 h in 7 mM PBS ($H_c = 0.2$). **(A)** Human RBCs were incubated with no additions (\circ); 5 mM NADH and 30 mM nicotinamide (\blacktriangle); 5 mM glucose (\blacktriangledown); 5 mM glucose, 5 mM NADH and 30 mM nicotinamide (\blacklozenge); 5 mM pyruvate, 5 mM NADH and 30 mM nicotinamide (\square); 1 mM iodoacetamide, 5 mM NADH and 30 mM nicotinamide (\blacktriangleleft); 5 mM glucose and 1 mM iodoacetamide (\blacktriangleright); and 5 mM glucose and 1.25 μM methylene blue (\blacksquare). **(B)** Pig RBCs were incubated with no additions (\circ); 5 mM NADH and 30 mM nicotinamide (\blacktriangle); 5 mM inosine (\blacktriangledown); 5 mM inosine, 5 mM NADH and 30 mM nicotinamide (\blacklozenge); 5 mM pyruvate, 5 mM NADH and 30 mM nicotinamide (\square). The metHb concentration was determined as detailed in §2.4.5. Data points represent the average of at least three experiments and error bars denote ± 1 s.d. Data were fitted with lines of best fit.

Similar trends were observed with pig RBCs (Figure 5.2B). Mature pig RBCs do not have glucose transporters and instead use inosine as their main metabolic fuel (Jarvis, *et al.*, 1980). As observed with human RBCs, in the absence of nutrients essentially no metHb was reduced. In the presence of extracellular NADH or inosine ~38% of the metHb was reduced after 8 h, however reduction with NADH occurred at a faster rate. The combination of inosine and extracellular NADH led to 89% of the metHb being reduced at 8 h, while pyruvate in the presence of NADH inhibited the amount of metHb reduced to just 10%.

5.3.2 Species dependency of metHb reduction in the presence of extracellular NADH

Although extracellular NADH increased the rate of metHb reduction in RBCs from humans and pigs, this was not the case for RBCs from all animal species. It was shown that some mammalian RBCs are unable to reduce metHb when supplied with extracellular NADH (Figure 5.3), but that for all RBCs tested pyruvate inhibited the extent of metHb reduction in the presence of NADH. Dog, human, grey kangaroo and pig RBCs were all able to reduce metHb when provided with NADH, while RBCs from cattle, horse and sheep showed limited metHb reduction under these conditions.

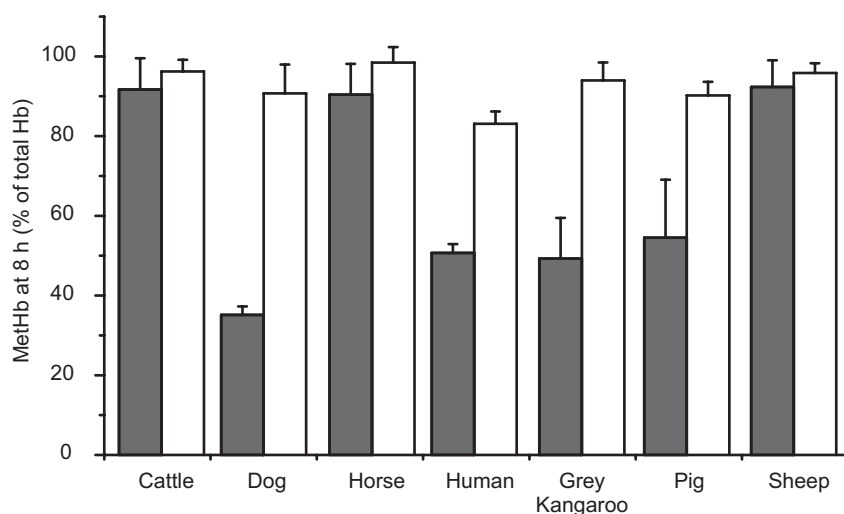


Figure 5.3: MetHb reduction by RBCs from a variety of different animal species. RBCs from cattle (n = 5), dog (n = 4), horse (n = 4), human (n = 3), grey kangaroo (n = 5), pig (n = 3) and sheep

(n = 3) were incubated with NaNO₂ to convert all Hb to metHb, then incubated at 37 °C for 8 h in 7 mM PBS (Hc = 0.2) with 5 mM NADH (■); and 5 mM NADH and 5 mM pyruvate (□). The metHb concentration at 8 h was determined using the method described in §2.4.5. Data represent the average of experiments on RBC from n individuals within each species. Error bars denote ± 1 s.d.

The lactate concentrations at 8 h were low in RBCs from animals that did not use NADH to reduce metHb (cattle, horse, sheep) and high in those that did use NADH (dog, human, grey kangaroo). Pyruvate concentrations were low across all species (Figure 5.4).

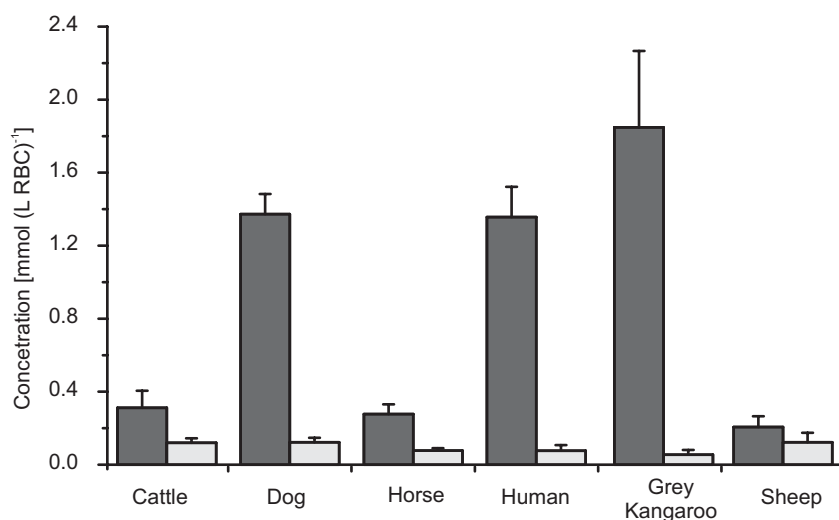


Figure 5.4: Lactate (■) and pyruvate (□) concentrations after 8 h incubation of RBCs containing methHb with 5 mM NADH. RBCs containing 100% metHb from cattle (n = 5), dog (n = 4), horse (n = 4), human (n = 3), grey kangaroo (n = 5), and sheep (n = 3) were incubated at 37 °C for 8 h in 7 mM PBS with 5 mM NADH. At 8 h the concentrations of lactate, and pyruvate were determined using the methods detailed in §2.4.2 and §2.4.6, respectively. Data represent the average of experiments on RBCs from n individuals within each species. Error bars denote ± 1 s.d.

5.3.3 Permeability of RBCs to NADH

To ensure that the effect of extracellular NADH on metHb reduction was not due to differences in membrane permeability [*adenyl-2,8-³H₂*]NADH was prepared (§2.3.2) and the partitioning of the radiolabel in the supernatant and cellular fractions examined (Figure 5.5). RBCs from all animal species tested showed minimal association of radioactivity with the cellular fraction; the majority of the radioactivity

was recovered in the supernatant (Figure 5.5). There was no difference between the partitioning in any of the species investigated. Hence, all RBCs tested appeared to be equally impermeable to NADH.

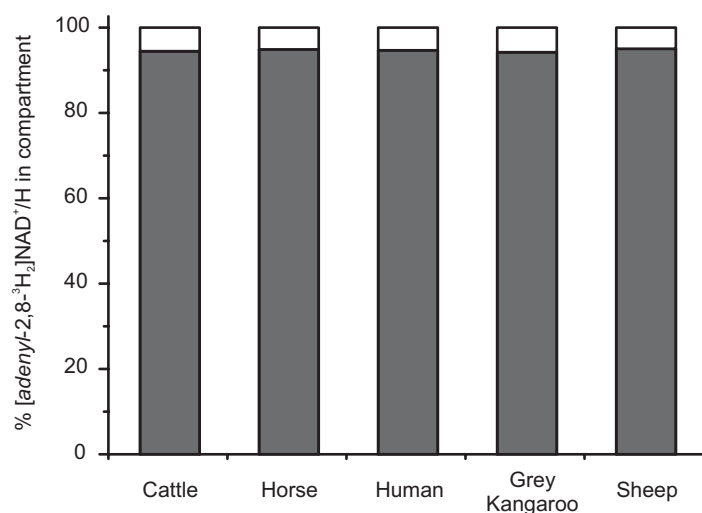


Figure 5.5: Percentage of radiolabel in supernatant (■) and cellular (□) fractions after 8 h incubation of RBCs containing methHb with [adenyl-2,8-³H₂]NADH. Cattle, horse, human, grey kangaroo, and sheep RBCs were incubated with NaNO₂ to convert Hb to methHb, and then incubated at 37 °C for 8 h in 7 mM PBS with 5 mM NADH/[adenyl-2,8-³H₂]NADH. The cellular and supernatant fractions were separated and the radioactivity measured as described in §2.4.4.

5.3.4 Oxidation of extracellular NADH by RBCs containing methHb

The NADH oxidation rate in the supernatants of RBC suspensions from the different animal species correlated with the ability of the RBCs to reduce methHb with extracellular NADH. RBCs from humans, grey kangaroos and pigs had higher rates of NADH oxidation, while those from cattle, horse and sheep had comparatively lower rates of NADH oxidation (Figure 5.6).

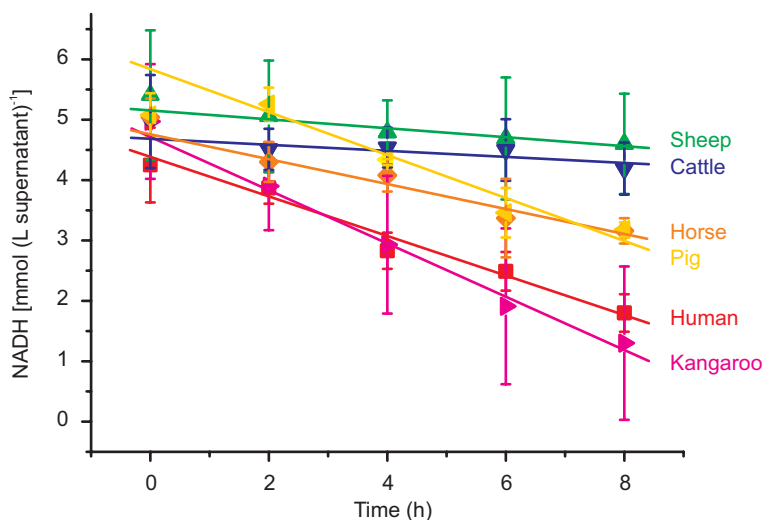


Figure 5.6: NADH oxidation rates in supernatants of methHb containing RBCs from various animal species. RBCs containing 100% methHb from cattle (n = 4, ▼), horse (n = 2, ◆), human (n = 3, ■), grey kangaroo (n = 2, ◄), pig (n = 2, ◄) and sheep (n = 2, ▲) were incubated at 37 °C for 8 h in 7 mM PBS with 5 mM NADH. The NADH concentration was calculated at intervals of 2 h from the absorbance at 340 nm and the molar extinction coefficient of NADH ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Data represent the average of experiments of RBCs from n individuals within each species. Error bars denote ± 1 s.d. Lines of best fit were applied to the data from 2–8 h.

5.3.5 Effect of extracellular LDH on NADH oxidation and methHb reduction rates

The NADH oxidation rate was compared with the methHb reduction rate in the presence and absence of nutrients and exogenous LDH for RBCs from animals with low (cattle, Figure 5.7) and high (pig, Figure 5.8) rates of methHb reduction in the presence of NADH. The methHb reduction and NADH oxidation rates were higher on the addition of nutrient and NADH for RBCs of both animal species. The methHb reduction rate was 9-fold higher for cattle RBCs incubated with both NADH and glucose, and was 2.4-fold higher for pig RBCs incubated with NADH and inosine, compared to NADH alone. The NADH oxidation rates in the presence of nutrients were 3.3 and 1.4-fold higher than those without, for cattle and pig RBCs, respectively. Additionally, the inclusion of LDH in the supernatant increased the methHb reduction rates in both the absence (cattle 5.1-fold, pigs 1.8-fold) and presence (cattle 1.6-fold, pigs 1.04-fold) of nutrients when compared to rates in the absence of exogenous LDH (Figures 5.7 and 5.8).

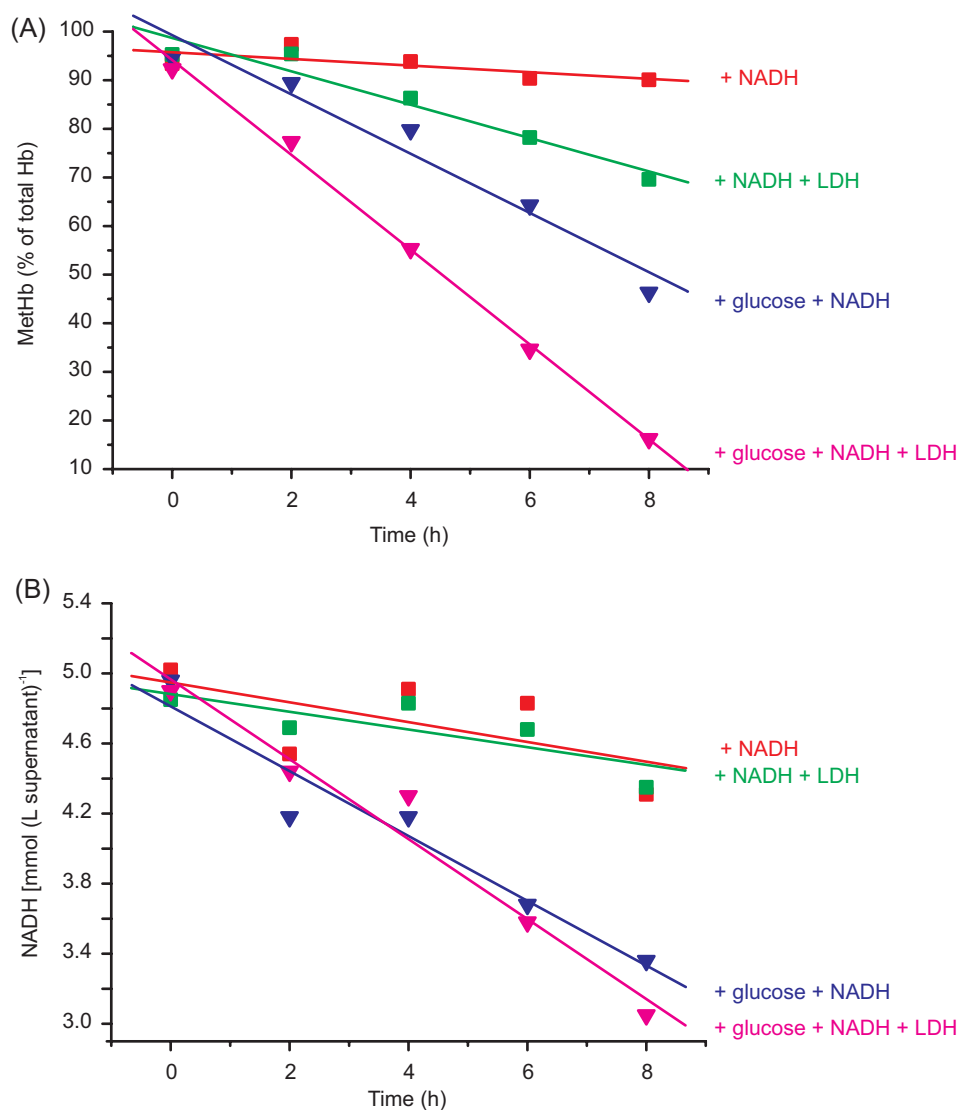


Figure 5.7: MethHb reduction (A) and NADH oxidation (B) in RBC suspensions and supernatants of cattle RBCs incubated with NADH and LDH. Cattle RBCs were incubated with NaNO_2 to convert Hb to metHb then incubated at 37°C for 8 h in 7 mM PBS ($\text{Hc} = 0.2$) with 5 mM NADH (■); 5 mM NADH and 3 IU LDH (■); 5 mM glucose and 5 mM NADH (▼); and 5 mM glucose, 5 mM NADH and 3 IU LDH (▼). The metHb concentration (A) was determined as detailed in §2.4.5. The NADH concentration (B) was calculated from the absorbance at 340 nm and the molar extinction coefficient of NADH ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Lines of best fit are shown.

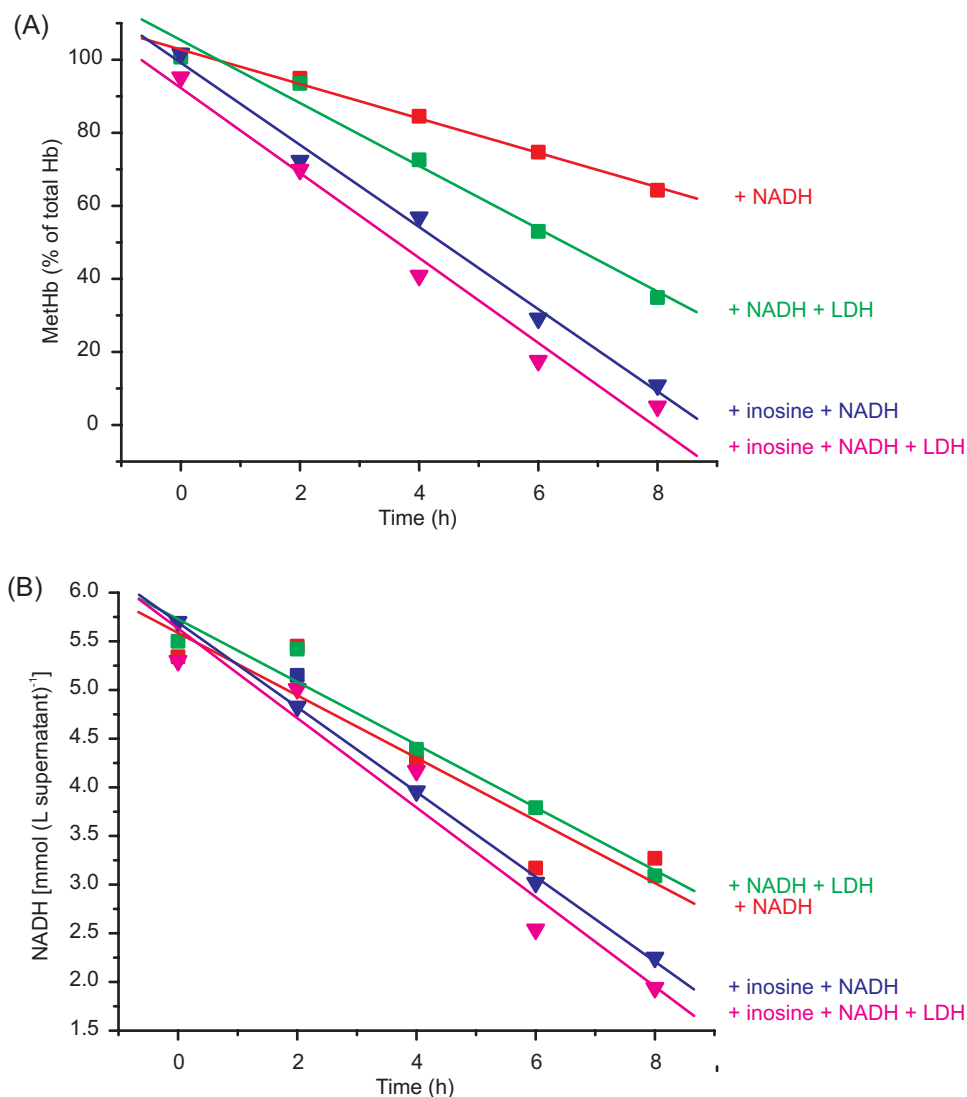


Figure 5.8: MethHb reduction (A) and NADH oxidation (B) in RBC suspensions and supernatants of pig RBCs incubated with NADH and LDH. Pig RBCs were incubated with NaNO_2 to convert Hb to methHb then incubated at 37 °C for 8 h in 7 mM PBS ($\text{Hc} = 0.2$) with 5 mM NADH (■); 5 mM NADH and 3 IU LDH (■); 5 mM glucose and 5 mM NADH (▼); and 5 mM glucose, 5 mM NADH and 3 IU LDH (▼). The methHb concentration (A) was determined as detailed in §2.4.5. The NADH concentration (B) was calculated from the absorbance at 340 nm and the molar extinction coefficient of NADH ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Lines of best fit are shown.

5.3.6 LDH release by RBCs and its effect on NADH oxidation and methHb reduction rates

The LDH activity in the supernatant fraction of RBCs containing methHb increased over the 8 h incubation for RBCs from all species tested (Figure 5.9). Supernatant

LDH activity was highest in incubations of RBCs from animals that showed high rates of metHb reduction in the presence of NADH.

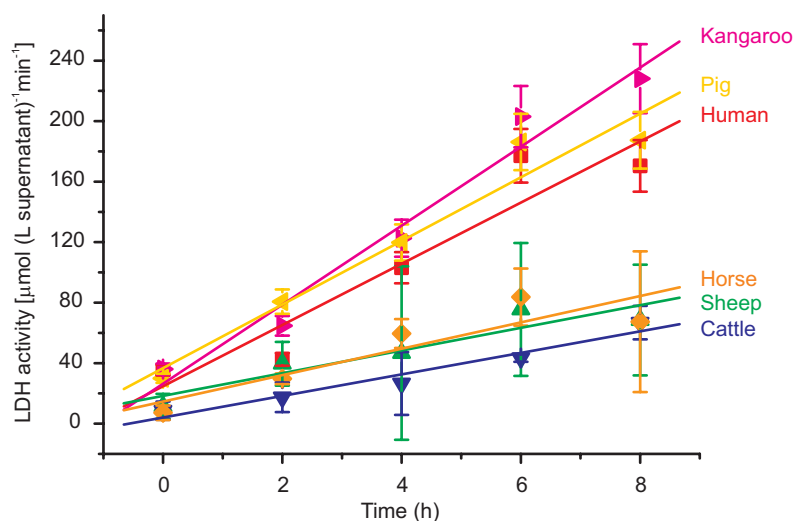


Figure 5.9: LDH activity in supernatants of metHb containing RBCs from various animal species. RBCs containing 100% MetHb from cattle (n = 3, ▼), horse (n = 2, ◆), human (n = 2, ■), grey kangaroo (n = 2, ▲), pig (n = 2, ▲) and sheep (n = 2, ▲) were incubated at 37 °C for 8 h in 7 mM PBS. The LDH activity in the supernatant was determined by the method described in §2.4.3. Data represent the average of experiments on RBCs from n individuals within each species. Error bars denote ± 1 s.d. Lines of best fit are shown.

Table 5.1: Comparison of the rate of metHb reduction, rate of NADH oxidation, and average supernatant LDH activity over the 8 h time period in RBCs from various mammalian species, incubated with 5 mM NADH for the 8 h. Error bars denote ± 1 s.d

Animal	MetHb at 8 h (% of total Hb)	NADH oxidation rate ($\mu\text{mol (L supernatant)}^{-1} \text{min}^{-1}$)	Supernatant LDH activity
Human	50.7 ± 2.2	5.46 ± 0.77	106 ± 5
Grey Kangaroo	49.3 ± 10.2	7.35 ± 1.39	131 ± 7
Pig	54.0 ± 14.5	5.93 ± 0.91	121 ± 6
Cattle	91.7 ± 7.8	0.83 ± 0.46	32.5 ± 1.6
Horse	90.4 ± 6.7	3.44 ± 0.58	49.5 ± 2.5
Sheep	92.3 ± 6.7	1.23 ± 0.25	48.4 ± 2.4

As shown in Table 5.1, RBCs from animal species which had a high capacity for reducing metHb in the presence of extracellular NADH also had relatively high supernatant NADH oxidation rates and relatively high LDH activity in the

supernatant. The LDH activity released into the suspension, presumably through haemolysis, was adequate to account for the observed NADH oxidation rate (Table 5.1). If LDH activity in the supernatant was comparatively low, NADH oxidation and metHb reduction rates were significantly attenuated.

5.4 Discussion

The major pathway for metHb reduction in the RBC under physiological conditions is via the *cyt b₅*-reductase-catalyzed NADH-MR system. Electrons are transferred from cytosolic NADH to *cyt b₅*-reductase, then to a soluble form of *cyt b₅* which reduces metHb (Figure 5.1A) (Abe and Sugita, 1979). Steady-state metHb levels are normally low, with less than 1% of the total Hb in the oxidized form at any one time (Jaffé and Hultquist, 1995). Cytosolic NADH, which is maintained in the reduced state by GAPDH, is essential for metHb reduction. The LDH reaction can also provide NADH for metHb reduction, but when metHb is less than 1%, NADH provides electrons for pyruvate reduction by LDH at the final step of RBC glycolysis (Jaffé and Hultquist, 1995). Glucose metabolism thus maintains the total NADH and NAD⁺ content, and in turn, the NADH-to-NAD⁺ ratio. Continual reduction of metHb requires the presence of metabolites which can lead to the generation of reduced pyridine nucleotides. Without glucose or an alternative metabolite, metHb is no longer reduced, NAD⁺ recycling to NADH at GAPDH no longer occurs, and NAD⁺ accumulates.

The results presented in this Chapter demonstrate that extracellular NADH, without entering the RBC (Figure 5.5), in both the presence and absence of nutrients, is able to reduce metHb in some animal species (Figure 5.3). In human and pig RBCs 5 mM NADH had a similar or greater capacity to reduce metHb than did 5 mM glucose or inosine (Figure 5.2). NADH added together with glucose or inosine had an additive effect on the rate of metHb reduction. The rates of metHb reduction in the presence of NADH are however significantly slower than the methylene blue facilitated reduction. In contrast, extracellular NADH did not significantly reduce metHb in horse, sheep and cattle RBCs (Figure 5.3).

Table 5.2: Enzyme activities of RBCs from animal species investigated^a.

Animal	Enzyme activity (IU (g Hb) ⁻¹)		
	LDH	NADH-MR	GAPDH
Cattle	6.63 ± 1.32 ¹	4.30 ²	46.52 ³
			14.34 ⁴
Dog	13.62 ± 3.48 ⁵	13.3 ± 2.6	24.2 ± 1.33 ¹
			11.57 ³
Horse	5.07 ± 0.91 ⁵	9.90 ²	9.27 ⁴
			60.14 ³
			19.54 ⁴
Human	200.0 ± 26.5	19.21 ± 3.85	57.20 ± 4.48 ⁷
			226 ± 41.90
Grey Kangaroo	52.47 ± 6.84	17.60 ± 0.9	
Pig	7.28 ± 2.20 ⁵	11.40 ²	48.79 ³
			11.25 ⁴
Sheep	5.16 ± 1.45 ⁵	9.60 ²	15.82 ⁴

^a Human enzyme activities were obtained from Beutler (1984), grey kangaroo enzyme activities were obtained from Agar *et al.* (2000) and the enzyme activities from all other animal species were obtained from Harvey (1989) and the following references therein: (1) Zinkl and Kaneko (1973), (2) Lo and Agar (1986), (3) Harkness *et al.* (1969), (4) Smith *et al.* (1965), (5) Kaneko (1973), (6) Kaneko *et al.* (1969), (7) Smith *et al.* (1972).

The activities of glycolytic and other enzymes in RBCs have been investigated in a number of animal species (Harvey, 1989; Agar, *et al.*, 2000). For many of these enzymes, there is wide variation in activity between species but generally small variations between individuals within a species (Agar, *et al.*, 2000). The LDH, NADH-MR and GAPDH activities for RBCs from the animals investigated in this Chapter are shown in Table 5.2. For each of the activities there is variation between the species, and in some cases discrepancies in enzyme activities previously reported by different laboratories. However, the trends in LDH activity: human > grey kangaroo > pig > dog > horse > cattle > sheep and NADH-MR activity: human > grey kangaroo > dog > pig > horse > sheep > cattle correspond reasonably well with the results presented here for the effect of NADH on methHb reduction: dog > grey kangaroo > human > pig > horse > cattle > sheep. These results suggest that these two enzymes may play a major role in the effect of extracellular NADH on methHb reduction.

The RBCs of species in which metHb was reduced by extracellular NADH produced elevated lactate concentrations, while those that did not, produced minimal lactate (Figure 5.4). In the absence of glucose, intracellular recycling of NADH is suspended, resulting in little, if any, metHb reduction and no build up of lactate. Hence, in RBCs from animals that can not employ extracellular NADH to reduce metHb, reducing equivalents are not available for either metHb reduction or the LDH reaction. In species that do utilise extracellular NADH to reduce metHb, the lactate production reflects availability of intracellular reducing agents. The mechanism of extracellular NADH enhancement of metHb reduction is different from the effect of glucose. Incubating nitrite-treated RBCs with glucose has been shown to result in the accumulation of pyruvate at an equivalent rate to the metHb reduction (Jaffé, 1981).

Incubation of RBCs with extracellular NADH (5 mM) and LDH (5 IU), the so-called 'pyruvate trap', has been shown previously to increase the rate of metHb reduction in human RBCs (Zerez, *et al.*, 1990). This is analogous to the observed haemolytic leakage of LDH in our RBC suspensions. The presence of NADH and LDH in the extracellular medium displaces the pseudo-equilibrium of the LDH reaction inside the cells via the transmembrane exchange of lactate back into the cells, hence increasing intracellular NADH concentrations and consequently metHb reduction rates (Figure 5.10). The low supernatant LDH activities of RBCs from some of the animal species limited the extent of metHb reduction by this mechanism. It was possible to enhance the reduction of metHb in the RBCs of these animals by the addition of 3 IU of exogenous LDH to the suspension (Figure 5.7). The marked increase in metHb reduction by cattle RBCs supplied with additional LDH suggests that the limiting factor for reduction of metHb mediated by extracellular NADH is the exogenous LDH activity, rather than the NADH-MR activity. The limited effect of exogenous LDH on metHb reduction rates in RBCs supplied with nutrients and exposed to extracellular NADH is consistent with this conclusion.

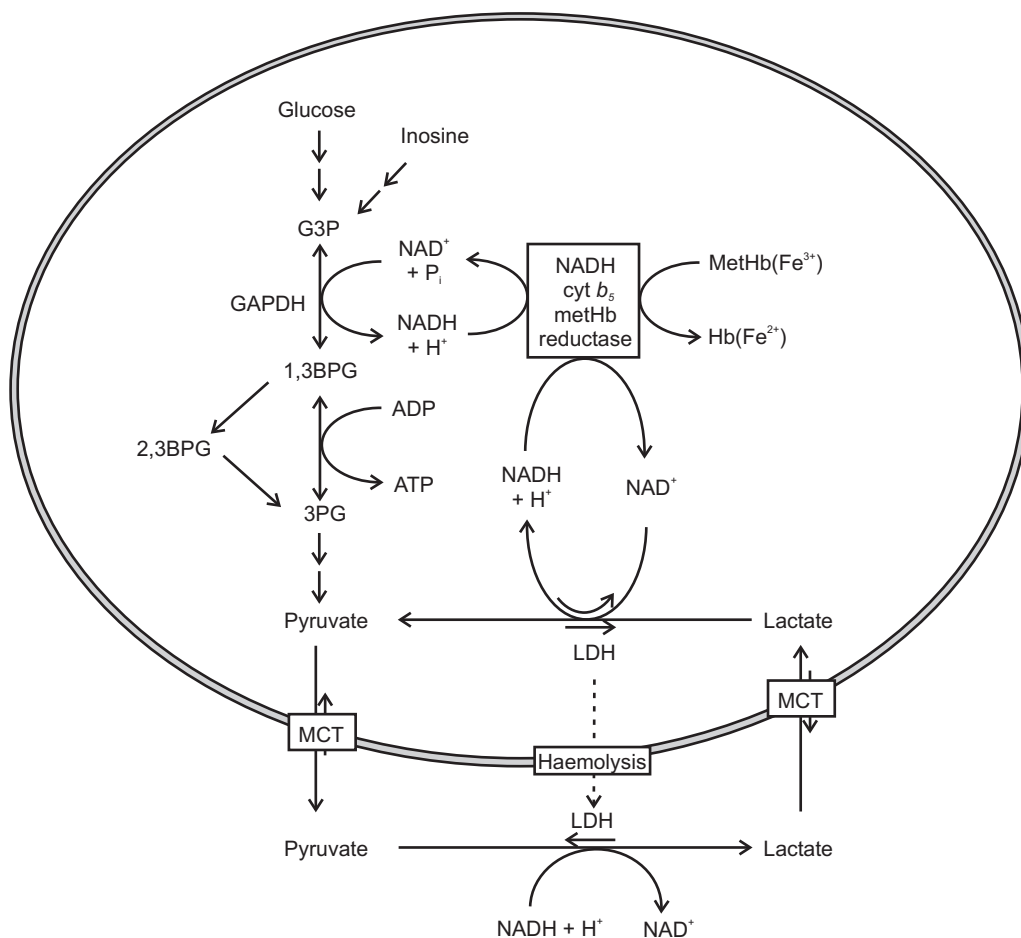


Figure 5.10: Mechanism to explain the effect of extracellular NADH on metabolism and methHb reduction in RBCs. Abbreviations used: 1,3BPG, 1,3-bisphosphoglycerate; 2,3BPG, 2,3-bisphosphoglycerate; G3P, glyceraldehyde 3-phosphate; MCT, monocarboxylate transporter; 3PG, 3-phosphoglycerate.

The inhibition of NADH-enhanced methHb reduction by pyruvate (Figure 5.3) in RBCs from all species suggests a competition between pyruvate and methHb for reducing equivalents from NADH. Pyruvate freely enters cells via the monocarboxylate transporter and so can increase its cytoplasmic concentration, altering the equilibrium of the intracellular LDH-catalysed reaction, resulting in oxidation of intracellular NADH by LDH, rather than by NADH-MR. Pyruvate is reduced extracellularly by the added NADH, as observed in the ¹H NMR experiments described in Chapter 4.

In conclusion, the experimental results presented in this Chapter do not support the hypothesis that the RBC plasma membrane contains an NADH-mediated electron

import system. Rather, extracellular NADH can be used to increase rates of metHb reduction in RBCs of some animal species by a mechanism that increases intracellular NADH concentrations indirectly. However, reasonably high supernatant LDH activity is necessary to elicit an effect. Increasing the intracellular NADH concentration, increases the metHb reduction rate, but not to the same extent as achieved with methylene blue, the conventional treatment for methaemoglobinaemia.