To my family,

With much love and thanks

"When you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of science."

William Thompson, Lord Kelvin

Popular Lectures and Addresses, 1891-1894

Magnesium in Cellular Energetics

A thesis submitted for the degree of
Doctor of Philosophy

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ABSTRACT

Most cellular magnesium is bound, yet it is the concentration of free magnesium, $[Mg^{2+}]_{free}$, in red blood cells that is vital in the regulation of enzyme activity and ion transport. It is unknown how changes in total blood magnesium affect the $[Mg^{2+}]_{free}$ within red blood cells or in tissue, because the presence of other cations, especially H^+ and potassium, K^+, affects the degree to which Mg^{2+} is bound. Consequently, this Thesis presents a new ^{31}P NMR spectroscopic method to measure $[Mg^{2+}]_{free}$ in blood, which analyses the changes in the phosphorus chemical shifts of ATP and 2,3-DPG using theoretical equations expressing the observed chemical shift as a function of pH, K^+ and $[Mg^{2+}]_{free}$, over the pH range of 5.75 to 8.5 and $[Mg^{2+}]_{free}$ range 0 to 5 mM. The equations were adjusted for the binding of haemoglobin to ATP and DPG, which required knowledge of the intracellular concentrations of ATP, DPG, K^+ and Hb. These equations enabled, for the first time, the simultaneous analyses of the chemical shifts of 3P-DPG and β-ATP to measure both intracellular pH and $[Mg^{2+}]_{free}$ in normal and sickle blood.

To simulate *in vivo* 100% oxygenated blood, samples were prepared for analysis by equilibration with a mixture of O_2 and CO_2, adjusted to give a pCO_2 of 40 mmHg and $pO_2 > 150$ mmHg. Under these conditions, normal whole blood had an intracellular pH of 7.20 ± 0.02 and a $[Mg^{2+}]_{free}$ of 0.41 ± 0.03 mM (n = 33). Further work determined blood pH and $[Mg^{2+}]_{free}$ for several clinical conditions including sickle cell anaemia, pre-eclampsia, hypoxia, patients with sub-arachnoid haemorrhage and chronic fatigue syndrome. This Thesis has demonstrated the potential of this new technique to evaluate the importance of $[Mg^{2+}]_{free}$ in the regulation of metabolite concentration and metabolic function, and to elucidate some of the properties of magnesium transport across the erythrocyte cell membrane.

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ABBREVIATIONS

Metabolites

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
2,3-DPG	2,3-diphosphoglycerate
CO_2	Carbon dioxide
Hb	Haemoglobin
Lac	Lactate
Mg^{2+}	Magnesium ion
$[Mg^{2+}]_{free}$	Intracellular free magnesium
Mg_T	Intracellular total magnesium
MgATP	Complex of ATP and a magnesium ion

Enzymes

ATPase	Adenosine 5'-phosphatase
DPGS	2,3-diphosphoglycerate synthase
HK	Hexokinase

Miscellaneous

2P – DPG	Phosphorus nucleus in the phosphate group attached to the second carbon in 2,3-DPG
3P – DPG	Phosphorus nucleus in the phosphate group attached to the third carbon in 2,3-

	DPG
α-ATP	Phosphorus nucleus in the α-phosphate group in ATP
β-ATP	Phosphorus nucleus in the β-phosphate group in ATP
γ-ATP	Phosphorus nucleus in the γ-phosphate group in ATP
f.i.d.	Free induction decay
Hct	Haematocrit
K_a	Acid dissociation constant
K_b	Association constant
K_d	Dissociation constant
MRS	Magnetic resonance spectroscopy
NMR	Nuclear magnetic resonance
PCA	Perchloric acid
PPP	Pentose phosphate pathway
PPA	Phenylphosphonic acid
pH_{ex}	Extracellular pH
pH_i	Intracellular pH
RBC	Red blood cell
r.f.	Radio frequency
T_1	Longitudinal relaxation time
T_2	Transverse relaxation time
TCA	Trichloroacetic acid

CHAPTER 1

Introduction

CHAPTER 1 – INTRODUCTION

Why study magnesium? It can be argued that magnesium, Mg, is one of the most important elements for our survival. Admittedly this title can be conferred to almost the whole periodic table depending on one's viewpoint, but as Mg forms the vital core to chlorophyll, it makes possible the all-important conversion of sunlight into retainable energy, leading to life as we know it.

It may seem more mundane to consider Mg's role in a physiological setting, but it nonetheless is as important in this context. After potassium, it is the second most abundant intracellular cation, and its diversity in existence has been recognised for many years; yet it is only recently that time has been devoted to elucidating the part it has to play in cellular function. Over the past several decades, we have begun to uncover the mysteries of how cytosolic ionised calcium, Ca^{2+}, is regulated and how it regulates cell function. The very fact that Mg^{2+}, as a similar, yet fundamentally different, divalent cation must interact with the same pathways as Ca^{2+}, implies that it too will be linked with cell function.

One of the reasons behind the lack of study is the difficulty involved in measuring Mg^{2+}. Thus the underlying aim of this work was to assess previous measuring techniques and to improve the most non-invasive technique that offered greatest potential, but yet had become increasingly criticised since its conception 20 years ago.

1.1 BIO-CHEMISTRY OF MAGNESIUM

1.1.1 Mg in the body

The human adult body contains approximately 24 g of Mg (1 mole) (Durlach, 1989), with muscle and tissue accounting for almost half of this and bone for slightly

more than half, where the Mg is adsorbed to the surface of hydroxyapatite crystals (Gunther, 1993). Only about 1% is found in red blood cells, with less than 0.5% in the plasma, at a concentration of approximately 0.9 mM (Murphy, 2000; Saris et al., 2000; Johnson, 2001; Romani and Scarpa, 2000; Huijgen et al., 1997; Durlach et al., 1989).

In 1977, the US National Academy of Sciences announced that "Mg deficiency appears to be causing 215,000 fatal heart attacks in the US each year, and perhaps as many as 20,000,000 worldwide" (Committee, 1977; Foster, 1994). This somewhat surprising statement stemmed from a demographic analysis of the level of Mg in drinking water correlated with cardiovascular mortality and morbidity (Durlach et al., 1989; Altura and Altura, 1996; Yang and Chiu, 1999; Ford, 1999). It was also seen that myocardial Mg level was significantly lower in soft water areas than in hard water areas (Maheswaran et al., 1999). It has since been estimated that 20% of the US population is Mg deficient (Durlach, 1989).

This deficiency is caused by a high Mg turnover coupled with ever decreasing Mg intake. The former is seen to be 15 times that of selenium, 30 times that of calcium and 400 times that of iron (Durlach, 1989). Losses of Mg occur through obligatory loss in urine. Approximately 75% of the total plasma Mg is filtered through the glomerula membrane in the kidney, the main organ responsible for regulation of Mg (see (Saris et al., 2000) for reviews). In contrast to Na and Ca, only 15% of this is reabsorbed in the proximal tubes, most (60-70%) in the thick ascending loop of Henle. Under normal conditions, 3-5% of the filtered Mg is excreted in the urine. Assuming the basic loss, the RDA for Mg is 6 mg/kg/day, or approximately 420 mg for the average adult male (Durlach, 1989; Altura and Altura, 1996). However, it is customary when determining Mg balances to ignore the dermoskeletal

and menstrual losses with an important Mg loss through sweat. When these are included, daily requirements are closer to 500-800 mg, with elite athletes needing as much as 1,500 mg/day (Durlach, 1989).

In fact, it is estimated that the average US person consumes less than 65% of the RDA (Altura and Altura, 1996; Marier, 1985; Morgan et al., 1985; Altura et al., 1997), and, as the body most readily absorbs Mg from aqueous sources, this has been partly attributed to decreases in Mg levels in drinking water. At the turn of the century, the average intake was nearer to 500 mg/day, however, then it was more common to use deep-well water, which accumulates much higher Mg concentrations (up to 100-150 mg/litre) as the water filters through many soil layers, for drinking and cooking (see Table 1.1 and Figure 1.1). Nowadays, most water is collected from surface reservoirs that typically contain concentrations of only 5-20 mg/l. Although whole seeds, unmilled grains, green leafy vegetables, legumes and nuts are rich in Mg, processing of these foods using low-Mg water will effectively remove most food Mg content (Saris et al., 2000). This, coupled with increased use of phosphate fertilizers (which actively bind Mg in the soil, thus preventing Mg absorption by plants), increased dairy products in the average diet (due to the drive to improve Ca intake), increases in dietary caffeine and alcohol (both diuretics and preventatives of renal Mg reabsorption), increasing obesity (leading to increased sweat rates and prevention of Mg absorption) and increasing levels of prolonged stress have led to an overall body Mg deficiency. It is interesting to note that human milk contains 30-40 mg/l to provide adequate Mg for the growing infant (Altura and Altura, 1996) and it has been suggested that this should also be the minimum concentration in drinking water (Durlach, 1989).

Table 1.1 Progressive decline in dietary intake in the USA of magnesium over the past 100 years (Altura and Altura, 1996).

Years	Mg Intake/Day (mg)
1900 – 1908	475 – 500
1909 – 1913	415 – 435
1915 – 1929	385 – 398
1935 – 1939	360 – 375
1947 – 1949	358 – 370
1957 – 1959	340 – 360
1965 – 1976	300 – 340
1978 – 1985	225 – 318
1987 – 1992	175 - 248

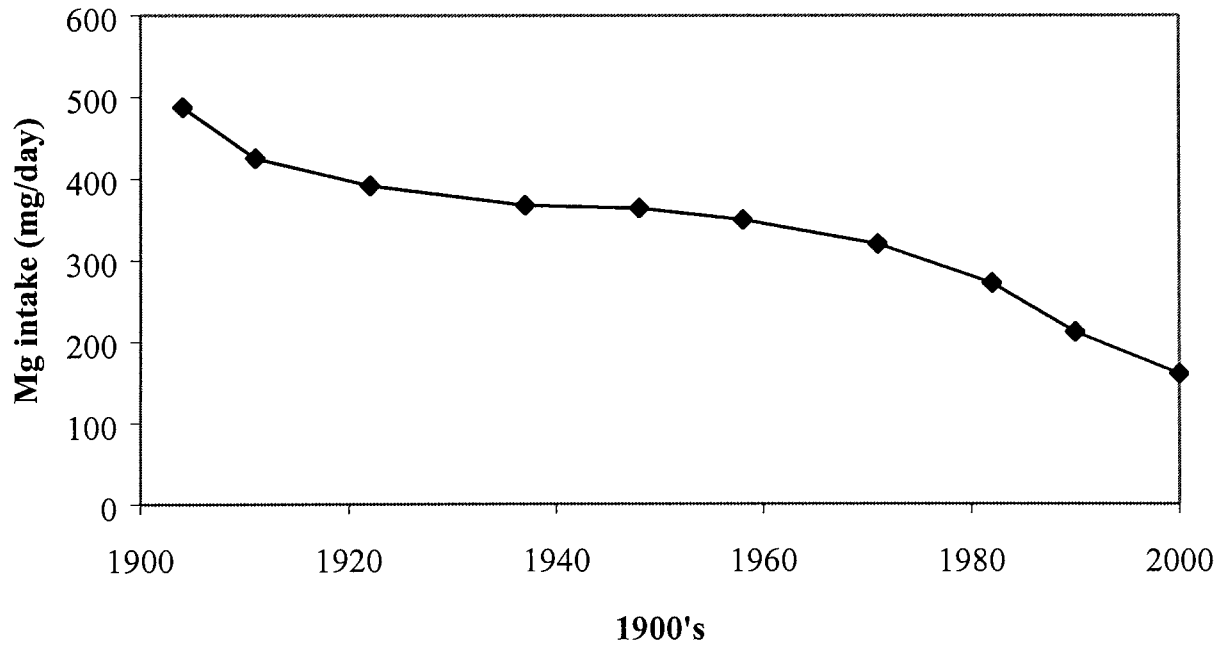


Fig. 1.1 Dietary intake in the USA of magnesium (Altura and Altura, 1996).

The important role of Mg in modulating cellular transport functions and receptors, signal transduction, enzyme activities, energy metabolism, nucleic acid and protein synthesis as well as protecting biological membranes makes Mg deficiency a potential health hazard. It is extremely important for the metabolism of Ca, K, P, Zn, Cu, Fe, Na, Pb, Cd, acetylcholine and nitric oxide and for the activation of thiamine

and therefore, for a very wide range of crucial body functions (Altura and Altura, 1996; Saris et al., 2000; Johnson, 2001). Anorexia, nausea, vomiting, lethargy and weakness are typical early symptoms of Mg deficiency. If severe Mg deficiency develops, paresthesia, muscular cramps, irritability, decreased attention span and mental confusion often occur. There is an accumulating body of evidence to suggest that dietary Mg deficiency plays an important role in the pathogenesis of ischaemic heart disease, congestive heart failure, sudden cardiac death, cardiac arrythmias, vascular complications of diabetes mellitus, pre-eclampsia/eclampsia, hypertension, atherosclerosis, stroke, impotence, peroxynitrite damage and fibromas, amongst many others (Saris et al., 2000; Johnson, 2001).

1.1.2 Physiology of Mg

In order to understand the effects of interactions with Mg in the body, it is helpful to recall some basic facts about it. Compared to Ca^{2+}, Mg^{2+} has a smaller ionic radius (0.065 nm vs. 0.099 nm), thus lending it a significantly higher charge density. This means its 6 coordination bonds in solution are stronger and less flexible than Ca^{2+}'s 6 or 8, and also that its hydration sphere is considerably larger, contrary, perhaps, to intuition, due to binding of water molecules beyond one coordination layer (Williams, 1970; Hille, 1984).

It has long been known that Mg is important for neurological and muscular function, hypomagnesemia leads to hyperexcitability due mainly to cellular Ca transport and signalling. Coupled with this, Mg activates more than 350 enzymes in the body (Altura and Altura, 1996; Romani and Scarpa, 2000) and is pivotal in the transfer, storage and utilization of energy. It is, in fact, not the total amount of Mg that is important, as much of it binds to suitable negatively charged groups, especially phosphates. Instead, the intracellular level of unbound Mg^{2+}, $[Mg^{2+}]_{free}$, regulates

intermediary metabolism through activation of rate-limiting enzymes. It is especially important for those enzymes that use nucleotides as substrates because, in general, it is the Mg-nucleotide complex that is the actual cofactor, while the free nucleotide (and free Mg^{2+}) acts to inhibit enzymatic action ((Noat et al., 1970) and see (Saris et al., 2000) for reviews). This can be understood when one considers how Mg can bind to ATP, for example. It has access to oxide ions on two phosphate groups and ATP's triphosphate chain can form a very stable, low energy conformer on complexing with Mg (Figure 1.2) (Ramirez and Marecek, 1980). This chain is then locked into one orientation, thus allowing greater ease of enzyme binding at a specific action site, which otherwise would be difficult if this phosphate chain were freely rotating. Once enzyme bound, the process of removing a phosphate group to release energy may begin.

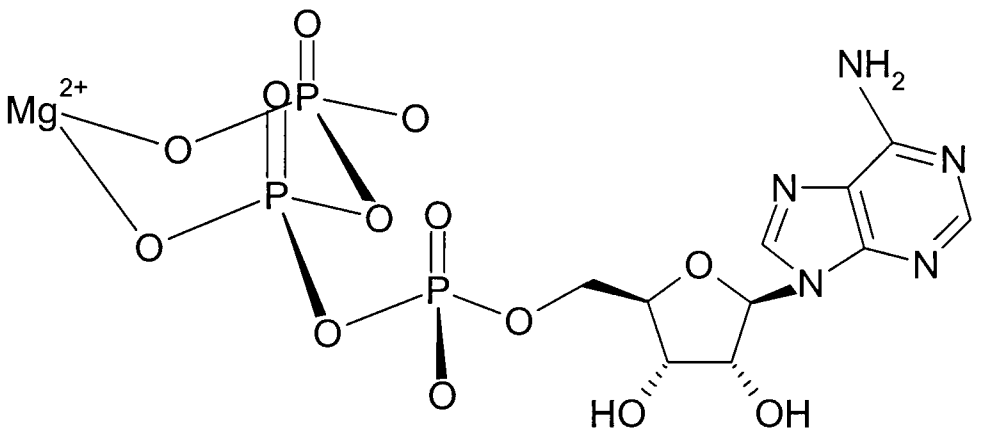


Fig 1.2 A proposed stable intermediate of adenosine triphosphate (ATP) bound to Mg.

This is true for phosphotransferases and hydolases such as ATPases, which are of central importance in the biochemistry of the cell, particularly in energy metabolism. One can include in this group hexokinase, phosphofructokinase, pyruvate kinase and enolase as especially important for red blood cell metabolism (Mulquiney et al., 1999). In addition to these, $[Mg^{2+}]_{free}$ is required for protein and

nucleic acid synthesis, the cell cycle, cytoskeletal and mitochondrial integrity and for the binding of substances to the plasma membrane (see for reviews (Altura and Altura, 1996)). Also, $[Mg^{2+}]_{free}$ frequently modulates ion transport by pumps, carriers and channels and thereby may modulate signal transduction and the cytosolic concentrations of Ca and K (Saris et al., 2000).

Given the widespread physiological requirement for Mg, one would expect the cytosolic $[Mg^{2+}]_{free}$ to be efficiently regulated. How this is achieved has yet to be fully understood. Ca^{2+} signalling is facilitated by large Ca^{2+} gradients (up to 10,000-fold) across the plasma and sarcoplasmic reticulum membranes (Murphy, 2000). In contrast, only small gradients of Mg^{2+} (generally a factor of 2 or less) are reported across the plasma or intracellular membranes (Flatman, 1991). In contrast to Ca^{2+} signalling, agonists are thought not to cause large (orders of magnitude) alterations in cytosolic $[Mg^{2+}]_{free}$. Even upon hormonal stimulation of heart and liver, which results in a 10-15% change in total cell magnesium, there is thought to be little or no change in cytosolic $[Mg^{2+}]_{free}$ (Murphy, 2000). The absence of detectable major changes in $[Mg^{2+}]_{free}$ and the extremely slow turnover of Mg across the cell plasma membrane in normal conditions has supported for more than 3 decades the assumption that $[Mg^{2+}]_{free}$ was kept constant at the level necessary for enzyme and channel function (Romani and Scarpa, 2000). But over the past decade new observations, coupled with better analyses, have begun to revert this way of thinking. It is now suggested that larger fluxes of Mg^{2+} can cross the plasma membrane in either direction, especially following a variety of hormonal and non-hormonal stimuli (Romani and Scarpa, 2000).

1.2 THE HUMAN ERYTHROCYTE

Of all cell types, the red blood cell is the most convenient and accessible to study. As a result, the human erythrocyte is the most extensively characterised in terms of its intermediary and secondary metabolism (Rapoport, 1968; Mulquiney et al., 1999; Mulquiney and Kuchel, 1999a; Mulquiney and Kuchel, 1999b), along with its membrane transport properties (Ellory et al., 1998).

The major function of the erythrocyte is to facilitate oxygen and carbon dioxide transport between the lungs and all other tissues, but it also has important functions for acid-base balance. Unlike most other cells in mammals, the erythrocyte has no nucleus, no endoplasmic reticulum and no mitochondria. These organelles are lost in the transformation from reticulocyte, the direct precursor of the erythrocyte, to the mature cell. The erythrocyte is therefore incapable of protein or lipid synthesis as a means to repair damage and retain its integrity. Instead there is a continuous breakdown of old erythrocytes and production of new ones. In the 100-120 days spent in circulation, an erythrocyte undergoes numerous gradual physical and metabolic changes (Ouwerkerk et al., 1989), decreasing its metabolic capacity and deformability.

In order to deliver oxygen to peripheral tissues, the erythrocyte must pass through narrow capillaries. In free suspension it is a biconcave discoid with a diameter of about 8 μm, but due to its deformability it can pass through capillaries with a diameter as small as 0.5 μm (Campbell et al., 1984; Ouwerkerk et al., 1989). The metabolic pathways outlined below, offering ATP and protection against oxidative damage, are necessary to retain this flexibility and shape.

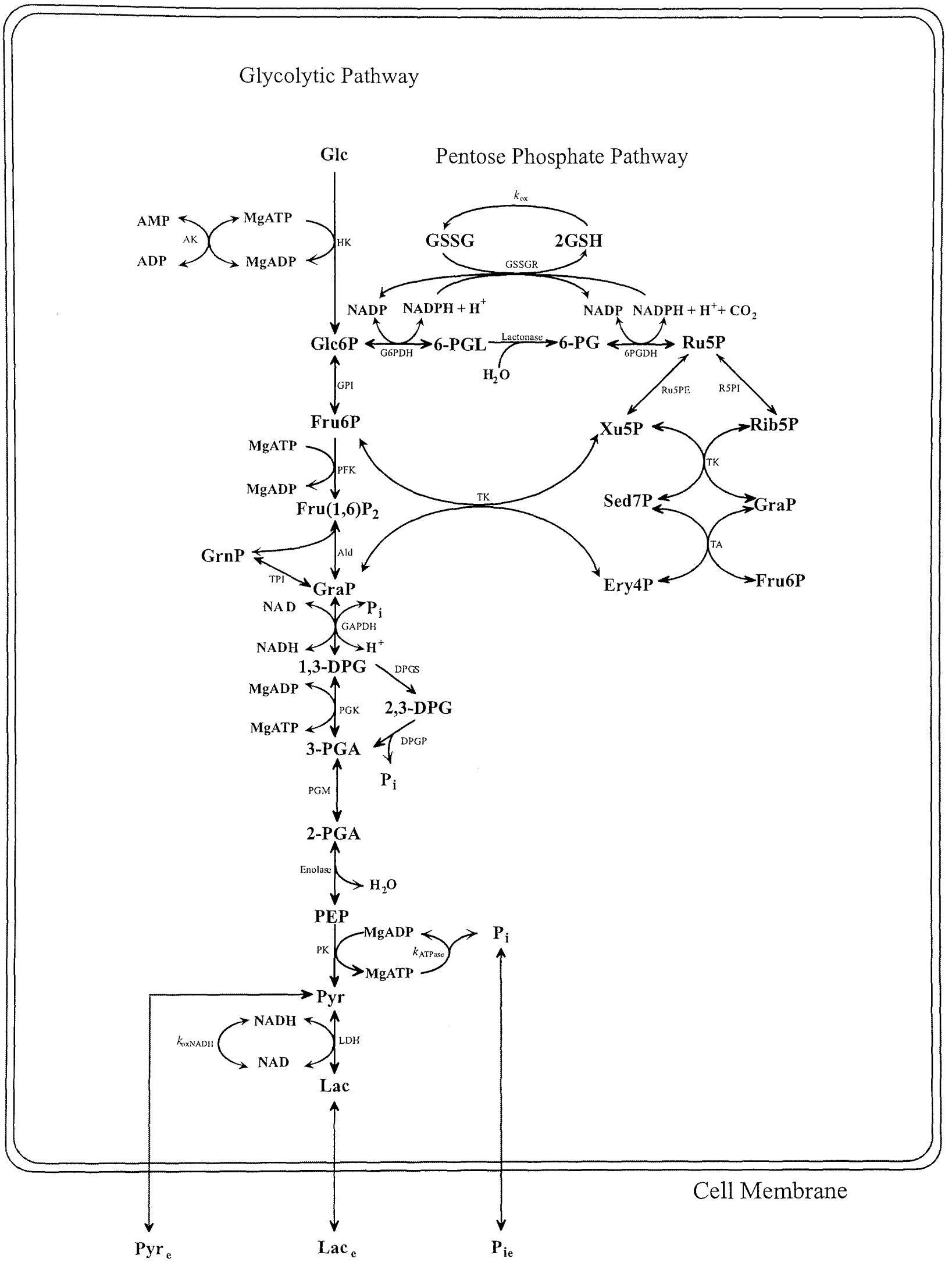

Glycolytic Pathway
Pentose Phosphate Pathway
Glc
AMP
MgATP
AK
HK
ADP
MgADP
k_{ox}
GSSG
2GSH
GSSGR
NADP
NADPH + H+
NADP
NADPH + H+ + CO2
Glc6P
G6PDH
6-PGL
Lactonase
H2O
6-PG
6PGDH
Ru5P
GPI
Ru5PE
R5PI
Fru6P
Xu5P
Rib5P
MgATP
PFK
MgADP
TK
Fru(1,6)P2
Sed7P
GraP
TK
GrnP
Ald
TA
TPI
Ery4P
Fru6P
GraP
NAD
Pi
GAPDH
NADH
H+
1,3-DPG
DPGS
MgADP
PGK
2,3-DPG
MgATP
DPGP
3-PGA
Pi
PGM
2-PGA
Enolase
H2O
PEP
MgADP
Pi
PK
k_{ATPase}
MgATP
Pyr
NADH
k_{oxNADH}
LDH
NAD
Lac
Cell Membrane
Pyr e
Lac e
Pi e

Fig 1.3 Reaction scheme of the major metabolic pathways in the human erythrocyte: glycolysis, the Rapoport-Luebering (2,3-DPG) shunt, and the pentose phosphate pathway.[a]

Enzymes		Metabolites	
AK	(adenylate kinase)	1,3-DPG	1,3-diphosphoglycerate
Ald	aldose	2,3-DPG	2,3-diphosphoglycerate
2,3-DPGP	2,3-DPG-phosphatase	Ery4P	erythrose-4-phosphate
2,3-DPGS	2,3-DPG synthase	$Fru(1,6)P_2$	fructose 1,6-diphosphate
Eno	enolase	Fru6P	fructose 6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase	Glc	glucose
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Glc6P	glucose 6-phosphate
GPI	glucosephosphate isomerase	GraP	glyceraldehyde 3-phosphate
HK	hexokinase	GrnP	dihydroxyacetone phosphate
k_{ATPase}	non glycolytic energy consumption	Lac	lactate
k_{ox}	reduction processes consuming GSH	Lac_e	extracellular lactate
k_{oxNADH}	reducing processes requiring NADH	P_i	inorganic phosphate
Lactonase	δ-gluconolactonase	P_{ie}	extracellular P_i
LDH	lactate dehydrogenase	PEP	phosphenolpyruvate
PFK	phosphofructokinase	2-PGA	2-phosphoglycerate
PGK	phosphoglycerate kinase	3-PGA	3-phosphoglycerate
6PGDH	6-phosphogluconate dehydrogenase	6-PG	6-phosphogluconate
PGM	phosphoglycerate mutase	6-PGL	6-phosphoglucolactone
PK	pyruvate kinase	Pyr	pyruvate
R5PI	ribose-5-phosphate isomerase	Pyr_e	extracellular pyruvate
Ru5E	ribulose-5-phosphate epimerase	Rib5P	ribose-5-phosphate
TA	transaldolase	Ru5P	ribulose-5-phosphate
TK	transketolase	Sed7P	sedoheptulose-7-phosphate
TPI	triose phosphate isomerase	Xu5P	xylulose-5-phosphate

[a] The assistance of Pete Mulquiney in preparing this Figure is gratefully acknowledged.

1.2.1 Metabolism

Erythrocytes, having no mitochondria, lack oxidative phosphorylation, but produce ATP solely by metabolising glucose to pyruvate or lactate through the Embden-Meyerhof pathway (Fig. 1.3). By the action of cation pumping ATPases, osmotic equilibrium, cell volume and intracellular pH are controlled. Glucose is also utilised in the pentose phosphate pathway to yield reducing equivalents in the form of NADPH, which is used for the conversion of oxidised glutathione to its reduced form. This pathway is important for the protection of the erythrocyte against oxidative damage to proteins and membrane lipids.

An important feature of glycolysis in erythrocytes is the possession of an alternative pathway for carbon flux via 1,3-diphosphoglycerate (1,3-DPG). This pathway, known as the 2,3-DPG or Rapoport-Luerbering shunt, bypasses phosphoglycerate kinase by converting 1,3-DPG to 2,3-DPG (Rapoport and Luebering, 1950; Rapoport and Luebering, 1951; Rapoport and Luebering, 1952). 2,3-DPG is an important modulator of haemoglobin oxygen affinity (Duhm, 1971; Meldon, 1985) and is usually present in erythrocytes in approximately equimolar concentration with haemoglobin, therefore playing an important role in blood oxygen transport.

Over the last 25 years many mathematical models of erythrocyte metabolism have been developed (Rapoport et al., 1974; Rapoport and Heinrich, 1975; Schauer et al., 1981; Werner and Heinrich, 1985; Lee et al., 1991; Ni and Savageau, 1996) though the most complete was by Mulquiney and co-workers (Mulquiney et al., 1999; Mulquiney and Kuchel, 1999a; Mulquiney and Kuchel, 1999b) who accounted for the effects of $[Mg^{2+}]_{free}$, H^{+} and haemoglobin on 2,3-DPG regulation and control.

1.2.2 Ion Transport

Overview

As mentioned above, the principal membrane transport systems should maintain osmotic equilibrium, cell volume and intracellular pH. These systems in the human red cell have received considerable attention (see for reviews (Lew et al., 1991; Ellory et al., 1998) with the red cell often being used as a convenient model system.

Of great importance to cell function is the powerful anion exchanger, AE1, or Band 3, transporting HCO_3^- or OH^- into the cell in return for Cl^-. This allows the cells to partition these penetrating ions according to the Donnan equilibrium, in the interests of osmotic and charge equality, and effectively clamps the membrane potential and intracellular pH, due to the rapid transport of Cl^- across the membrane. This can be described by the equation:

$$r = [Cl^-]_i / [Cl^-]_{ex} = [HCO_3^-]_i / [HCO_3^-]_{ex} = [OH^-]_i / [OH^-]_{ex} = [H^+]_{ex} / [H^+]_i \quad (1.1)$$

where the subscripts i and ex denote intracellular and extracellular, respectively.

The ratio, r, is mainly determined by the intracellular concentration of non-penetrating charged molecules, such as haemoglobin, ATP and 2,3-DPG (Duhm, 1971) and physiologically is approximately 0.7 (E_m = -12 mV). However, the value of r is also dependent on the extracellular pH (Duhm, 1971).

Na^+ and K^+ fluxes govern volume regulation in the erythrocyte (Mairbaurl and Hoffman, 1992), and transport systems involving the principal monovalent cations are shown in Fig 1.4. In general, erythrocytes have a low cation permeability, with cell ionic composition and ionic homeostasis thought to follow the classical 'pump-leak' model. Of interest, the Na^+/K^+ pump maintains a strong inwardly directing Na^+ gradient, consuming ATP to do so, while any Ca^{2+} entering the cell is pumped out by

the ATP-driven Ca^{2+} pump. Erythrocytes do contain a KCl cotransporter (KCC1), which can maintain cell volume by allowing efflux of KCl along the outwardly directing K^+ gradient, shortly followed by water, and thereby causing a volume decrease. This is activated by several physiological signals, such as cell swelling, low pH, low $[Mg^{2+}]_{free}$, urea, raised temperature and oxygen tension (Joiner et al., 1998; Gibson et al., 1998; Campbell et al., 1999; Flatman and Creanor, 1999; Gibson et al., 2001). However this transporter remains cryptically quiescent (Ellory et al., 1983; Hall and Ellory, 1986) in the normal mature human red cell. Thus mature human red cells appear to lack effective volume regulatory mechanisms, unlike the nucleated red cells of fish and birds, and indeed other mammals, such as sheep, dog and horse (Delpire and Lauf, 1991).

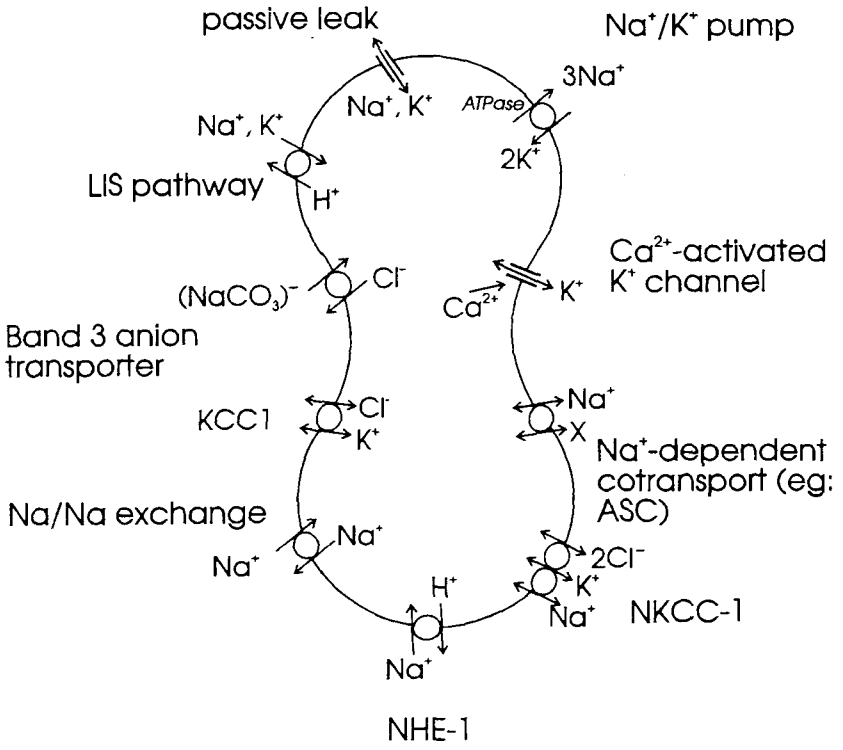


Fig. 1.4 Transport systems involving the principal monovalent cations.

1.2.3 Mg Transport

Magnesium homeostasis is less well understood, but there appear to be ATP and Na^+ dependent transporters for Mg^{2+} (Gunther, 1981; Flatman, 1988). Since

efflux of Mg occurs against the electrochemical gradient, there must be an energy-coupled mechanism for the extrusion of Mg. In erythrocytes, at least, this is achieved mainly by antiport against Na^+ at the expense of the Na^+ gradient. Mg influx into the cell is thought to occur mainly by diffusion from the slightly higher free concentration in the extracellular space (typically 0.7 over 0.4 mM) (Flatman, 1991; Flatman, 1992; Flatman and Smith, 1996).

It has recently been established that net Mg^{2+} efflux and net Mg^{2+} influx are separate and regulated pathways, establishing homeostasis of $[Mg^{2+}]_{free}$ (Gunther, 1993).

1.3 pH REGULATION

Blood pH is important to cellular function in all cell types through its influence on enzyme kinetics. In erythrocytes, it plays an important role, with both metabolism and ion transport responding to changes in pH. In particular, by altering the charges on specific acid residues on haemoglobin, pH significantly affects its oxygen affinity. As pH falls, oxygen affinity falls, so facilitating O_2 release in metabolically active tissues where the released CO_2 lowers the pH. This is reversed in the lungs where the loss of CO_2 leads to increases in pH and hence increased oxygen affinity by haemoglobin. This relationship is known as the Bohr effect. Also of importance for the red cell, is that pH (and oxygen tension) is an important external effector of 2,3-DPG metabolism (see (Mulquiney et al., 1999) for reviews).

Furthermore, as protons are positively charged, they will compete with $[Mg^{2+}]_{free}$ for the same binding sites. This has important consequences for both the measurement of $[Mg^{2+}]_{free}$ and its regulation within the cell.

As mentioned above, the intracellular pH is in fact ultimately governed by the external conditions, through AE1. As a result, it is important to consider what

controls external pH as this will have an important bearing on the intracellular conditions. The normal pH_{ex} of arterial blood is 7.4, with a range of 7.36 – 7.44, although the range compatible with life is about 6.8 – 7.7 (Campbell et al., 1984). In the interstitial fluid around metabolically active tissue, the pH_{ex} is between 7.18 – 7.36, a good estimate for mixed venous blood pH_{ex} (Campbell et al., 1984).

There is no single receptor or centre which is specifically sensitive to pH_{ex} and which integrates all aspects of its regulation. However, there are three main mechanisms by which the body can regulate changes in pH_{ex}:

1) Buffer systems within the body (bicarbonate-carbonic acid)

2) The renal regulation of H^+ excretion

3) The respiratory regulation of CO_2 excretion.

1.3.1 Buffer systems

The first of these is immediately available and can be represented by the first equilibrium:

$$HCO_3^- + H^+ \Leftrightarrow H_2CO_3 \Leftrightarrow CO_2 + H_2O \qquad (1.2)$$

And, through the second equilibrium, by varying the volume of CO_2 excreted through the lungs, changes in pH_{ex} can be resisted. The other important buffer system for blood is haemoglobin, particularly as its buffering power varies with oxygenation. Upon deoxygenation, haemoglobin is a stronger base and thus removal of O_2 from the blood simultaneously provides a buffer for much of the H_2CO_3 added to it from the tissue.

1.3.2 Renal regulation

Changes in pH_{ex} over a longer time course can be resisted by action of the kidney. There are four basic types of disturbance:

1) Respiratory acidosis

This occurs when the ratio of alveolar ventilation to CO_2 production falls, characterised by hypercapnia. Over time, the kidney compensates for the fall in pH_{ex} by excreting H^+ and retaining HCO_3^-.

2) Metabolic acidosis

The primary change is an increase in H^+ and a fall in HCO_3^-. The pH_{ex} decrease immediately causes an increase in pulmonary ventilation via the peripheral chemoreceptors, and hence leads to a decrease in pCO_2 to restore pH_{ex} values.

3) Respiratory alkalosis

Excessive ventilation will lower alveolar pCO_2 (hypocapnia) and increase pH_{ex}. The kidney reacts by excreting more HCO_3^- and reabsorbing more H^+. Thus HCO_3^- falls along with pH_{ex}. Also, the primary increase in pH_{ex} stimulates glycolysis in the blood, leading to the production of lactate and H^+, thus helping to restore the pH_{ex}.

4) Metabolic alkalosis

The primary change is a fall in H^+ and a rise in HCO_3^-. The kidney responds by excreting the latter, although one would also expect to see a decrease in ventilation to help increase pCO_2. However, this is not often observed.

1.3.3 Respiratory regulation

The most important chemical stimulus for breathing is the arterial pCO_2 as sensed by medullary chemoreceptors. In normal subjects breathing air at sea-level the pulmonary ventilation is adjusted to keep the arterial pCO_2 at 40 mmHg. There are no cells in the brain that adjust the ventilation in response to changes in the O_2 tension of the blood. However, there are chemoreceptors in the aortic and carotid bodies which respond to hypoxaemia and stimulate the breathing, but only when the arterial pO_2

falls below about 60 mm Hg. Both receptors can have their sensitivity temporarily or chronically re-set in either direction. For example if ventilation is chronically increased, eg by hypoxia at altitude, the medullary chemoreceptors become adjusted down to the lower pCO_2 and will then resist any tendency for it to rise.

Finally, unless the proportion of ventilation to blood-flow in all alveoli is both uniform and appropriate to the mixed venous and inspired gas composition, the arterial gas composition cannot be normal. The only way in which oxygenation of the blood may be corrected in the presence of ventilation/blood-flow inequality is for total ventilation to increase. The net result of this hyperventilation is improvement in and possible normalisation of arterial pO_2 but at the expense of an abnormally low arterial pCO_2.

1.4 MEASUREMENTS OF pH AND Mg IN BLOOD

In determining these important parameters, it is helpful to divide the measurements into extra- and intracellular, the former being more straightforward than the latter, where invasiveness and perturbation of the system in question must be considered.

1.4.1 pH

Extracellular

The use of a standard glass electrode is sufficient to measure pH_{ex}, if the temperature is adequately controlled (Bartschi et al., 1970; Ashwood et al., 1983), and clinically this is achieved using a blood gas analyser, which measures pCO_2 simultaneously. However most physiological studies of erythrocytes use washed cells suspended in a phosphate buffer. This buffer is calibrated prior to use to give an appropriate pH_{ex} at the experimental temperature.

Intracellular

The first reported method for determining pH_i was the freeze-thaw technique (Funder and Wieth, 1966), in which separated, washed erythrocytes were haemolysed by repeated freezing and thawing in liquid nitrogen and a water bath at 37°C. The resulting haemolysate could then be tested using a standard electrode. Despite its reproducibility, clearly this is an invasive technique and pH_i measurements by the non-invasive method of ^{31}P NMR spectroscopy have become increasingly favoured. The first of these was in the landmark study of erythrocytes by Moon and Richards in 1973 (Moon and Richards, 1973). The method relies on the fact that the resonance frequency of ^{31}P in many phosphates is strongly dependent on pH; if a titration curve of a given phosphate compound in the intracellular milieu is known, a determination of pH from a measured resonance frequency is possible. Both endogenous compounds, such as inorganic phosphate, ATP and, in blood, DPG (Lam et al., 1979), and non-physiologic or exogenous compounds, such as methylphosphonate, have been used (see (Radda and Seeley, 1979) for reviews) with good reported comparisons with the freeze-thaw technique (Petersen et al., 1987). However, the accuracy of the pH measurements depends on the understanding of the extent to which factors other than pH influence ^{31}P resonance frequencies (see (Roberts et al., 1981) for reviews). Such factors include temperature (Raftos et al., 1986), ionic strength (Roberts et al., 1981), oxygen tension (Labotka, 1984) and $[Mg^{2+}]_{free}$ (Roberts et al., 1981; Golding and Golding, 1995).

1.4.2 Mg

Extracellular

In plasma, Mg can be found in three fractions; in an ultrafiltrable fraction consisting of ionised Mg^{2+} (70-80%), complex bound Mg (1-2%), and in a protein-

bound non-ultrafiltrable fraction (20-30%) (Saris et al., 2000; Lewenstam et al., 1994). The reference range for total Mg concentration in adult blood plasma is 0.65-1.05 mM, and for ionised Mg^{2+} 0.55-0.75 mM. Despite the fact that ionised fractions of potassium, sodium and calcium are now most frequently requested in routine clinical analysis, until recently the measurement of $[Mg^{2+}]_{free}$ was not covered by any direct method, despite, as mentioned above, this is the biologically active species.

Determination of total Mg can be achieved by a number of techniques. Photometry using a number of chromogenic reagents, such as xlyidyl blue, calmagite, methyltymol, magon, and titan yellow are frequently used (Tietz, 1990). Another frequently used technique is atomic spectroscopy either by flame emission or by absorption after electrothermal atomisation (AAS).

Ionised Mg can now be measured potentiometrically using a Mg-selective electrode. These have been difficult to develop, the size of the hydrated Mg^{2+} ion causing problems for specificity, but it was first achieved in 1990 and electrodes have been improved since (Lewenstam et al., 1994), though careful calibrating and sampling are needed for accurate measurements.

Intracellular

Total Mg in erythrocytes can be measured in a similar manner to plasma, using either photometry or atomic spectroscopy. Analysis may be performed on either packed erythrocytes or on whole blood after acid extraction (see reviews in (Millart et al., 1995)) followed by subtraction of the plasma value. The reference range for total Mg in erythrocytes is 1.65-2.65 mMol/litre cell, although it is preferred to express this relative to cell water, and therefore give an absolute concentration, assuming 70% of the cell is water. The range then becomes 2.4-3.8 mM. It is

generally considered that 3.29 ± 0.34 mM is normal (Millart et al., 1995; Mulquiney and Kuchel, 1999a).

Previously, $[Mg^{2+}]_{free}$ was estimated by studying the position of the equilibrium adenylate kinase (Rose, 1968) or aconitase hydratase (Veloso et al., 1973; Veech et al., 1994), the apparent equilibrium constants of which depend upon the $[Mg^{2+}]_{free}$. However, cells must be at equilibrium, accurate measurements of low concentrations of metabolites are required and it must be assumed that the total concentrations are kinetically active, which can be questioned.

A number of modern techniques are available to measure $[Mg^{2+}]_{free}$. These include Mg-selective electrodes (Rouilly et al., 1990; Zhang et al., 1997), metallochromic indicators (as described above)(Scarpa, 1982), zero point titrations for plasma membrane permeabilisation using the ionophore A23187 (Flatman and Lew, 1977), fluorescent probes, such as furaptra, Mag-fura-2, Mag-indo-1 (Jung and Brierley, 1991; Freudenrich et al., 1992), and ^{31}P, using endogenous phosphates as above (Gupta et al., 1978), and ^{19}F, using exogenous compounds such as Mg-APTRA, (Levy et al., 1988; London, 1991) NMR spectroscopy. The use, drawbacks and comparisons of these have been reviewed by many (Alvarez et al., 1987; Geven et al., 1991; London, 1991; Murphy, 1993; Millart et al., 1995; Romani and Scarpa, 2000; Saris et al., 2000). One problem is the interference from cytosolic free Ca^{2+}, Na^{+}, and pH, especially for the metallochromic indicators. However, there are many other considerations; microelectrodes and fluorescence probes only measure the activity of Mg^{2+} ions, not the concentrations; zero-point titrations do not give a direct measure of Mg^{2+}, but operate by inference; and both of these involve perturbation of the cellular system under study, by invasion and competitive Mg-chelating.

For completeness, it is worth adding one further possible NMR technique, that of ^{25}Mg NMR spectroscopy. In theory, this would be the most direct, non-invasive measurement. However, it too is beset with problems. Firstly, the natural occurrence of the ^{25}Mg isotope is quite low at 10%, its resonance frequency is very insensitive to the formation of complexes and, as a quadrupolar nucleus, its spectroscopic peaks are very broad. Despite this, some work on erythrocytes has been moderately successful (Bock et al., 1991) and been improved more recently (Shien et al., 1999). Perhaps this interesting area can be developed further as spectroscopic analysis improves.

Given that all techniques have their drawbacks, it seems logical to use the most non-invasive, ^{31}P NMR spectroscopy, using endogenous phosphorus containing compounds. If this can be developed to the required precision and accuracy, there is the real hope that it can be used for the ideal measurement, an *in vivo* determination of intracellular $[Mg^{2+}]_{free}$.

1.4.3 Summary

Despite the wide range of measurement techniques (or perhaps because of), the reported values of $[Mg^{2+}]_{free}$ in oxygenated erythrocytes vary at least three-fold, from 0.2 – 0.6 mM. The measurement of changes in $[Mg^{2+}]_{free}$ are more challenging than, for example, those in Ca^{2+} because, although a 5- to 10-fold increase in cytosolic Ca^{2+} is common, such a large percentage change in $[Mg^{2+}]_{free}$ would be unlikely because of the much higher basal $[Mg^{2+}]_{free}$. A 1 μM rise in Ca^{2+} is easily measured against the 100 nM basal Ca^{2+}, whereas a 1 μM rise in $[Mg^{2+}]_{free}$ is lost in the 0.5 to 1 mM basal $[Mg^{2+}]_{free}$. Add to this the fact that Mg^{2+} is well-buffered and it would require a very large flux to allow for a large percentage change in $[Mg^{2+}]_{free}$, and one can understand why only small changes in $[Mg^{2+}]_{free}$ are observed. However, even

though the changes in $[Mg^{2+}]_{free}$ are small on a percentage basis, they may have physiological consequences given the widespread importance of $[Mg^{2+}]_{free}$ to cellular systems.

1.5 AIMS

As indicated above, the general aim of this work was to develop and improve the ^{31}P NMR spectroscopic method for determining intracellular free Mg^{2+}, $[Mg^{2+}]_{free}$, in human erythrocytes. More specific aims were as follows:

- To develop standard titration curves of ^{31}P NMR chemical shifts of endogenous phosphate groups over ranges of pH and Mg^{2+}.
- To accurately fit these curves with a mathematical model of chemical shifts using equations based on the complex equilibria present for the phosphate groups within erythrocytes.
- To overcome reported associated problems with the technique using refinements to this model.
- To verify the new model by comparing results and predictions with other established techniques.
- To use the model to analyse experimentally measured chemical shifts in normal human erythrocytes.
- To determine intracellular $[Mg^{2+}]_{free}$ and pH in a number of clinical settings, including hypoxia, sickle cell anaemia, pre-eclampsia and sub-arachnoid haemorrhage.
- To analyse the regulation of $[Mg^{2+}]_{free}$ and its relationship with red blood cell metabolism.

CHAPTER 2

Materials and Methods

CHAPTER 2 – MATERIALS AND METHODS

2.1 MATERIALS

Sigma Chemical Co. (St Louis, MO, USA)

Adenosine 5'-triphosphate (ATP, free acid, high purity)

Arabinogalactose (Stractan II)

D-2,3-diphosphoglycerate (2,3-DPG, free acid)

Ethlylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)

Haemoglobin (human)

N-[2-hydroxyethyl]-3-piperazine-N'-[2-ethansulphonic acid] (HEPES)

3-[N-Morpholino] propanesulphonic acid (MOPS)

Phosphocreatine (PCr, dipotassium salt)

Aldrich Chem. Co. (Milwakee, WI, USA)

Phenyl phosphonic acid (PPA)

Dowex Exchange Resin (IWT-TMD-8 ion exchange resin)

Merck (Poole, UK/Darmstadt, Germany)

Ethylene glycol

Magnesium Chloride ($MgCl_2.6H_2O$)

Ethylenediaminetetraacetic acid (EDTA)

Heparin (5,000 U mL^{-1})

BOC gasses (Chatswood, NSW)

Carbon monoxide, CO

5% Carbon dioxide/95% Oxygen (CO_2/O_2)

Calbiochem (Nottingham, UK)

Ionophore, A23187 ($C_{29}H_{37}N_3O_6$)

2.2 PREPARATION OF BUFFERS AND SOLUTIONS

2.2.1 pH

Most pH measurements were made using a Activon Model 109 pH/mV meter with an Activon AEP-432 glass membrane pH electrode (Activon, Granville, NSW)

2.2.2 Osmolality

Osmolality was measured using a vapour pressure osmometer (Wescor Instruments, Model 5100C, Logan, UT, USA). The osmometer was routinely calibrated using a NaCl standard of 300 mOsm kg^{-1}.

2.2.3 Adjustment of association constants as required in Section 3.2

Three steps were required to adjust the published acid dissociation constants (K_a) or metal binding constants (K_b), from a given temperature and ionic strength (I) to another temperature and ionic strength. Firstly, the Debye-Huckel theory was used to adjust the constants to zero ionic strength, by applying the equation

$$K^0 = K^I \times \frac{\gamma(\text{products})}{\gamma(\text{reactants})} \qquad (2.1)$$

where γ, the activity coefficient, was calculated from the charge (z) and ionic strength using:

$$\ln \gamma = \frac{-(Az^2 I^{\frac{1}{2}})}{(1 + BI^{\frac{1}{2}})} \qquad (2.2)$$

where the constant A is 1.17582 at 298.15 and 1.20294 at 310.15 K and B is ~ 1.6 (Veech et al., 1994). Ionic strength was calculated from the concentration (c), and charge using:

$$I = \tfrac{1}{2} \sum_i [c_i] z_i^2 \qquad (2.3)$$

Secondly, a combination of Kirchoff (eq 2.4) and Van't Hoff (eq 2.5) laws was used to adjust the constants to 310.15 K:

$$^{T}\Delta H = {}^{298.15}\Delta H + \int_{298.15}^{T} \Delta_r C_p \, dT \qquad (2.4)$$

where $\Delta_r C_p$ is the change in heat capacity of reactants and products and T is the final temperature, in this case 310.15 K, equivalent to body temperature, and

$$d(\ln K)/dT = \Delta H/RT^2 \qquad (2.5)$$

where the gas constant, R, is 8.31441 J K^{-1} mol^{-1}. Combining equations 2.4 and 2.5 gives:

$$\int_{298.15}^{T} d\ln K = \int_{298.15}^{T} ({}^{298.15}\Delta H/RT^2)dT + \int_{298.15}^{T} (\Delta_r C_p/RT)dT$$

$$- \int_{298.15}^{T} (\Delta_r C_p \times 298.15/RT^2)dT \qquad (2.6)$$

assuming heat capacities are constant with respect to temperature. Thirdly, having adjusted to the appropriate temperature, the Debye-Huckel theory was used again to return to the required ionic strength. The required constants, adjusted to 310.15K and an ionic strength of 0.25, are shown in Table 2.1.

Table 2.1 Acid dissociation constants (upper section) and equilibrium constants (lower section) at 37°C, I = 0.25 M and K^+ = 0.2 M used in Eq. 2.8 to generate the required amounts of $[Mg]_T$ for the titration solutions.

Reaction	$_{app}{}^{37}K^{0.25}$	Ref
$HATP^{3-} \Leftrightarrow H^+ + ATP^{4-}$	3.13×10^{-7} M	(Clarke et al., 1996)
$H_2ATP^{2-} \Leftrightarrow H^+ + HATP^{3-}$	1.91×10^{-4} M	(Clarke et al., 1996)
$H_2PO_4^- \Leftrightarrow H^+ + HPO_4^{2-}$	1.70×10^{-7} M	(Veech et al., 1994)
$HDPG^{4-} \Leftrightarrow H^+ + DPG^{5-}$	9.85×10^{-9} M	(Achilles et al., 1972)
$H_2DPG^{3-} \Leftrightarrow H^+ + HDPG^{4-}$	3.39×10^{-7} M	(Achilles et al., 1972)
$Mg^{2+} + ATP^{4-} \Leftrightarrow MgATP^{2-}$	15.72×10^3 M^{-1}	(Clarke et al., 1996)
$Mg^{2+} + HATP^{3-} \Leftrightarrow MgHATP^-$	1.08×10^2 M^{-1}	(Veech et al., 1994)
$Mg^{2+} + HPO_4^{2-} \Leftrightarrow MgHPO_4$	7.48×10^1 M^{-1}	(Veech et al., 1994)
$Mg^{2+} + DPG^{5-} \Leftrightarrow MgDPG^{3-}$	2.92×10^3 M^{-1}	(Achilles et al., 1972)
$Mg^{2+} + HDPG^{4-} \Leftrightarrow MgHDPG^{2-}$	2.44×10^2 M^{-1}	(Achilles et al., 1972)

2.2.4 Solutions for standard titration curves

Solutions were prepared as previously described (Clarke et al., 1996) and contained 5 mM each of $K_2ATP{:}2H_2O$, $Na_5DPG{:}3.5H_2O$ (Sigma), and $KH_2PO_4{:}K_2HPO_4$ (British Drug House/Merck, Poole), at 310.15K and an ionic strength of 0.25 M. The pH of the solutions ranged from 5.75 to 8.50 and the total magnesium varied from 0 to 15.9 mM. Ionic strength was adjusted to 0.25 M by adding KCl and the pH was adjusted using 1 M KOH or HCl. In total, 130 samples were prepared, with 13 pH values, at 10 free magnesium concentrations, $[Mg^{2+}]_{free}$. Using the relevant equilibrium constants (Table 2.1) for each particular ionic species,

the amount of total $MgCl_2$, $[Mg]_T$, added to achieve a desired $[Mg^{2+}]_{free}$ was calculated using the equation:

$$[Mg]_T = [Mg^{2+}]_{free} \left[1 + \sum_i \left(\frac{[A]_{Ti} \times K_{bi}}{fA_i} \right)\right] \quad (2.7)$$

where f is a ratio of the ionised ligand, A^{z-}, to the total ligand, A_T, $fA_i = [A]_{Ti} / [A^{z-}]$. For the calibration solutions containing the three magnesium binding ligands, ATP, DPG and P_i, the $[Mg]_T$ required for each solution was calculated from the equation:

$$[Mg]_T = [Mg^{2+}]_{free}\left[1 + \frac{[P_i]_T \times K_{bMgPi}}{fP_i} + \frac{[ATP]_T \times \left(K_{bMgATP} + \frac{[H^+]}{K_{aHATP}} - K_{bHATP} \right)}{fATP} + \frac{[DPG]_T \times \left(K_{bMgDPG} + \frac{[H^+]}{K_{aHDPG}} - K_{bHDPG} \right)}{fDPG}\right] \quad (2.8)$$

Solution of Equation 2.8, and the simultaneous equations involved to provide the correct ionic strength and pH (see Appendix I), was achieved using Mathematica™ (Wolfram Research, Champaign, IL). Using this method, $MgCl_2$ was added to give solutions containing $[Mg^{2+}]_{free}$ of 0, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.25, 2.50 and 5.00 mM.

2.2.5 Buffers

MOPS-buffer for erythrocyte suspensions for pH measurements

Solutions were prepared containing 140 mM NaCl, 10mM MOPS, 5mM glucose and the pH was adjusted to 7.4 at 37°C using 1 M KOH or HCl. Osmolarity of the solution was maintained at 300 mOsm kg^{-1}.

HEPES-buffer for washing erythrocytes in experiments using Stractan II density separation and the ionophore, A23187

Solutions were prepared containing 5 mM KCl, 145 mM NaCl, 0.05 mM EGTA, 0.2 mM $MgCl_2$, 10 mM Na-HEPES and the pH was adjusted to 7.4 at 37 °C using 1 M KOH or HCl. Osmolarity of the solution was maintained at 300 mOsm kg^{-1}.

2.3 HAEMATOLOGICAL TECHNIQUES

2.3.1 Measurement of pH, pCO_2 and pO_2 of blood samples

The pH, pCO_2 and pO_2 were confirmed before and after NMR analysis using a blood gas analyser (Radiometer ABL 330).

2.3.2 Preparation of erythrocyte suspensions for pH measurements

Freshly drawn venous blood was obtained from consenting adult donors by venipuncture. Following centrifugation (3000 x g, 10 min, 4°C), the plasma supernatant and buffy coat were removed by aspiration. The cells were resuspended in ~4 volumes of cold MOPS-buffered saline and centrifugally washed a further three times. Before NMR analysis, the cell suspension was gently bubbled with carbon monoxide (100% CO), air (approx 75% N_2 : 25% O_2), carbogen (95% O_2 : 5% CO_2) or nitrogen (100% N_2) for ~10 min.

2.3.3 Preparation of whole human blood samples

Blood from volunteers (8-10 ml) was collected by venipuncture into lithium-heparinised syringes and sickle blood (5 ml) was collected from consenting HbSS patients (approved by the Ethics Committee, Medical School, University of Birmingham). To simulate *in vivo* oxygenated blood, whole blood samples were

prepared for analysis by equilibration with a mixture of O_2 and CO_2 (95%:5%), adjusted to give a final pCO_2 of 40 mmHg and pO_2 > 150 mmHg, thus ensuring complete oxygenation. To minimise cell degradation, time from phlebotomy to analysis was kept to a minimum (1-3 h) during which blood samples were stored on ice.

2.3.4 Measurement of Haematocrit

The haematocrits (Hct) of blood samples were determined following centrifugation at 13 000 x g for 10 min in a microhaematocrit centrifuge. A Hawksley microhaematocrit reader (Hawksley and Sons Ltd., UK) was used to measure the haematocrit. The measurements were made in triplicate and the average of these multiplied by 0.97 to allow for the volume of suspension medium trapped between the packed cells (Dacie and Lewis, 1975).

2.4 NMR METHODS

2.4.1 Measurement of Longitudinal Relaxation Time (T_1)

T_1 measurements of physiological phosphorus-containing compounds were determined for sample solutions using a standard inversion-recovery method:

$$d_1 - \{\pi/2_x - d_2 - \pi_y - d_2 - \pi/2_x\} - vd - \pi/2_x - \text{acquisition}$$

where d_1 represents the relaxation time (x 5 T_1s (Harris and Newman, 1976)), d_2 a 3 μs delay, and vd a variable delay. The terms enclosed in the braces represent a composite π pulse. The magnitudes of the T_1s were determined using the Varian T_1 calculation subroutine. Table 2.2 shows the T_1 values of ^{31}P in selected compounds.

Table 2.2 T_1 values of ^{31}P in selected compounds

Compound	T_1 value [a] (s)
PPA	3.75 ± 0.02
3P – DPG	2.85 ± 0.01
2P – DPG	2.70 ± 0.01
P_i	2.41 ± 0.02
α - ATP	0.55 ± 0.02
β - ATP	0.46 ± 0.01
γ - ATP	0.39 ± 0.02

[a] T_1 values were calculated according to Section 2.4.1 at 37 °C on the Varian spectrometer described in 2.4.2, using the T_1 calculation subroutine on eight spectra. Relaxation delay of 40s was used, and the measurements made on samples containing the metabolite with osmolality adjusted to ~300 mOsm kg^{-1} and pH ~7.2.

2.4.2 ^{31}P NMR Spectroscopic Analyses of Standard Solutions

^{31}P NMR spectra of the solutions were acquired using a 9.4 Tesla, Oxford Instruments wide-bore superconducting magnet interfaced with a Varian Inova spectrometer operating at a phosphorus frequency of 161.9 MHz. A 10 mm probe was used, with the sample temperature set to 310.15 K. The homogeneity of the magnetic field was optimised by shimming on the ^{1}H free induction decay to give ^{1}H spectral linewidths of 9 ± 2 Hz. For each sample, a 90° pulse (18.5 μs) was used with an interpulse delay of 15 s, a spectral width of 8 kHz and no proton decoupling. The delay time was based on pre-determined T_1 values. Each spectrum consisted of 128 summed transients. Up to 256 transients were acquired for samples in which the β-phosphorus of ATP was especially broad.

A capillary containing approximately 50 μl of 0.5 M phenylphosphonic acid (PPA) was used as an external chemical shift reference, standardised relative to phosphocreatine at 0.00 ppm. Prior to Fourier transformation, the signal/noise ratio was increased by multiplying the ^{31}P NMR free induction decays by an exponential

function sufficient to generate a line broadening of 1 Hz, and the time domain signals were zero filled once. The NMR1 program (Tripos, St Louis, MO) was used to fit peak areas, linewidths and chemical shifts of spectral resonances.

2.4.3 ^{31}P NMR spectroscopy of human red blood cells

^{31}P NMR spectra of the cells were acquired using the magnet and spectrometer described above. The whole blood (~ 4.5 ml), prepared as above, was sealed in a 10 mm NMR sample tube together with an external reference capillary containing 50 µl of 0.3 M PPA and brought to 37 °C before ^{31}P NMR spectroscopic analysis. A 10 mm probe was used, with the sample temperature set to 310.15 K. The homogeneity of the magnetic field was optimised by shimming on the ^{1}H free induction decay to a linewidth of 30 ± 2 Hz (normal cells) or 23 ± 2 Hz (sickle cells). A 45° pulse was used with an interpulse delay of 0.52 s to give peaks of a high signal to noise ratio (Marjanovic et al., 1993). The spectral width was 8 kHz, with an acquisition time of 0.272 s (4.5 k data points) and 2048 transients were summed. No proton decoupling was used. Prior to Fourier transformation, the signal/noise ratio was increased by multiplying the ^{31}P NMR free induction decays by an exponential function sufficient to generate a line broadening of 7 Hz, and the time domain signals were zero filled once, giving a digital resolution of 1.1 Hz per point. The NMR1 programme (Tripos, St Louis, MO) was used to fit peak areas, linewidths and chemical shifts of spectral resonances.

Note that pulse angles in the experiments on red blood cells were chosen so as to maximise the signal-to-noise ratio of the phosphorus resonances. This was done using the Ernst angle theorem, incorporating the T_1 values of Table 2.2. As a result, the nuclear populations of 3P 2,3-DPG were not 'fully relaxed', i.e. partially

saturated. When concentrations were being quantified using the intensities of the signals obtained, a pre-determined 'saturation-factor' was used to allow for the diminished intensities caused by saturation.

2.4.4 Sample temperature calibration

Sample temperature was measured according to the method of Bubb et al. (Bubb et al., 1988), by acquiring a single pulse proton spectrum of a sample containing a capillary filled with ethylene glycol. This was confirmed by inserting a standard mercury thermometer into the sample whilst in the magnet, and after 30 minutes of equilibration, the thermometer was quickly removed and read.

2.5 CHEMICAL ANALYSES

Total ATP, DPG and Mg concentrations (in plasma and TCA extracts of whole blood) were determined using Sigma Diagnostic kits. Mg content in erythrocytes was determined by subtracting the plasma value from the blood measurement, after allowing for the haematocrit value.

Immediately after the NMR measurement, samples were taken for determination of ATP and DPG concentrations using diagnostic kits (Sigma Chemical Co., St Louis, MO). Total Mg concentrations in plasma and TCA extracts of whole blood were determined in duplicate using a diagnostic kit (Sigma Chemical Co., St Louis, MO). The total erythrocyte Mg concentrations were then calculated by subtracting the plasma total Mg from the whole blood measurement. Concentrations are presented as moles per litre cell water, assuming a 95% packing efficiency and 70% cellular water content (Mulquiney and Kuchel, 1997b).

Lactate content of plasma from whole blood samples was determined according to the method of Bergmayer (Bergmayer, 1986).

2.6 STATISTICAL ANALYSES

Data are presented as means ± standard deviations unless otherwise stated. Significance was tested using analysis of variance, with repeated measures where appropriate, with $p < 0.05$ considered significant.

2.7 METHODS USED IN CHAPTER 4

2.7.1 Haemoglobin solutions to test effect on metabolite NMR 'visibility'

Solutions were prepared containing 2 mM $K_2ATP{:}2H_2O$, 7 mM $Na_5DPG{:}3.5H_2O$ (Sigma), 190 mM KCl, 3 mM $MgCl_2{:}6H_2O$ with and without 6 mM human Hb (Sigma), and the pH was adjusted to 7.2 at 37 °C using 1 M KOH or HCl.

2.7.2 Ionophore A23187

Methods for measuring $[Mg^{2+}]_i$ in red cells using the ionophore A23187 at various $[Mg]_{T,i}$ are based on those of Flatman and Lew (Flatman and Lew, 1977; Flatman, 1980). Red cell $[Mg]_{T,i}$ was varied by equilibration with a range of extracellular concentrations of Mg^{2+}, $[Mg]_{T,ex}$, using the ionophore A23187 to allow permeation of the membrane. At each $[Mg]_{T,i}$, $[Mg^{2+}]_i$ was calculated from the measured values of the extracellular concentration of ionised Mg^{2+}, $[Mg^{2+}]_{ex}$, and the Donnan ratio, *r*.

Preparations of red blood cells

Plasma was removed from fresh heparinised blood and stored on ice, while the red cells were washed three times at 4 °C in 10 volumes of wash solution (solution A) containing: 80 mM KCl, 70 mM NaCl, 10 mM Hepes-Na and 0.02 mM EDTA, pH 7.55. After each centrifugation (15 min at 3800g), the supernatant and the remaining buffy coat were removed by aspiration. Cells were then made up to 10% haematocrit

in an incubation solution (solution B) containing: 76 mM KCl, 67 mM NaCl, 20 mM Hepes-Na, 0.1 mM EGTA-Na and 10 mM inosine, pH 7.55 with sufficient amounts of 100 mM $MgCl_2$ to give final concentrations of $[Mg]_{T,ex}$ of approximately 0.1, 0.2 and 0.3 mM. Inclusion of EGTA was essential to prevent elevation of cell Ca^{2+} during the incubation with A23187. Otherwise, a rapid decline in cell ATP following maximum activation of the Ca^{2+} pump has been reported (Raftos et al., 1999).

The cell suspensions were then placed in a 37 °C water bath, and stirred rapidly while A23187 (10 mM in ethanol) was added to final concentration of 10 μM. Incubation was continued for 20 min, by which time Mg^{2+} was fully equilibrated across the cell membrane (Flatman, 1980; Raftos et al., 1999).

After incubation, the cells were harvested by centrifugation and washed three times at 4 °C in 10 volumes of solution A, including 5% stored plasma (Raftos and Lew, 1995), to remove the ionophore. The packed cells were then resuspended in stored plasma to a haematocrit of 40-50%. These 'whole blood' samples were then prepared for NMR analyses as described above. The time taken from phlebotomy to NMR analyses was 4-6 h.

The whole experiment was repeated in triplicate, using blood from three separate volunteers, thus generating 9 data sets.

Evaluation of $[Mg^{2+}]_i$

The calculations, described here in brief, were used previously by Flatman and Lew (Flatman, 1980). The value for $[Mg^{2+}]_{ex}$ was the $[Mg]_{T,ex}$ measured in the supernatant (using chemical assay, Section 2.5) corrected for the amount bound to EDTA using the Chelator algorithm (Schoenmakers et al., 1992). Once Mg^{2+} is equilibrated across the cell membrane in the presence of A23187,

$$\frac{[Mg^{2+}]_i}{[Mg^{2+}]_{ex}} = \left(\frac{[H^+]_i}{[H^+]_{ex}}\right)^2 = \left(\frac{[Cl^-]_{ex}}{[Cl^-]_i}\right)^2 = \frac{1}{r^2}$$

so that

$$[Mg^{2+}]_{ex} = r^2 \times [Mg^{2+}]_i$$

where r is the Donnan ratio (Section 1), physiologically ~0.7 (Duhm, 1971). This ratio was also evaluated from the ratio of H_{ex}/H_i, with both parameters measured at the time of NMR analysis, as described above.

2.8 METHODS USED IN CHAPTER 6

2.8.1 Stractan II preparation

Stock solution

Arabinogalactan (Stractan II) was dissolved 1:1 by weight in double distilled water using a tall beaker over a magnetic stirrer hot plate. This was boiled gently until fully dissolved. The beaker was covered loosely with a clean Petri dish to decrease evaporation. After cooling, several spoonfuls of amberlite MB-3 (Dowex exchange resin) were added to remove contaminant osmoticants. This mixture was then left overnight in a cold room at 4 °C.

The amberlite was removed by filtration under vacuum and the osmolality of a small sample was measured after a 1:1 dilution with HEPES buffer (with a calibrated osmolality of 300 mOsm kg^{-1}). This measurement required the equation:

$$Os = 2Om - Ob$$

where Os is the osmolality of the Stractan solution, Ob is the osmolality of the buffer, and Om is the osmolality measured of the 1:1 mix of Stractan and buffer.

Unless Os was between 100-140 mOsm kg^{-1}, the amberlite treatment was repeated until this was the case. Typically, three or four such treatments were required. The total volume of the remaining solution, Vt, was measured.

Measurement of the density of Stractan stock solution

After Os <140 mOsm kg^{-1}, a 10 ml syringe with a fixed stop at approximately 5 ml and a long 19 gauge needle was used to accurately weigh approximately 5 ml of double distilled water 5 times. Assuming the water density was 1.00 g/ml, the volume weighed was calculated, V. After thoroughly drying the apparatus, the same volume of Stractan stock was weighed repeatedly, with the reproducibility of the measurement within the ± 0.002 g range. The density of the Stractan stock, Ds, was calculated by dividing the measured weight by V.

Calculation of the volume of water, Vw, in the stock

Using the nomenclature: density, D; weight, W; volume, V; water, w; Stractan, s; limiting density of Stractan (the most mass of Stractan dissolved in 1 ml water), D' = 1.56 g/ml; buffer, b; by definition:

$$Ds = (Ws+Ww)/(Vs+Vw) \tag{2.9}$$

and:

$$D' = Ws/Vs \tag{2.10}$$

Then, assuming Dw=1 g/ml, numerically Ww = Vw, and replacing Ws = D'Vs in Eq. 2.9 & 2.10:

$$Ds = ((D'Vs/Vw)+1)/((Vs/Vw)+1) \tag{2.11}$$

therefore:

$$Vs/Vw = (Ds-1)/(D'-Ds) \tag{2.12}$$

Defining Vt = Vs+Vw and r = Vs/Vw

$$Vw = Vt/(r+1) \qquad (2.13)$$

Adjusting the osmolality of Stractan stock

After estimating Vw from equation 2.13, sufficient HEPES-Na was dissolved in the stock to give a final concentration of 20 mM in Vw. $MgCl_2$ and EGTA from concentrated stock solutions were added to give final concentrations in Vw of 0.2 mM and 0.1 mM, respectively. The pH was adjusted to 7.4 at 37°C using 10 M NaOH. All additions were made under vigorous magnetic stirring to prevent dilution. The solution was then filtered through 0.45 μM Millipore filters under vacuum to sterilise the mixture. Then, 3g of pre-cleaned albumin was dissolved per 100 ml Vt.

Ds was measured as described above, and Vw and Os were estimated as before. The weight of NaCl required to increase Os to 300 mOsm kg^{-1} was calculated and 70% of this was added as anhydrous salt. After dissolving, the Os was re-measured and again 70% of the required NaCl was added. This process was repeated until Os reached 300 mOsm kg^{-1}; typically two or three more steps were needed.

The final Ds of the Stractan stock was measured to be 1.196 g/ml. This was kept frozen at –20°C until required.

Preparation of Stractan solutions of any desired density

Total volumes, Vt, of various densities, Dx, were required to separate red blood cells from patients with sickle cell anaemia, as described below in Section 2.10.2. These were prepared using the following equations:

From conservation of mass:

$$VtDs = VsDs + VbDb \qquad (2.14)$$

Replacing Vb by Vt-Vs:

$$Dx = (Vs/Vt)Ds + (1-(Vs/Vt))Db \quad (2.15)$$

Defining f = Vs/Vt:

$$f = (Dx-1)/(Ds-1) \quad (2.16)$$

f was calculated according to the Dx required using Eq 2.16. Although it would then have been straightforward to calculate the necessary volumes of Stractan stock and the volume of buffer required to mix with the stock to produce a final Vt Stractan solution of density Dx, in practice it was more precise to measure out the required quantities by weight. The same large syringe with long 19 gauge needle was used, and the necessary weights were calculated by:

$$Ws = DsVs = fDsVt \quad (2.17)$$

This was then topped up with buffer to give a final weight of:

$$Wt = DxVt \quad (2.18)$$

2.8.2 Separation of red blood cells from patients with sickle cell anaemia by density

Cells were fractionated on discontinuous density gradients of Stractan II (arabinogalactan) as described previously (Corash et al., 1974) with minor modifications (Ortiz et al., 1986) (Lew V.L., Personal Communication) (see Section 2.10.1 above). Two 3 ml iso-osmotic Stractan density layers were used, at 1.090 and 1.099 g/ml, with a cushion of 1.196 g/ml. Blood (20 ml) was collected from consenting HbSS patients and washed with a buffer containing 5 mM KCl, 145 mM NaCl, 0.05 mM EGTA, 0.2 mM $MgCl_2$, 10 mM Na-HEPES, pH 7.4. Following centrifugation at 3000 g for 10 min, the plasma was removed and stored on ice, and the buffy coat was carefully removed and discarded. For each experiment, packed cells from 2 patients were pooled to give a total of 8-10 ml of packed cells. This was divided into 2 equal volumes, which were layered on the Stractan gradient in 13.2 ml

Beckman ultracentrifugation tubes. After ultracentrifugation (52,000g for 40 min at 4 °C), three layers were harvested, light (D < 1.090 g/ml), medium (1.090 g/ml < D < 1.099 g/ml) and dense (D > 1.099 g/ml), with the dense layer containing most of the irreversibly sickled cells, ISC's. The density-fractionated red cells were washed three times using the above HEPES buffer, before being resuspended in pooled plasma from the same two patients, to give final haematocrits of 35-50%. These 'whole blood' samples were then prepared for NMR analyses as described above. The time taken from phlebotomy to NMR analyses was 5-7 h.

2.9 METHODS USED IN CHAPTERS 8 AND 9

2.9.1 Patients with sub-arachnoid haemorrhage (SAH)

Inclusion criteria

Patients suffering with SAH, as confirmed by either Computer Topography (CT) or Lumbar Puncture; admitted within 3 days of the ictus, with World Federation of Neurosurgeons Grading System, WFNS, grade 3 or higher and/or Fisher grade 2 or higher.

Treatment protocol

Patients (n = 3) received an intra-venous loading dose of 16 mmol $MgSO_4$ in 10ml 0.9% NaCl over 30 minutes. An intra-venous maintenance dose was administered of 64 mmol $MgSO_4$ in 48ml 0.9% NaCl over 24 hours for 10 days. Twice daily serum magnesium levels were analysed to ensure that the blood magnesium level was maintained at 2.5 mM. A minimum 4 hourly blood pressure and pulse was recorded, along with alternate daily transcranial Doppler

ultrasonography to record middle cerebral artery (MCA) velocity. A daily neurological examination was performed.

Sampling of blood

When possible, an 8ml blood sample was collected from the patient before treatment. Once treatment began, one 8 ml blood sample was taken, if possible once every 12 hours, and at least once every 24 hours.

2.9.2 Pregnant mothers with and without complications of pre-eclampsia

Inclusion criteria for patients with pre-eclampsia

Patients were included in the study if they were over 18 years old, were able to understand English, had no psychiatric history and had given informed consent. They were also required to be willing and able to give urine and/or blood samples and to have the outcome of their pregnancy followed up and documented. Their diastolic blood pressure must have been ≥ 90 mmHg on 2 separate occasions within a 24 hour period and they must have had ≥ 500 mg protein at least twice on dipsticking consecutively or ≥ 100 mg/l protein/creatinine ratio.

Exclusion criteria for patients with pre-eclampsia

Patients were excluded from the study if they were educationally disadvantaged or unsupported, or if they were private patients. They were also excluded if there had been more than a trace of proteinuria before 20/40 or if they had been diagnosed with insulin dependent diabetes.

Inclusion criteria for mothers with uncomplicated pregnancies

Normal pregnant subjects were included in the study, matched according to age (± 4 years but over 18 yrs old), weight, and term of gestation. They were required to understand English, have no psychiatric history and have given informed consent. They were also required to be willing and able to give urine and/or blood samples and to have the outcome of their pregnancy followed up and documented. They had singleton pregnancies without known fetal abnormality and had diastolic blood pressure consistently below 90 mmHg.

Exclusion criteria for mothers with uncomplicated pregnancies

Normal subjects were excluded from the study if they were educationally disadvantaged or unsupported, or if they were private patients. They also should have had no history of drug/substance abuse, no treatment received to promote conception, not more than 5 years of infertility associated with their pregnancy and not have had a systolic blood pressure ≥ 140 mmHg or a diastolic pressure ≥ 90 mmHg. They should also have had a normal previous obstetric history, and in particular no previous pregnancy should have been complicated with pre-eclampsia or hypertension.

Sampling of blood

Blood samples (9 ml) were taken from consenting patients with pregnancies complicated by pre-eclampsia (n = 7) and from consenting normal pregnant subjects (n = 6), as defined in the above Section. Blood samples were also analysed from control subjects, consisting of non-pregnant females, within the same age range of the normal and pre-eclampsic patients (n = 6).

CHAPTER 3

Measuring $[Mg^{2+}]_{free}$ and pH using ^{31}P NMR spectroscopy

CHAPTER 3 – MEASURING $[Mg^{2+}]_{FREE}$ USING ^{31}P NMR SPECTROSCOPY

The first high resolution NMR spectroscopic studies of cells appeared in the early 1970s (Eakin et al., 1972; Moon and Richards, 1973). Since that time NMR spectroscopy has become a widely used to tool for monitoring biochemical reactions and quantifying levels of metabolites (for reviews see (Radda and Seeley, 1979; Gadian et al., 1979; Gupta et al., 1984)). The benefit of NMR spectroscopy comes from its ability to monitor a range of metabolites simultaneously, in real time, from a wide variety of biological samples in a non-destructive manner. Of particular interest to this work is that Moon and Richards (Moon and Richards, 1973) demonstrated the ability of ^{31}P NMR spectroscopy to monitor 2,3-DPG, P_i and ATP concentrations in erythrocytes. They showed that the changes in chemical shift of the resonances from these compounds could be used to monitor changes in pH. It was quickly seen that H^+ was not the only cation that would exert an effect on chemical shift, and it was reported that ATP resonances could be used to estimate $[Mg^{2+}]_{free}$ (Gupta et al., 1978a). Since that time the accuracy and precision of measurements has been improved, and NMR spectroscopy's ability to measure a chemically labile species, rather than a total amount, is of great importance to understanding metabolism.

However, there are a number of potential errors associated with using the observed NMR signal from ATP to determine $[Mg^{2+}]_{free}$:

1) uncertainty in the binding constant between ATP and magnesium (Garfinkel and Garfinkel, 1984),
2) the effect of intracellular ionic content, especially K^+ concentration and ionic strength, on this interaction (Golding and Golding, 1995; Golding et al., 1996)

3) the effect of metabolite-haemoglobin interactions (Mulquiney and Kuchel, 1997a).

Gupta and co-workers (Gupta et al., 1978b), the first to use this method, addressed the first two problems by determining an apparent MgATP binding constant, $^{app}K_b$, from the difference between the endpoints of the chemical shifts of the magnesium bound and unbound α and β phosphorus nuclei of ATP, $\delta_{\alpha\beta\text{-MgATP}}$ and $\delta_{\alpha\beta\text{-ATP}}$ (Gupta and Moore, 1980; Gupta et al., 1983b; Gupta et al., 1983a). Gupta and co-workers considered that "an advantage of using an apparent binding constant, instead of 'intrinsic' constants measured under different conditions, but corrected considering all relevant equilibria, is that no assumptions need to be made about the nature of the equilibria involved as long as intracellular ionic conditions are well simulated" (Gupta et al., 1984). It is true that, given the important caveat, this approach decreased the number of equilibria that had to be considered and overcame the need to rely on adjusted published values of $^{app}K_b$, which varied 100-fold (Goldberg and Tewari, 1991). The effect of this variation when determining $[Mg^{2+}]_{free}$ is demonstrated in Table 3.1. However, as with any simplification, problems are associated with this approach, which include the possible difference in ionic strength between solutions and *in vivo* conditions, the accuracy of the chemical shift endpoints required to determine $^{app}K_b$ (Mosher et al., 1992) and the assumption that $\delta_{\alpha\text{-ATP}}$ does not vary. On top of this, as inferred by Gupta and co-workers, the measured apparent constant is only valid if analysing cells under the same conditions as the measurement. This may be limiting given that different clinical states may not produce the same intracellular environments, and a new apparent constant would have to be measured or calculated for each case.

Because the chemical shifts of ^{31}P NMR phosphorus peaks depend on many different factors (Roberts et al., 1981), accurate analysis should take all factors into account, which has become possible with the increased capacity of computation. Previous analyses using phosphorus chemical shifts have been used to obtain *either* pH (Tehrani et al., 1982) *or* $[Mg^{2+}]_{free}$ (Gupta et al., 1978a) in red blood cells. Only one approach used the ^{31}P resonances of ATP to determine *both* pH and $[Mg^{2+}]_{free}$ (Williams et al., 1993), but this averaged all ATP species with an apparent binding constant and made no allowance for the effect of haemoglobin and K^+ binding upon the observed chemical shift.

Table 3.1 Values of calculated $[Mg^{2+}]_{free}$ and corresponding chemical shifts of β-ATP, using the extreme literature values of 1,690 M^{-1} and 128,000 M^{-1} for the MgATP binding constant (Hotta et al., 1961; Noat et al., 1970), with total [ATP] of 5 mM and ionic strength of 0.25 M, at 37°C.

	K_b = 1,690 M^{-1}		K_b = 128,000 M^{-1}	
Total [Mg] (mM)	$[Mg^{2+}]_{free}$ (mM)	$\delta_{\beta\text{-ATP}}$ (ppm)	$[Mg^{2+}]_{free}$ (mM)	$\delta_{\beta\text{-ATP}}$ (ppm)
4	1.038	16.78	0.044	16.18
4.5	1.303	16.64	0.124	15.93
5.1	1.668	16.49	0.519	15.81

In this Chapter, a new approach is followed to allow for the combined effects of the competing cations in the multiple equibria that exist within the red blood cell. As mentioned above, the previous technique used to analyse spectra began by measuring an association constant for Mg^{2+} to ATP under physiological conditions. As problems became reported with this, the technique was adapted over the years, adding in extra factors to the equations used to analyse NMR spectra to overcome the

problems. However, one can only keep adjusting for so long before it seems more sensible to begin again from a different angle, taking account of the problems that lay ahead. The new approach presented here aimed to create equations to analyse spectra that involve intrinsic, or absolute, binding constants for all the important equilibria. Despite the extra complexity, this gives the technique greater flexibility and potential for development in the future. How this new approach deals with the reported problems is discussed.

3.1 ANALYSIS OF ^{31}P NMR SPECTRA – THEORY

It is not intended here to give a review of the theory of NMR with quantum mechanical considerations, but instead to discuss the interpretation of observed spectral peaks, assuming that suitable spectra can be, and have been acquired.

3.1.1 The origin of changing chemical shifts

Following excitation of a phosphorus nucleus in a magnetic field using a radio frequency pulse, the nucleus will release its excitation energy as it returns to a lower energy state ("relaxation"). This energy will be at a precise frequency (the resonance frequency) that is dependent on the chemical environment of the nucleus. For example, each of ATP's three phosphorus nuclei (α, β and γ) resonate at different frequencies and when represented on a spectrum, three peaks are seen, one at each frequency, or more commonly referred to as 'chemical shift', δ_α, δ_β and δ_γ. When ATP binds to Mg, the nuclei's chemical environment changes and therefore the chemical shift changes.

Considering the resonance from β-ATP, when both ATP^{4-} and $MgATP^{2-}$ are present, if either the NMR timescale were short enough, or the rate of exchange slow enough, each phosphorus species would be observed, producing two peaks with

intensities reflecting the population of the two species in the equilibrium. More generally, as is the case for this interaction at 37°C and ionic strength of 0.25 M, the exchange is fast, and a weighted average of the two signals is observed, according to the relative populations of the two species. The signal average is directly dependent on the rate of exchange (Figure 3.1).

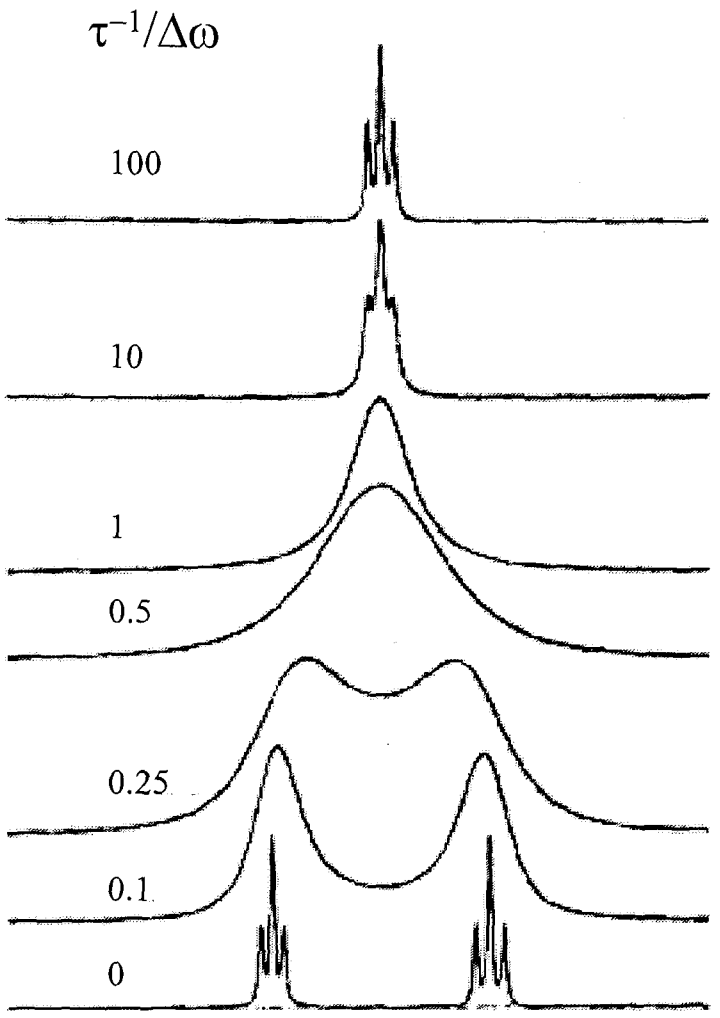


Fig. 3.1 Computer simulated lineshapes for a 2-site exchange with equal populations, in which the lineshapes in the absence of exchange are given by 1:2:1 triplets, as for β-ATP and β-MgATP. The lineshapes were calculated at different exchange rates τ^{-1} expressed as multiples of the chemical shift difference $\Delta\omega$ (in rad s^{-1}) (Vasavada et al., 1984).

Because the average also reflects the position of equilibrium, the extent to which the peak is shifted towards one or other of the limits of the change in chemical shift for the two species ATP^{4-} and $MgATP^{2-}$ (end points) indicates the relative amounts of bound/unbound ATP. Typical spectra of ATP and MgATP are shown in Figure 3.2 showing the changing chemical shift as total Mg is varied.

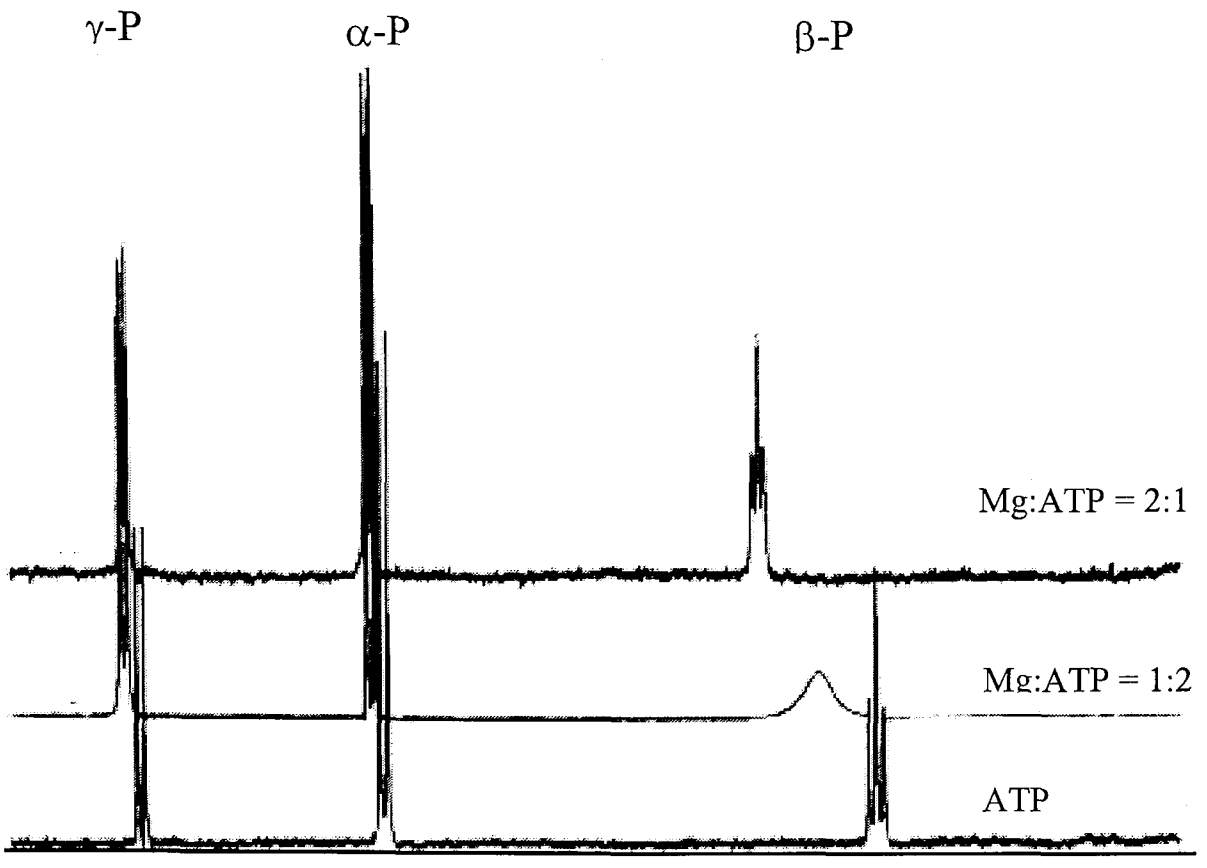


Fig. 3.2 ^{31}P NMR spectra of ATP, Mg:ATP = 1:2, and Mg:ATP = 2:1, showing the the chemical shifts of γ, α and β resonances (Vasavada et al., 1984).

From the spectra, the lack of a clear triplet for β ATP at intermediate values of magnesium indicates that the exchange occurs at an intermediate rate, summarised by:

$$\delta_{obs} = \frac{[MgATP]}{[ATP]_T} \times \delta_{MgATP} + \frac{[ATP]_T - [MgATP]}{[ATP]_T} \times \delta_{ATP} \qquad (3.1)$$

Of course, Mg^{2+} is not the only cation that binds to ATP^{4-}; in the red cell particularly, the two important competitors for binding are H^+ and K^+, and simultaneous binding is possible. In an ideal world, the NMR timescale would be short enough to prevent any signal averaging, for then each ATP species would give rise to a distinct peak whose intensity is directly proportional to the concentration in the cell (Figure 3.3). Unfortunately, this is not the case and instead only one peak is visible, the weighted average of all species present.

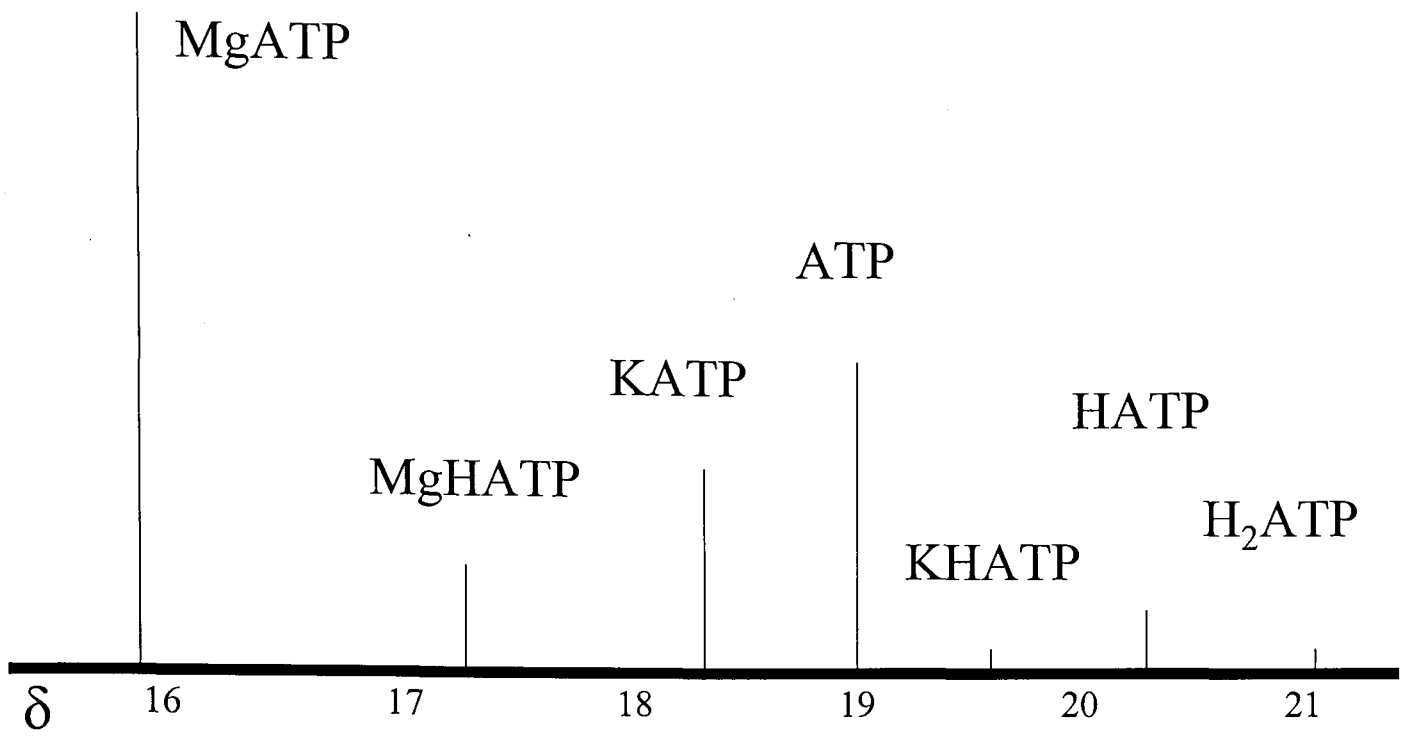


Fig. 3.3 A diagrammatic representation of a ^{31}P NMR spectrum of β-ATP in a physiological solution with no signal averaging. The height, or intensity, of each peak is directly proportional to the concentration of each species.

This is also the case for the two DPG peaks, the other NMR visible metabolite in the erythrocyte. The challenge, therefore, is to calculate the concentrations of all species that contribute to the observed signal. To do this, the positions of the multiple equilibria, and therefore the association/binding constants, must be known, along with the chemical shifts of each intermediate species of ATP and DPG.

In this study, it was achieved by first acquiring spectra of standard titration solutions consisting of the important phosphorus-containing red cell constituents that can be seen by ^{31}P NMR. As the aim was to measure pH and $[Mg^{2+}]_{free}$ in the red cell, these parameters were varied in the solutions. Mathematical equations that relate the observed chemical shift to the concentrations of the intermediates in the multiple equilibria were 'fitted' to the experimental data, using the intermediates' binding constants and chemical shifts as the fitting parameters. Once evaluated, these

equations could be used to analyse chemical shifts observed in red blood cells and determine the amounts of all intermediates in the cell, including H^+ and $[Mg^{2+}]_{free}$.

3.2 STANDARD TITRATION SOLUTIONS – PROPERTIES AND ANALYSIS

3.2.1 Important properties of the solutions

When preparing the solutions, as described in Section 2.2, the ionic strength was held constant at a physiological value. This value, 0.25 M, was higher than the usually assumed value in the literature of 0.15 M but was more likely given the concentrations of metabolites and Hb in erythrocytes. Experimental evidence for this value has been reported (Veech et al., 1979). However, the solutions were not an exact replication of the intracellular environment and, to achieve the necessary ionic strength, a greater concentration of KCl was required than that normally present *in vivo*. Typically [KCl] was about 190 mM, although minor deviations were required to prevent the ionic strength of the solutions varying over the range of pH and $[Mg^{2+}]_{free}$. This was an important difference from previous work, where the assumption had been made that a certain concentration of KCl necessarily determines the ionic strength, which is not the case physiologically in the presence of highly charged metabolites.

One important constituent not included in the solutions was haemoglobin. This was omitted as its many interactions with metabolites are poorly understood, and its inclusion would have complicated the determination of the required parameters of binding constants and chemical shifts. However, once the equations had been described in terms of the metabolites, the effect of haemoglobin could then be included, as described below.

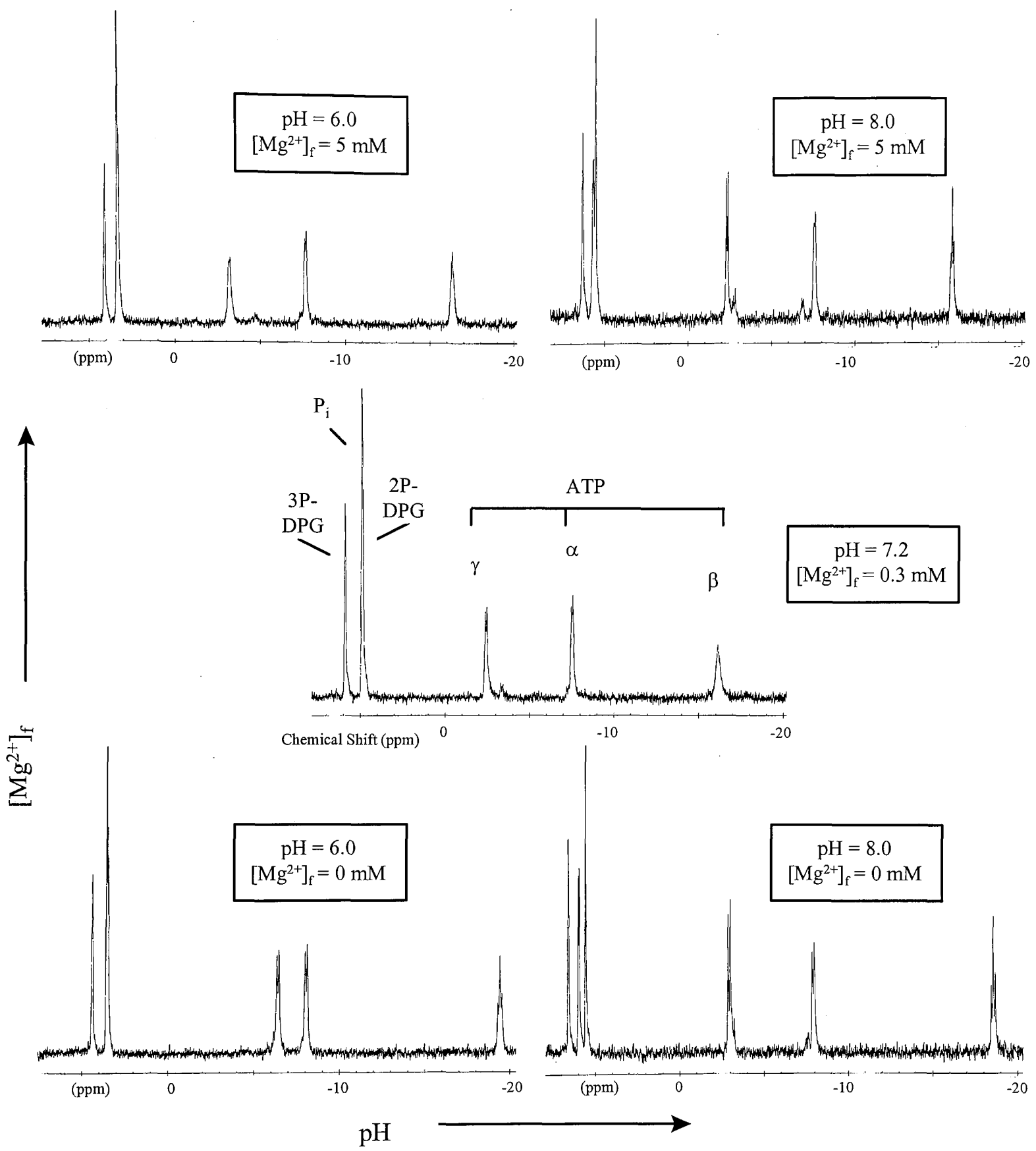


Fig. 3.4 Spectra acquired of the standard solutions over the range of pH, 5.75 to 8.5, and $[Mg^{2+}]_{free}$, 0 to 5 mM. A spectrum typically expected for blood at physiological conditions of a pH of 7.2 and $[Mg]_{free}$ 0.3 mM is centred.

It was not important when preparing the solutions to include the same concentrations of metabolites as seen physiologically. Again, this was because their values could be introduced to the equations once the binding constants and chemical shifts had been determined.

3.2.2 Analysis of the standard titration solutions

Typical ^{31}P NMR spectra of the titration solutions acquired between a pH of 6 and 8, and $[Mg^{2+}]_{free}$ between 0 and 5 mM are shown in Fig. 3.4. The ATP, DPG and P_i peaks increased in chemical shift as pH increased, reflecting a decrease in electronic shielding upon proton association. The maximal chemical shift changes are given in Table 3.2. Increasing $[Mg^{2+}]_{free}$ altered the δ of all ATP peaks (β by 3.26 ppm and γ by 3.72 ppm, both more than α, 0.46 ppm), but led to only slight changes in those of P_i (0.03 ppm) and 3P- and 2P- DPG (0.34 and 0.76 ppm, respectively). The chemical shifts of the 2P-DPG and P_i peaks were more sensitive to changes in pH than to changes in magnesium (3.15 and 2.35 ppm, respectively), whereas those of the β and γ phosphate peaks of ATP were equally sensitive to both pH and $[Mg^{2+}]_{free}$. At the physiological pH of 7.2 and $[Mg^{2+}]_{free}$ of 0.3 mM, the β-ATP triplet could not be fully resolved due to line broadening caused by chemical exchange, but the peak could nonetheless be used for chemical shift determination. The P_i peak overlapped with the 2P-DPG doublet, thus neither could be used for pH or $[Mg^{2+}]_{free}$ determination in red blood cells. Instead, it was necessary to use the 3P-DPG peak.

From the plot of the chemical shift of 3P-DPG, it can be seen that δ_{3P} did not significantly change with $[Mg^{2+}]_{free}$, other than at high pH (> 7.8) and very low $[Mg^{2+}]_{free}$ (< 0.1 mM). This suggested that δ_{3P} could be used as a good indicator of

physiological pH. The chemical shift of the β-ATP changed significantly with both pH and $[Mg^{2+}]_{free}$ in the physiological region to allow an accurate determination of pH and $[Mg^{2+}]_{free}$ from the observed chemical shifts. A change in chemical shift of ~ 0.05 ppm corresponded to a 0.015 mM change in $[Mg^{2+}]_{free}$ at constant pH.

Table 3.2 The changes in chemical shifts of the resonance frequencies of phosphorus-containing metabolites over the range of pH and $[Mg^{2+}]_{free}$.

Phosphate Group	pH 5.75 to 8.5 (ppm)	$[Mg^{2+}]_{free}$ 0 to 5 mM (ppm)	Total variance observed (ppm) 5.75 pH 0 mM Mg_f to 8.5 pH 5 mM Mg_f
α-ATP	0.37	0.46	0.59
β-ATP	2.54	3.26	3.82
γ-ATP	4.16	3.72	4.66
3P-DPG	2.79	0.34	2.79
2P-DPG	3.15	0.76	3.15
P_i	2.35	0.03	2.35

3.2.3 Determination of ATP and DPG intermediates' binding constants and chemical shifts by theoretical fitting of the observed chemical shifts.

The chemical shifts for the 3P–DPG and β-ATP peaks were plotted against pH and $[Mg^{2+}]_{free}$, generating a 3D surface. This was then fitted to an equation using the nonlinear fit algorithm in Mathematica™ with the main parameters being the chemical shifts of the H^+, K^+ and Mg^{2+} bound forms of β–ATP and 3P–DPG, and the binding constants of each species to H^+, K^+ and Mg^{2+}.

The equation used for fitting was based on the principle that the observed chemical shift (δ_{obs}) could be described by combining the chemical shifts of all the

relevant forms of a substance present in the equilibrium and weighting their contribution to the observed chemical shift according to the ratio of their population to that of the total substance present (Veech et al., 1994):

$$\delta_{obs} = \frac{[X_{species\,1}]}{[Total\,X]} \times \delta_{species\,1} + \frac{[X_{species\,2}]}{[Total\,X]} \times \delta_{species\,2} + \cdots \qquad (3.2)$$

Hence for ATP, assuming all ATP to consist of several species such that:

$$[ATP]_T = [ATP^{4-}] + [HATP^{3-}] + [H_2ATP^{2-}] + [MgATP^{2-}] + [MgHATP^{-}] + [KATP^{3-}] + [KHATP^{2-}]$$

the theoretical chemical shift can be described by:

$$\delta_{obs} = \frac{\left[ATP^{4-}\right]x\,\delta_{ATP} + \left[HATP^{3-}\right]x\,\delta_{HATP} + \left[MgATP^{2-}\right]x\,\delta_{MgATP} + \left[MgHATP^{-}\right]x\,\delta_{MgHATP} + \left[KATP^{3-}\right]x\,\delta_{KATP} + \left[KHATP^{2-}\right]x\,\delta_{KHATP} + \left[H_2ATP^{2-}\right]x\,\delta_{H2ATP}}{[ATP]_T} \qquad (3.3)$$

Using the equilibrium constants (assuming activity coefficients of unity), the concentrations of all forms can be expressed relative to the concentration of the completely ionised form (f's in Eq. 2.7). Taking these and substituting in the above equation, the observed chemical shift was represented by:

$$\delta_{obs} = \left(\frac{\delta_{ATP} + \left(\frac{[H^+]}{K_{aATP}}\right)\delta_{HATP} + \left(K_{bMgATP} \times [Mg^{2+}]\right)\delta_{MgATP} + \left(\frac{K_{bMgHATP} \times [Mg^{2+}][H^+]}{K_{aATP}}\right)\delta_{MgHATP} + \left(K_{bKATP} \times [K^+]\right)\delta_{KATP} + \left(\frac{K_{bKHATP} \times [K^+][H^+]}{K_{aATP}}\right)\delta_{KHATP} + \left(\frac{[H^+]^2}{K_{aHATP} \times K_{aATP}}\right)\delta_{H_2ATP}}{1 + \left(\frac{[H^+]}{K_{aATP}}\right) + \left(K_{bMgATP} \times [Mg^{2+}]\right) + \left(\frac{K_{bMgHATP} \times [Mg^{2+}][H^+]}{K_{aATP}}\right) + \left(K_{bKATP} \times [K^+]\right) + \left(\frac{K_{bKHATP} \times [K^+][H^+]}{K_{aATP}}\right) + \left(\frac{[H^+]^2}{K_{aHATP} \times K_{aATP}}\right)} \right) \quad (3.4)$$

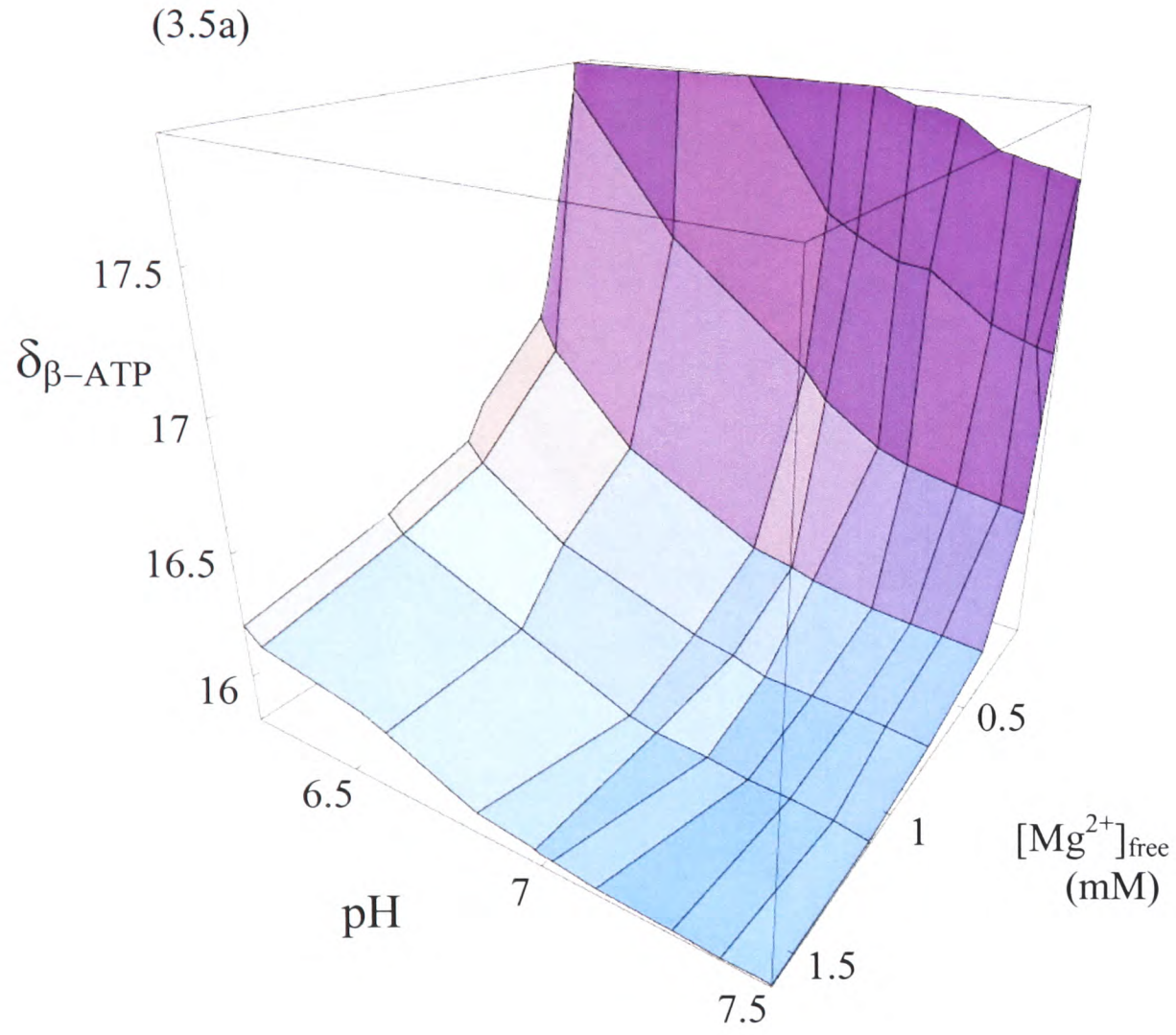
Similarly for DPG, the following equation describing the observed chemical shift for DPG was formulated, assuming the forms present were DPG^{5-}, $HDPG^{4-}$, H_2DPG^{3-}, $MgDPG^{3-}$, $MgHDPG^{2-}$, $KDPG^{4-}$ and $KHDPG^{3-}$:

$$\delta_{obs} = \left(\frac{\delta_{DPG} + \left(\frac{[H^+]}{K_{aDPG}}\right)\delta_{HDPG} + \left(K_{bMgDPG} \times [Mg^{2+}]\right)\delta_{MgDPG} + \left(\frac{K_{bMgHDPG} \times [Mg^{2+}][H^+]}{K_{aDPG}}\right)\delta_{MgHDPG} + \left(K_{bKDPG} \times [K^+]\right)\delta_{KDPG} + \left(\frac{K_{bKHDPG} \times [K^+][H^+]}{K_{aDPG}}\right)\delta_{KHDPG} + \left(\frac{[H^+]^2}{K_{aHDPG} \times K_{aDPG}}\right)\delta_{H_2DPG}}{1 + \left(\frac{[H^+]}{K_{aDPG}}\right) + \left(K_{bMgDPG} \times [Mg^{2+}]\right) + \left(\frac{K_{bMgHDPG} \times [Mg^{2+}][H^+]}{K_{aDPG}}\right) + \left(K_{bKDPG} \times [K^+]\right) + \left(\frac{K_{bKHDPG} \times [K^+][H^+]}{K_{aDPG}}\right) + \left(\frac{[H^+]^2}{K_{aHDPG} \times K_{aDPG}}\right)} \right) \quad (3.5)$$

The substitution of $[H^+]$ to 10^{-pH} was used to make the equations more convenient. 'Goodness' of fit may be determined by χ^2, where $,\chi^2 = \sum_i |F_i - f_i|^2$ with F_i the value of the i^{th} data point and f_i is the value obtained from the fit.

Using the observed β-ATP peak chemical shifts (δ_β), a 3D surface curve was generated as a plot of pH and $[Mg^{2+}]_{free}$ with the section relevant to physiological conditions expanded (Fig. 3.5a). Eqn. 3.4 was fitted to these data and plotted in Fig. 3.5b. Using the observed 3P-DPG peak chemical shifts (δ_{3P}), a 3D surface curve was generated as a plot of pH and $[Mg^{2+}]_{free}$ (Fig. 3.6a). Eqn. 3.5 was fitted to these data and plotted in Fig. 3.6b. The final values of the fitting parameters of chemical shifts and explicit equilibrium constants are given in Table 3.3.

The process of fitting the parameters listed in Table 3.3 used the 'NonlinearFit' algorithm in Mathmatica. However, this algorithm would not find real (i.e. chemically possible) solutions if all parameters were allowed to vary simultaneously. Therefore in practice, three or four parameters were allowed to vary within manually defined minima and maxima, whilst holding the others constant, and an initial estimate for the fitting curve was produced. This curve was then compared to the experimentally observed data both manually and using χ^2 for reference, and the same three or four parameters were then varied again within improved minima and maxima to produce a better estimate for the fitting curve. This optimising process was continued iteratively across all the parameters until the closest fit was achieved. A final value for χ^2 was obtained for this fit by allowing all parameters to vary simultaneously within relatively small, manually defined minima-maxima ranges. The 'NonlinearFit' algorithm also determined the standard errors for the parameters in this manner.



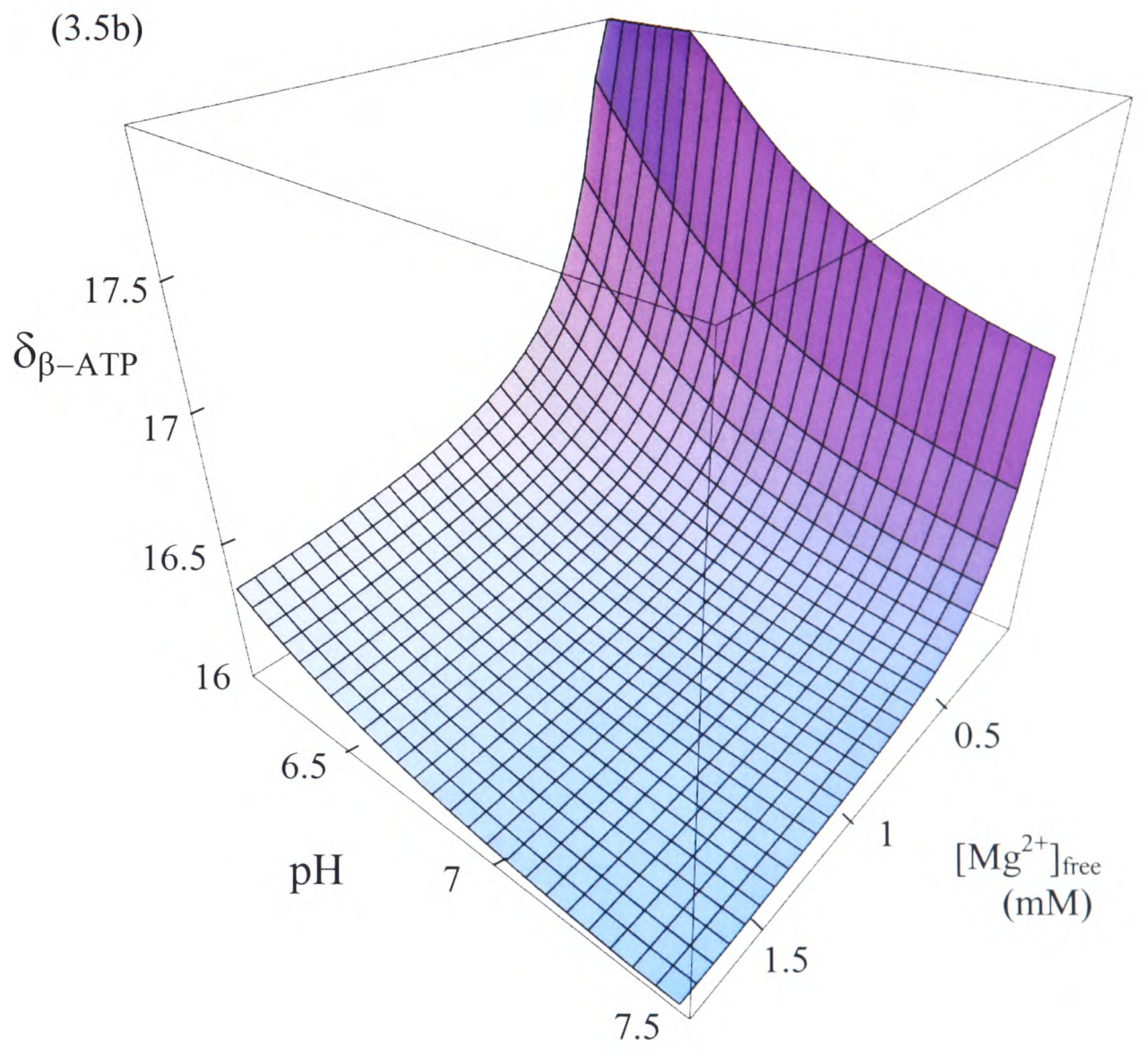


Fig. 3.5 Plot of the observed chemical shift (3.5a) and the curve produced by the fitting equation (3.5b) for β-ATP against pH and $[Mg^{2+}]_{free}$, expanded to show the physiological region.

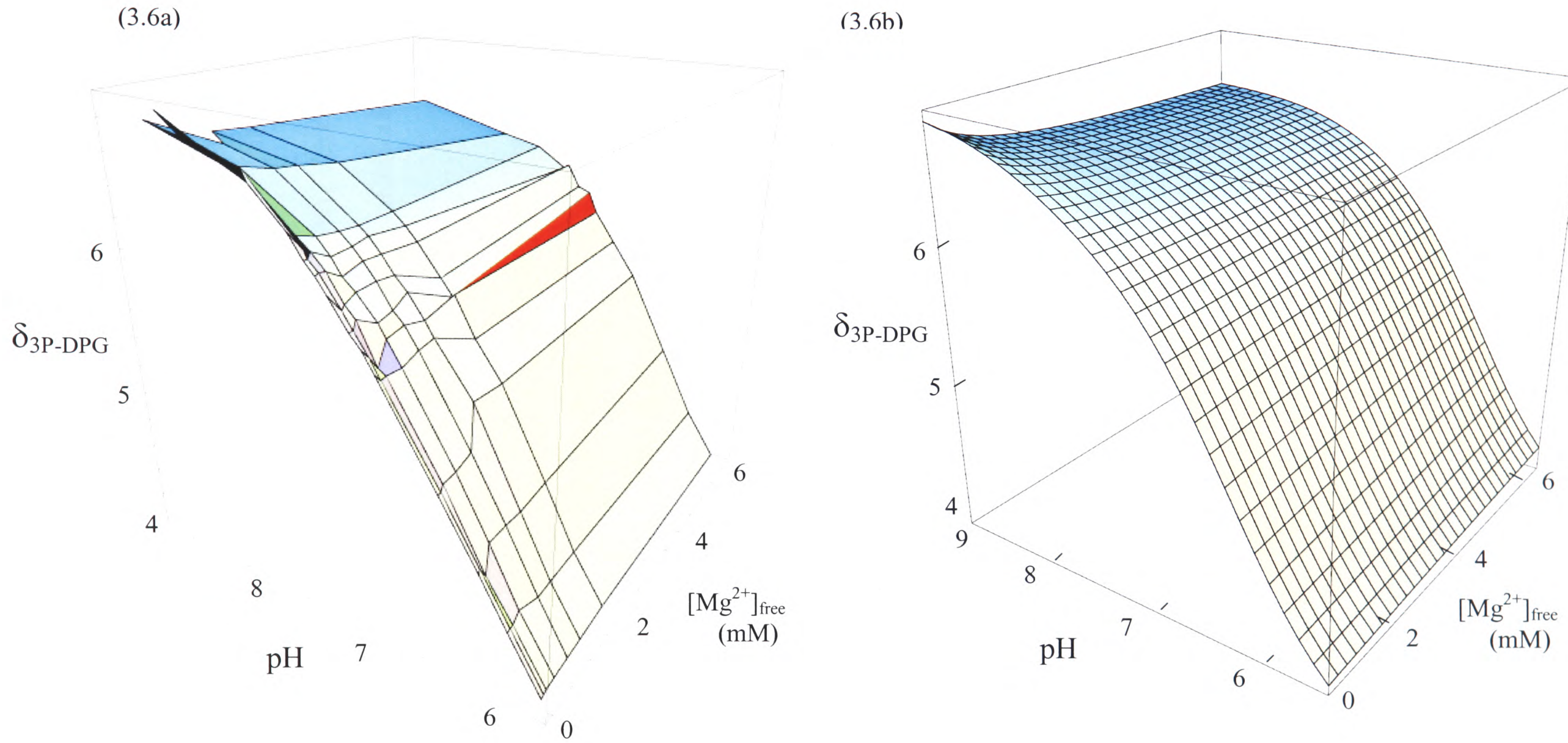


Fig. 3.6 Plot of the observed chemical shift (3.6a) and the curve produced by the fitting equation (3.6b) for 3P-DPG against pH and $[Mg^{2+}]_{free}$.

Table 3.3: Parameters used to produce the best fit of the theoretical chemical shift to that of the observed, including interactions with potassium. Chemical shifts are presented as ppm relative to phosphocreatine at 0 ppm. Data are means ± SEM.

Parameter	$\delta_{\beta\text{-ATP}}$	$\delta_{3P\text{-DPG}}$
K_{aX}	$2.00 \pm 0.06 \times 10^{-7}$ M	$7.80 \pm 0.07 \times 10^{-8}$ M
K_{aHX}	1.91×10^{-4} M *	$4.46 \pm 0.05 \times 10^{-7}$ M
K_{bMgX}	$7.50 \pm 0.2 \times 10^{4}$ M^{-1}	$2.40 \pm 0.06 \times 10^{3}$ M^{-1}
K_{bMgHX}	$5.50 \pm 0.64 \times 10^{3}$ M^{-1}	$3.78 \pm 0.01 \times 10^{2}$ M^{-1}
K_{bKX}	$1.20 \pm 0.25 \times 10$ M^{-1}	$1.30 \pm 0.02 \times 10$ M^{-1}
K_{bKHX}	4.00 ± 3.18 M^{-1}	$1.08 \pm 0.02 \times 10$ M^{-1}
δ_X	19.10 ± 0.15	6.90 ± 0.02
δ_{HX}	20.60 ± 0.13	5.69 ± 0.02
δ_{H2X}	21.50 *	3.13 ± 0.02
δ_{MgX}	15.69 ± 0.02	6.35 ± 0.01
δ_{MgHX}	17.40 ± 0.11	3.79 ± 0.05
δ_{KX}	18.36 ± 0.07	6.74 ± 0.01
δ_{KHX}	19.60 ± 0.02	4.74 ± 0.01

* Errors not calculable for region studied

3.3 ADDRESSING REPORTED PROBLEMS OF USING ^{31}P NMR TO MEASURE $[Mg^{2+}]_{FREE}$

As mentioned above, there are a number of potential errors associated with using the observed NMR signal from ATP to determine $[Mg^{2+}]_{free}$. However, this new approach can avoid many of these issues.

3.3.1 Explicit vs apparent equilibrium constants

An important feature of this work was the use of explicit equilibrium constants over apparent constants, as used previously (Gupta et al., 1978a; Ouwerkerk et al., 1989; Resnick, 1992; Resnick, 1995). This allowed the ready adaptation of the technique to a wide range of conditions, either in red blood cells or other tissues. However, this, of course, placed pressure on the need for a greater number of accurate constants for the approach to be reliable. The original method of Gupta et al (Gupta et al., 1978a) attempted to overcome the need for accurately knowing a wide range of constants by measuring an apparent dissociation constant for MgATP, $^{app}K_{dMgATP}$, under conditions thought appropriate to the *in vivo* situation. However there are a number of problems associated with such an assumption, which are discussed below.

Ionic Strength

Firstly, as only a physiological concentration of potassium, K^+, was included by Gupta in the *in vitro* solutions, along with variable levels of ATP and Mg, $^{app}K_{dMgATP}$ would be different from that *in vivo* due to a different ionic strength caused by the presence of other charged species, such as DPG, *in vivo*. The *in vitro* solutions used by Gupta et al (Gupta et al., 1978a) contained a range of ionic strengths from 0.16 to 0.19 M. The addition of 7 mM DPG would increase the ionic strength to 0.24 M and, assuming $^{app}K_{dMgATP}$ was measured to be 38 μM at 0.17 M ionic strength, this would be ~ 60 μM *in vivo*. Because the calculated $[Mg^{2+}]_{free}$ was directly proportional to $^{app}K_{dMgATP}$ this would have increased $[Mg^{2+}]_{free}$ 1.5-fold.

Chemical shift endpoints

Secondly, an accurate assessment of $^{app}K_{dMgATP}$ using ^{31}P MRS requires an accurate knowledge of both chemical shift endpoints (δ_{ATP} and δ_{MgATP}). Although

δ_{ATP} may be relatively straightforward to determine, δ_{MgATP} is more difficult due to the presence of the presence of Mg_2ATP, whose chemical shift will affect the overall observed chemical shift (Mosher et al., 1992).

α-ATP is not constant

Thirdly, rather than absolute chemical shifts, the difference between $\delta_{\alpha\text{-ATP}}$ and $\delta_{\beta\text{-ATP}}$ was used to determine $^{app}K_{dMgATP}$, under the assumption that neither is particularly affected by pH near neutrality. This was based on ^{31}P NMR studies (Cohn and Hughes, 1962) that have shown that Mg^{2+} induced the greatest chemical shift on β-ATP and the least on α-ATP. This was interpreted as indicating that Mg^{2+} binds mainly to the β and γ phosphate groups (Pecoraro et al., 1984) possibly via a cyclic 6 membered transition state that is energetically favoured (Fig. 1.2), and has little interaction with the α residue. So much so, and especially at physiological pH, that subsequent determination of intracellular $[Mg^{2+}]_{free}$ has depended on the changing difference of the chemical shift of β-ATP from that of α-ATP, $\delta_{\alpha\beta}$, in many tissue types (Gupta et al., 1978a; Gupta and Moore, 1980; Gupta et al., 1984). Since the first ^{31}P NMR investigations of intracellular conditions in red cells, it has become common practice to use the α-ATP peak as an internal chemical shift standard. However it can be seen (Fig 3.4) that its chemical shift was not only variable over the range of pH and Mg studied but also the chemical shifts of α-ATP and β-ATP changed differently as pH and Mg were varied, especially at the magnesium levels used to estimate $^{app}K_{dMgATP}$ (Vasavada et al., 1984). This challenged the reliability of the use of $\delta_{\alpha\beta}$ to determine accurately pH and $[Mg^{2+}]_{free}$. Therefore, in this study, all chemical shifts were measured relative to an external capillary standard.

The new approach in this study ensured that the above problems were not encountered.

3.3.2 Metabolite associations with K^+

Although assuming an $^{app}K_{dMgATP}$ might, in theory, alleviate the need to know the exact effect that intracellular ionic content would have on binding interactions, it does place restrictions on how readily this method may be applied in a range of conditions. A new binding constant would have to be determined for each new intracellular condition, including changes in pH and K^+. A change in the potassium concentration would exert an effect not only on K_{bMgATP}, by changing the ionic strength and by competitively binding to ATP, but also an effect on the observed chemical shift by changing the amount of potassium-bound species (Golding and Golding, 1995) present in solution.

Assuming both KX and KHX forms exist for both ATP and DPG improved the fit of the model to the experimental data significantly, with χ^2 decreasing by 90% and 50% for β-ATP and 3P-DPG curves, respectively. The fact that this improved the model was justification alone for explicitly specifying the interactions with K^+. However, it also allowed the separation of ionic strength from $[K^+]$, which then gives the technique greater flexibility of application.

One sacrifice for the increased adaptability and accuracy of using the new equations was that, now that more than two competitively binding species (H^+, Mg^{2+} and now K^+) for two given ligands (ATP and DPG) had been introduced, absolute concentrations of both those ligands and one of the binding species must be known before the equations could be solved. Therefore, in order to calculate pH and

$[Mg^{2+}]_{free}$ in red blood cells, the amounts of total ATP, DPG and K^+ needed to be known, as well as the necessary binding constants for K^+.

As a verification of the results from theoretical curve fitting, the new value for the explicit $MgATP^{2-}$ binding constant of 7.50×10^4 M^{-1} was in excellent agreement to that calculated by Mulquiney et al (Mulquiney and Kuchel, 1998), which approximated to 8.0×10^4 M^{-1} following appropriate adjustments of pH and ionic strength.

3.3.3 Metabolite associations with haemoglobin

ATP and DPG, along with other phosphorylated metabolites in human erythrocytes, bind reversibly with Hb. DPG has been shown to bind to deoxyHb at a central cavity between the two β-chains on the two-fold symmetry axis of the Hb tetramer (Arnone, 1972). Four positively charged groups on each of the two β-chains (the adjacent α-amino group of His 2 and its imidazole side group, and the side groups of Lys 82 and His 143) create a pocket of eight positive charges of which the negatively charged DPG is stereochemically complementary. On oxygenation the central cavity narrows, becoming too small to accommodate DPG. The site of DPG binding to oxyHb is still uncertain, but two possibilities appear to exist; DPG may interact with positively charged residues at the entrance to the central cavity, or another site or sites may exist. There is evidence that the binding site is close to that of deoxyHb (Gupta et al., 1979; Russu et al., 1990). Given the similar pH dependence of the binding of DPG and ATP, as well as other anions (Garby et al., 1969; Rapoport et al., 1972) plus the fact that DPG decreases the binding of other metabolites and vice versa (Garby et al., 1969; Berger et al., 1973; Hamasaki and Rose, 1974), it has

been assumed that ATP and other metabolites have the same binding site on both oxy- and deoxyHb as does DPG (Mulquiney and Kuchel, 1997b).

These interactions are dependent on pH (Garby et al., 1969; Rapoport et al., 1972; Mulquiney and Kuchel, 1997b) and oxygenation state of Hb (Bunn and Forget, 1986; Gerber et al., 1973) (Hamasaki and Rose, 1974; Gupta et al., 1978b) and significantly affect the free and bound concentrations of metabolites within the cells. Clearly these need to be included in a complete model of red blood cell equilibria. The pH dependence will partly be allowed for by explicitly specifying [ATP]/[HATP] and [DPG]/[HDPG]. As deoxyHb is paramagnetic, with the central haem Fe^{2+} ion in a high spin electronic configuration, its presence would complicate the NMR analysis of erythrocytes. Therefore, it was decided to perform experiments on fully oxygenated blood, where the haem Fe^{2+} would be low spin and diamagnetic. This also discounts any concern over the oxygenation state, as there would be if mixed venous blood were to be used (see Section 3.4). Therefore, only metabolite interactions with oxyHb needed to be considered.

At first, Gupta et al (Gupta et al., 1978a) did take into account the effect that metabolite-haemoglobin interactions would have on the equilibria. However, by 1983 (Gupta et al., 1983b), this complexity in the method was lost, perhaps because, according to their equilibrium constants, Hb bound MgATP and ATP equally strongly and this would allow the Hb effect to be ignored (Mulquiney and Kuchel, 1997a). Perhaps also this added complexity, coupled with the lack of computational power at the time, made solving the necessary multiple equilibria too difficult to be achieved routinely. A disregard of the effect of Hb has existed ever since (Resnick et al., 1984; Bock et al., 1985; Bock et al., 1987; Resnick et al., 1987; Woods et al., 1988; Jelicks et al., 1989; Ouwerkerk et al., 1989; Resnick et al., 1990; Resnick et al., 1993;

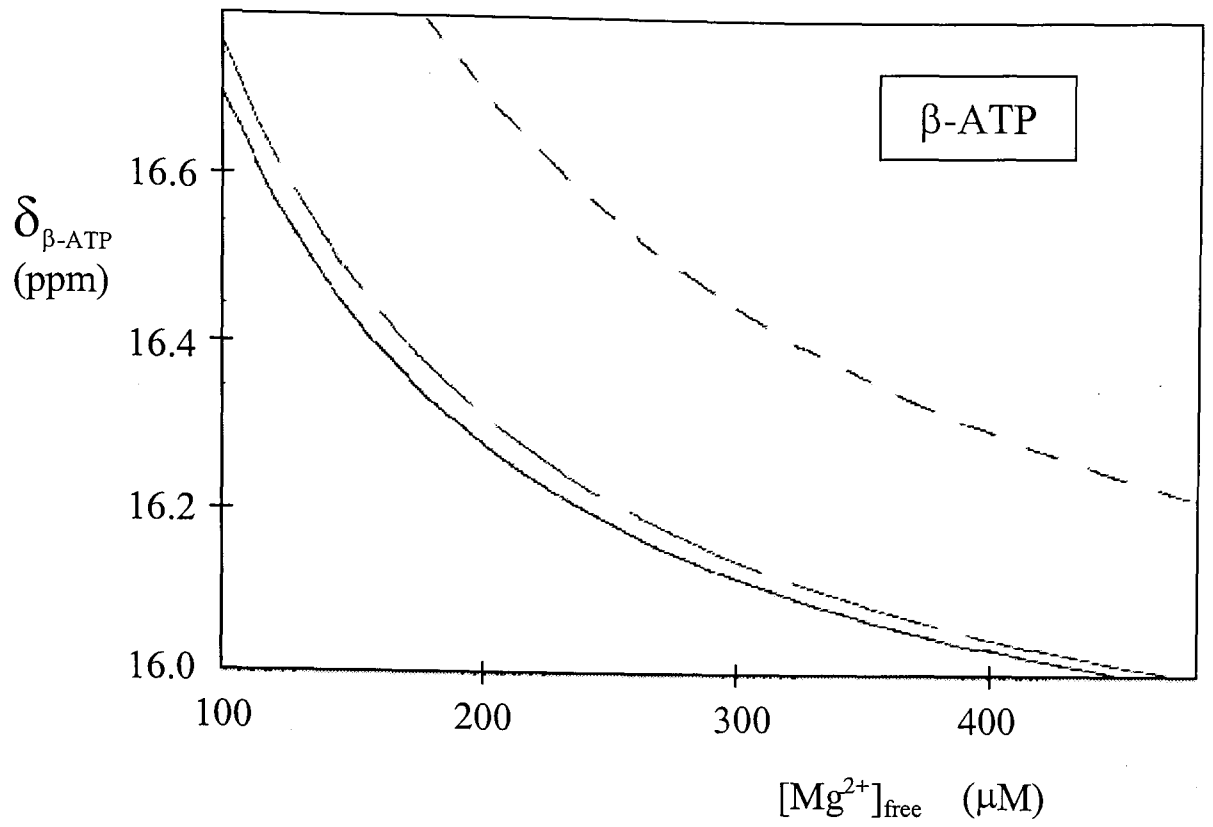
Barbagallo et al., 1997), until 1997, when the effect of metabolite-Hb interactions was reinvestigated (Mulquiney and Kuchel, 1997a; Mulquiney and Kuchel, 1997b).

The standard titration solutions did not include haemoglobin because the extent of DPG and ATP binding to haemoglobin is uncertain and such effects would complicate the analysis of the interactions between H^+, Mg^{2+}, K^+, ATP^{4-} and DPG^{5-}. However, after the observed experimental chemical shifts were modelled exactly by equations describing the effect of all intermediates as functions of H^+ and Mg^{2+}, these equations could be altered to allow for Hb binding. Although each equilibrium is independent of every other, and therefore the addition of Hb cannot, *per se*, alter the Mg binding ATP equilibrium, the observed chemical shift was not independent as it intrinsically reflects a weighted average of *all* species present. Therefore the effect of Hb binding on the observed chemical shifts of both DPG and ATP must be taken into account. This was achieved, to a first approximation, by assuming that the chemical shifts of ATP^{4-}, $MgATP^{2-}$ and DPG^{5-} did not change when each species bound to Hb. This was reported as true within 5% error in oxygenated erythrocytes (Gupta et al., 1978b; Mulquiney and Kuchel, 1997b). Thus, Equation 3.1 for ATP became:

$$\delta_{obs} = \frac{[MgATP] + [HbMgATP]}{[ATP]_T} \times \delta_{MgATP} + \frac{[ATP]_{free} + [HbATP]}{[ATP]_T} \times \delta_{ATP} \qquad (3.6)$$

These equations also required the binding constants for Hb binding to DPG, ATP and MgATP. There were only two known sets of constants (Berger et al., 1973; Gupta et al., 1978b), which largely agreed except for the value of MgATP binding to Hb (Table 3.4). This alters the positions of the equilibria to different degrees and therefore the fitted curve by a different amount when the effect of Hb binding was combined in the fitting equations. The different effects that the two sets of constants had on the analysis for pH_i and $[Mg^{2+}]_{free}$ are shown in Fig 3.7 (a & b) for a total ATP of 2 mM, total DPG of 5 mM and total Hb of 7 mM.

(a) At pH = 7.2



(b) At $[Mg^{2+}]_{free}$ = 0.3 mM

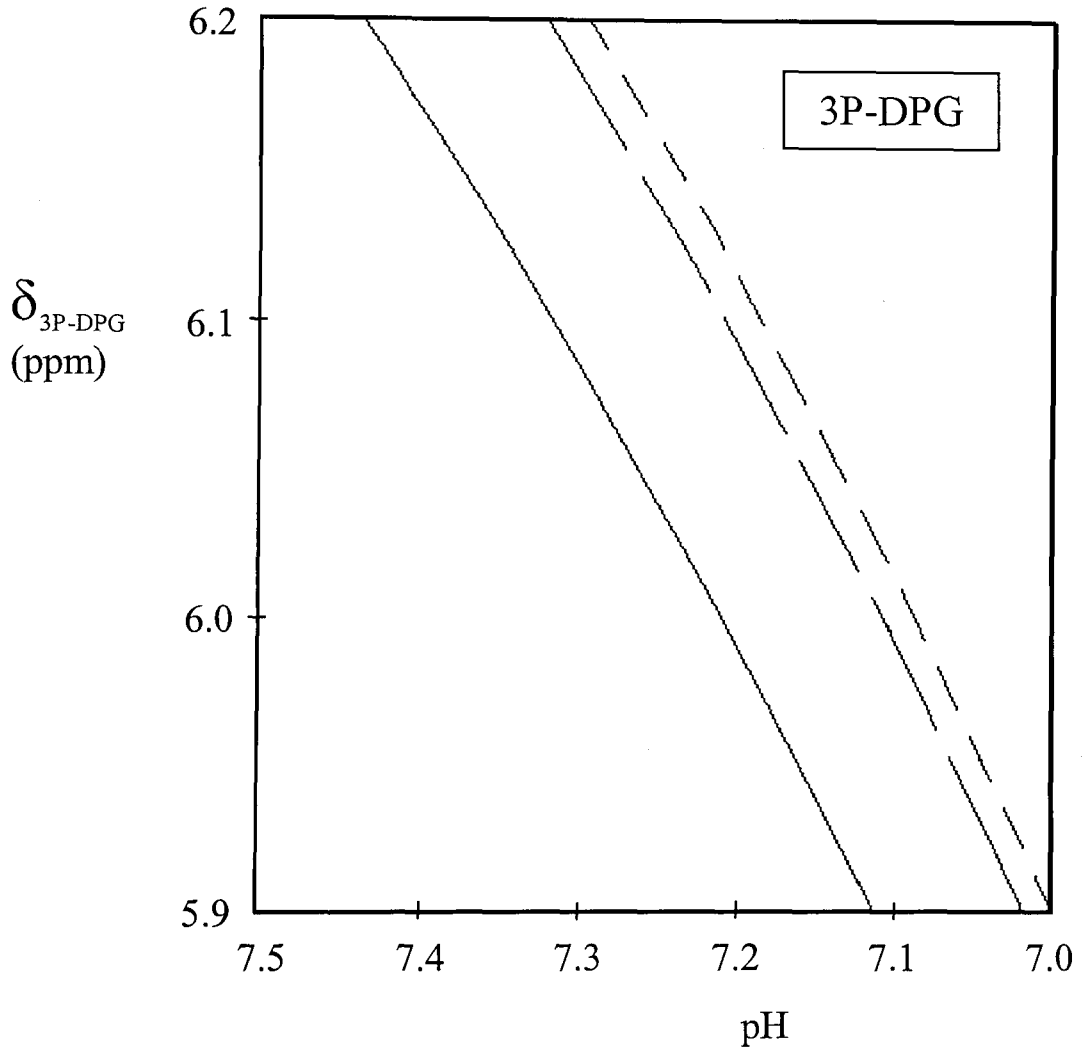


Fig. 3.7a & b Effects of allowing Hb binding to ATP, MgATP and DPG on the theoretical chemical shift, (a) β-ATP and (b) 3P-DPG, with no Hb (solid line), with 2 mM ATP, 5 mM DPG and 7 mM Hb and using equilibrium constants from either Gupta et al. (long dash) (Gupta et al., 1978b), or Berger et al. (short dash) (Berger et al., 1973).

Table 3.4 Association constants for Mg^{2+} and oxygenated haemoglobin complexes of ATP and DPG at pH 7.2, 37 °C and I ~0.15 M from two different workers.

Association Constant	Gupta et al. (1978)	Berger et al. (1973)
$K_{oxyHbATP}$ (M^{-1})	2.92×10^2	3.6×10^2
$K_{oxyHbMgATP}$ (M^{-1})	1.9×10^2	3.9×10
$K_{oxyHbDPG}$ (M^{-1})	2.11×10^2	2.5×10^2

3.3.4 Metabolite associations with K^+ and haemoglobin

For a complete description of the erythrocyte, the effects of both K^+ and Hb should be taken into account. However, once K^+ was specified in the Mg–ATP equilibrium, it also needed to be specified in the ATP–Hb equilibrium. Both Berger et al. (Berger et al., 1973) and Gupta et al. (Gupta et al., 1978b) measured apparent Hb binding constants using a physiological $[K^+]$ and therefore there were no data describing binding between KATP/KHATP and Hb. However, by estimating realistic binding constants for these intermediates (and for HATP), whilst ensuring that the overall binding of 'ATP' (ATP_{free}, HATP, KATP, KHATP) to Hb remained equal to that measured by Berger et al., a possible adjustment to the theoretical equations could be made (Equations 3.7 & 3.8) that included the effect that Hb binding had on the equilibria for all species of ATP and DPG and hence on the overall observed chemical shifts (Appendix II). Including the effects of both K^+ and Hb provided the most complete model of erythrocyte conditions.

Having formulated these equations, they were solved using the Mathematica algorithm presented in Appendix II. This algorithm calculated an initial estimate of chemical shift from starting values of pH and $[Mg^{2+}]$free. After comparison with the

observed chemical shift, small changes were made to the trial values pH and $[Mg^{2+}]_{free}$ to improve the estimate. This iteration continued until the changes to pH and $[Mg^{2+}]_{free}$ were less than 0.0001 pH units and 0.0001 mM, respectively.

Once solved, the concentrations of all intermediates in the erythrocyte were known and could be used to predict the total Mg in the cell, Mg_T, calculated by summing the concentrations of all Mg-containing species. This was used in Section 4 to validate the technique.

3.4 MEASURING $[Mg^{2+}]_{FREE}$ AND pH IN NORMAL ERYTHROCYTES

3.4.1 Comparison between different methods of analysis

The observed chemical shifts of 3P-DPG and β-ATP in normal erythrocytes (n = 33) were analysed using the newly developed technique to calculate pH_i, $[Mg^{2+}]_{free}$ and Mg_T. These results were compared with the results from analyses using several other methods and are presented in Table 3.5.

The difference in Hb binding constants lead to discrepancies in the calculated values of pH_i, $[Mg^{2+}]_{free}$ and Mg_T. It can be seen that pH_i, $[Mg^{2+}]_{free}$ and Mg_T were determined to be 7.20, 0.407 mM and 3.30 mM, respectively, using constants from Berger et al. (Berger et al., 1973), but 7.24, 0.220 mM and 2.55 mM using those from Gupta et al. (Gupta et al., 1978b). It was decided to use constants from Berger et al. because errors have recently been noted with the methodology used by Gupta et al. (Mulquiney and Kuchel, 1997b), the Mg_T calculated by the model is closer to the reported erythrocyte total found experimentally and finally because pH_i is closer to that expected under physiological conditions (Tehrani et al., 1982).

Table 3.5 Analysis of spectral data from normal human erythrocytes with a comparison of results using several methods listed below.

Analysis Method	pH_i	$[Mg^{2+}]_{free}$ (mM)	Mg_T (mM)
1	7.2	0.242 ± 0.015	-
2	7.37 ± 0.02	0.181 ± 0.025	-
3	7.20 ± 0.02	0.260 ± 0.021	2.50 ± 0.10
4	7.24 ± 0.02	0.220 ± 0.020	2.55 ± 0.11
5	**7.20 ± 0.02**	**0.407 ± 0.030**	**3.09 ± 0.30**

Method 1: The most commonly used method, in which δ_{ATP} and δ_{MgATP} endpoints are calculated from *in vitro* solutions, along with an apparent MgATP dissociation constant, $^{app}K_{dMgATP}$. It was then assumed that pH = 7.2 and:
$[Mg^{2+}]_{free} = {}^{app}K_{dMgATP} \times (\delta_{ATP} - \delta_{obs})/(\delta_{obs} - \delta_{MgATP})$ (Gupta et al., 1983b)

Method 2: Analysis using the theoretical equations presented here assuming no interaction with Hb or K^+.

Method 3: Analysis after including interactions between DPG and ATP with K^+ using parameters listed in Table 3.3 and assuming no interaction with Hb.

Method 4: Analysis using the Mathematica program presented in Appendix II, which included all possible interactions between H^+, K^+, Mg^{2+}, Hb, ATP^{4-} and DPG^{5-} using constants from Gupta et al. (Gupta et al., 1978b).

Method 5: Analysis using the Mathematica program presented in Appendix II, which included all possible interactions between H^+, K^+, Mg^{2+}, Hb, ATP^{4-} and DPG^{5-} using constants from Berger et al. (Berger et al., 1973). This analysis was used in all further experiments in this study.

3.4.2 The external medium

As explained above, it was necessary to ensure that, before NMR analysis, the Hb in the erythrocytes was fully oxygenated. However, of what the external medium should consist was initially unclear. The majority of previous work removed the plasma from the whole blood samples then washed and resuspended the erythrocytes in suitable isotonic pH-buffered medium. This buffer was often phosphate based (using MOPS), but some workers found this had adverse effects on $[Mg^{2+}]_{free}$ and

instead used a HEPES-based buffer (Bookchin et al., 1984; Lew et al., 1991; Raftos et al., 1999). Cells required in the fully oxygenated state have then been either aerated with CO, 100% O_2, air or occasionally carbogen (95% O_2:5% CO_2).

To determine the best approach, pilot experiments were performed analysing erythrocytes under a range of conditions suspended in isotonic MOPS-buffered saline, MBS, and their own plasma. The results of these studies are presented in Table 3.6 with all analyses using oxyHb association constants as determined by Berger et al. (Berger et al., 1973) unless otherwise indicated (i.e. using the analysis method 5 from Table 3.5).

The results showed a considerable variation in both pH_i and $[Mg^{2+}]_{free}$ according to the different sample preparations. In particular, using the common technique of washing and suspending in MBS, after aerating with CO, the pH_i was more acidic than the expected physiological value of 7.2. When the cells were left suspended in their own plasma, in general the pH_i was also acidic relative to what might be expected. The only exception was when the venous sample was analysed directly, with no aeration, and using oxyHb association constants, which gave a pH of 7.33. However, when deoxyHb constants were used to analyse the same sample data, the calculated pH_i value was very acidic, at 6.9. But as venous blood represents a mixture of oxygenation states, the true pH_i would lie between these two extremes.

As plasma is a bicarbonate-buffered system, one would expect aeration with carbogen to give physiological pH (5% of atmospheric pressure = 38 mmHg, equivalent to that of arterial blood where plasma pH is normally ~7.4). However, this was not the case, as full equilibration with 5% CO_2 lead to a very acidic pH_i. When the plasma was analysed under these conditions, the pCO_2 was in excess of 75 mmHg, with a corresponding pH_{ex} of around 7.1. This could not be confirmed by comparison

with published results, as no correlation between % CO_2 in the gas used for aeration and external pH has been reported. Most workers used 5% CO_2 apparently with the assumption that this produced physiological conditions.

Table 3.6 pH_i, $[Mg^{2+}]_{free}$ and Mg_T in different preparations of erythrocytes.

Medium	Aeration Gas	pH_i	$[Mg^{2+}]_{free}$ (mM)	Mg_T (mM)
MBS (n=16)	CO	7.06 ± 0.04	0.52 ± 0.05	3.30
Plasma (n=3)	none	7.33 ± 0.05 6.90$^+$	0.66 ± 0.04 1.00$^+$	4.00 3.60$^+$
Plasma (n=5)	CO	7.09 ± 0.04	0.51 ± 0.05	3.30
Plasma (n=4)	Air	7.15 ± 0.03	0.47 ± 0.06	3.25
Plasma (n=5)	O_2/CO_2	6.93 ± 0.04	0.51 ± 0.05	3.22

$^+$ indicates that the analysis used the deoxyHb association constants of Berger et al. (Berger et al., 1973)

Consequently, it was decided to analyse blood samples in as close to *in vivo* conditions as possible and, in particular, to simulate arterial blood. Therefore erythrocytes were left suspended in their own plasma, which was equilibrated to provide a pCO_2 of 40 mmHg and a pO_2 >150 mmHg. This had the advantage of ensuring the haemoglobin was diamagnetic, but also useful as only arterial blood can affect the chemoreceptors in the brain or carotid arteries to alter breathing, and thus have a bearing on the pathology of a clinical state.

3.5 SUMMARY – MODEL OF THE RED BLOOD CELL

The chemical shifts of the intermediate species were calculated from the above fitting equations and were used in new multiple equilibria equations, which included the binding of oxygenated haemoglobin to ATP^{4-}, $MgATP^{2-}$ and DPG^{5-}. To achieve this, it was assumed that the chemical shifts of each species were not affected by

binding to Hb (Gupta et al.,1978a; Rink et al., 1982). Binding constants for oxyHb were taken from Berger et al. (Berger et al., 1973) (Table 3.3). Therefore:

$$\delta_{\beta\text{-ATP}} = \left(\begin{array}{l} \left(\left(\frac{[\text{MgATP}]+[\text{HbMgATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{MgATP}} \right) + \left(\left(\frac{[\text{ATP}_{\text{free}}]+[\text{HbATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{ATP}} \right) \\ + \left(\left(\frac{[\text{HATP}]+[\text{HbHATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{HATP}} \right) + \left(\left(\frac{[\text{MgHATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{MgHATP}} \right) \\ + \left(\left(\frac{[\text{KATP}]+[\text{HbKATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{KATP}} \right) \\ + \left(\left(\frac{[\text{KHATP}]+[\text{HbKHATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{KHATP}} \right) + \left(\left(\frac{[\text{H}_2\text{ATP}]}{[\text{ATP}_{\text{Tot}}]} \right) \text{x } \delta_{\text{H2ATP}} \right) \end{array} \right) \tag{3.7}$$

and:

$$\delta_{\text{3PDPG}} = \left(\begin{array}{l} \left(\left(\frac{[\text{MgDPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{MgDPG}} \right) + \left(\left(\frac{[\text{DPG}_{\text{free}}]+[\text{HbDPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{DPG}} \right) \\ + \left(\left(\frac{[\text{HDPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{HDPG}} \right) + \left(\left(\frac{[\text{MgHDPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{MgHDPG}} \right) + \left(\left(\frac{[\text{KDPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{KDPG}} \right) \\ + \left(\left(\frac{[\text{KHDPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{KHDPG}} \right) + \left(\left(\frac{[\text{H}_2\text{DPG}]}{[\text{DPG}_{\text{Tot}}]} \right) \text{x } \delta_{\text{H2DPG}} \right) \end{array} \right) \tag{3.8}$$

(The above equations are simplified from the Mathematica program presented in Appendix II)

After determination of chemical shifts of β-ATP (δ_β) and 3P-DPG (δ_{3P}) from acquired spectra of normal red blood cells, the equations were solved simultaneously to calculate pH and $[Mg^{2+}]_{free}$ in red blood cells using the Solve algorithm in Mathematica (Appendix II). This method required the total amounts of ATP, DPG, Hb and K^+ to be entered in the algorithm before a solution could be found.

Erythrocyte total ATP and DPG concentrations were assayed as described above, and Hb and K^+ concentrations were assumed to be 7 mM (Gupta et al., 1978b; Mulquiney and Kuchel, 1997b) and 160 mM (Werner and Heinrich, 1985), respectively. It was important that a relatively large concentration of K^+ be included for its effects on the observed chemical shift to be accounted for, but its precise value was not important (± 5 mM), as its association constants were so low relative to that for $[Mg^{2+}]_{free}$ that small changes did not significantly affect the analysis of erythrocytes.

Total Mg in the erythrocyte predicted by the model was calculated by summing the concentrations of all Mg-containing species. Total Mg was also assayed for comparison (refer to Section 4 for further details). The complete results from the analysis of normal erythrocytes are presented in Table 3.7.

Table 3.7 Analysis of normal erythrocytes at 37°C suspended in plasma (n = 33). Intracellular total Hb and K^+_f were assumed to be 7.0 and 160 mM, respectively.

	Parameter	Value
NMR Chemical Shifts (ppm) relative to PCr at 0.00	$\delta_{3P\text{-}DPG}$	6.17 ± 0.02
	$\delta_{2P\text{-}DPG}$	5.28 ± 0.02
	$\delta_{\gamma\text{-}ATP}$	2.51 ± 0.02
	$\delta_{\alpha\text{-}ATP}$	7.59 ± 0.01
	$\delta_{\beta\text{-}ATP}$	16.306 ± 0.037
External Medium	pCO_2	39.8 ± 1.5 mmHg
	pO_2	> 150 mmHg
	pH_{ex}	7.39 ± 0.03
	Haematocrit	45 ± 3 %
Assayed values (mM/cell water)	ATP	1.85 ± 0.27
	DPG	7.70 ± 0.47
	Plasma Mg_T	0.94 ± 0.12
	Erythrocyte Mg_T	3.30 ± 0.39
Calculated values (mM/cell water)	Mg_T	3.09 ± 0.30
	pH_i	7.20 ± 0.02
	$[Mg^{2+}]_{free}$	0.41 ± 0.03
	ATP_f	0.045
	HATP	0.014
	H_2ATP	4.7×10^{-6}
	MgATP	1.367
	MgHATP	0.032
	DPG_f	0.983
	HDPG	0.795
	H_2DPG	0.112
	MgDPG	0.960
	MgHDPG	0.122
	K_T	163.5
	KATP	0.086
	KHATP	0.009
	KDPG	2.029
	KHDPG	1.379
	Hb_f	5.367
	HbATP	0.043
	HbKATP	0.032
	HbHATP	0.016
	HbKHATP	0.002
	HbMgATP	0.220
	HbDPG	1.319
	$Hb(ATP)_T$	0.094
Other	Ionic strength	0.23
	$r = H_{ex}/H_i$	0.65 ± 0.03

Standard deviations are given where the parameter was routinely recorded.

3.5.1 Important features of this work

Ionic strength

Ionic strength plays a very important part in determining the positions of equilibria and it is therefore important that association constants in models of erythrocytes are appropriate to the intracellular conditions. Although this is difficult to analyse precisely in erythrocytes due to the high protein concentration, the value is higher than normally assumed in the literature (0.15 M). The use of 0.25 M for the standard titration solutions was justified here as the new model predicted a value of 0.23 (assuming that Hb_f had a charge of 5+, referring to the central binding cavity, which can be considered important for the interactions with the metabolites). The actual value may be a little higher than this when the charges on intracellular enzymes and proteins are included. This is the first model that allows for a physiologically accurate high intracellular ionic strength.

Use of external chemical shift

Although there was only a small variation in the chemical shift of α-ATP in normal erythrocytes, the fact that it did change over the entire range of pH and $[Mg^{2+}]_{free}$ in the titration solutions meant that it was not suitable as an internal reference chemical shift and could not be used for the determination of K_{bMgATP}.

Use of explicit association constants

By using a large range of standard solutions it was possible to improve the determination of the many association constants and chemical shifts for all metabolite intermediates. One additional useful result from this work would be the use of the determination of association constants. Solutions are often required in experimental

practice to mimic the conditions found *in vivo*. These frequently require an accurate $[Mg^{2+}]_{free}$ in ATP-containing solutions, but all too often this is calculated using equilibrium constants that are inappropriate for the conditions used. This is partly due to constants being quoted as apparent ones, thus making it difficult to adjust them over a range of conditions. However, in this work, explicit constants have been determined which readily allow the generation of a specific $[Mg^{2+}]_{free}$ in an ATP containing solution over any range of pH, K, ATP, DPG, Hb, temperature and ionic strength. Furthermore, this technique could be used in other cell types if a replacement peak chemical shift were used instead of DPG, ideally inorganic phosphate.

Inclusion of all intermediates in analysis of observed δ_{3P} and δ_{β}

The interactions of H^+, K^+ and Hb with ATP and DPG and their effect on the analysis of the observed chemical shift have been included in the determination of pH and $[Mg^{2+}]_{free}$. This has improved the flexibility and widened application of this NMR technique as allowance can be made for variations in these parameters caused by different clinical conditions.

Use of 'arterial' whole blood

Very few methods aim to reproduce arterial blood and yet this was clinically the most important state to analyse as red blood cells were in a natural environment and devoid of adverse effects of washing and suspending cells in non-physiological buffers.

Effects of haemoglobin

This is the first NMR analysis to include the effects of haemoglobin since 1978. Although some reasonable estimates of specific association constants and

chemical shifts have been made, if these were to be experimentally determined in the future, they could be readily inserted into the equations.

3.5.2 Limitations of this work

Sensitivity of the NMR measurement

Firstly, determining the β-ATP chemical shift may not be that accurate due to the width of the peak caused by exchange broadening. Coupled to this, requiring the sensitivity of small changes in $[Mg^{2+}]_{free}$ to be reflected in observable changes in the β-ATP chemical shift means that $[Mg^{2+}]_{free}$ can only be measured with a degree of accuracy between 0.15 – 0.8 mM. But this may be the physiological limit, probably for the equivalent reason of sensitivity; i.e. small changes in metabolites need to have a precise effect on $[Mg^{2+}]_{free}$ levels. If $[Mg^{2+}]_{free}$ was very low (<0.1 mM), a very small change in the concentration of ATP would give a very large (relative) change in $[Mg^{2+}]_{free}$; conversely, if $[Mg^{2+}]_{free}$ was much larger (> 1mM) a large change in the concentration of ATP would only produce a relatively small change in $[Mg^{2+}]_{free}$.

Omission of some metabolite intermediates

Not every metabolite interaction has been included in the model, for example both MgKATP and MgKDPG may exist in solution but were not included in the above formulations. However the benefit of including these was outweighed by the fact that the appropriate association constants would have had to have been estimated, leading to unnecessary sources of error, given the very small influence that their presence would have had on the observed chemical shift. Also, at the extremes of the standard titration solutions both Mg_2ATP and $Mg(ATP)_2$ have been reported to form in significant quantities, though the latter seemed unlikely from charge considerations.

But neither was included in the fitting models as only negligible amounts would be present under physiological conditions.

Hb binding of metabolites

Hb–metabolite binding has not been well characterised, and this model specifies interactions with separate metabolite species, KATP, HATP, KHATP which were not known. But reasonable estimates have been used, verified by the fact that the overall K_{bHbATP} was the same. Also, changes with pH of Hb binding to individual metabolite species has not been taken into account. However the effect of such changes over pH 7.1 - 7.2 would be minor.

Assumption of no change in chemical shift on metabolite association to oxyHb

As has been mentioned, the assumption of there being no change in δ_{ATP}, δ_{MgATP} or δ_{DPG} when each metabolite binds to Hb was true within a 5% error. This was low enough for the effect of a change to be discounted here. In fact, it was difficult to calculate the change in each chemical shift from reported data as only the change in the observed chemical shift was given when Hb was added to a solution of the metabolite. The ensuing change in observed chemical shift could be due to changes in the positions of the multiple equilibria, affected by Hb preferentially binding some forms of the metabolite over others (and alterations in the ionic strength of the solutions). This can only be fully studied when the exact binding interactions of Hb have been determined. It is therefore better at present to assume that there was no change in the chemical shifts.

Model is only for the analysis of oxygenated red blood cells

The model could be extended to measure deoxygenated cells, but the paramagnetic of deoxyHb and its likely changes to metabolite chemical shifts on

binding would pose problems. It would be preferable to measure oxygenated cells to characterise the intracellular environment, including total Mg, then change the constants in the equations to reflect binding by deoxyHb, and thereby make a prediction about the deoxygenated state.

Estimation of K^+ interactions

The strengths of the interactions with K^+ have been estimated, achieved by modelling the observed data from the standard titration solutions, which did not contain a large variance in concentration of K^+. As a result, the accuracy of the estimation may be limited; mathematical fitting of the K^+ parameters would have been accurate if more points over a wider range had been analysed. However to have done so, and to have kept the ionic strength constant, the total concentrations of the other constituents would have had to have been altered. This would have introduced further complications to the analysis, and was probably not necessary, given that the results of the K^+ interactions were in good agreement with previously published data.

Other issues

1) The haemoglobin concentrations may now mean that the solutions within the red cell should be regarded as non-dilute (compared with the 'dilute' standard titration solutions). As such, the Debye-Huckel laws for association constants would no longer hold, and this analysis would no longer be valid. For the present model it was assumed that the values of association constants obtained at lower Hb concentrations remain valid for the concentrations of Hb found in the erythrocyte.

2) There have been other DPG binding sites reported on Hb (Lennon et al., 1994). No account was taken of this in this work.

3) Other Mg binding is possible (by Cl^- or HCO_3^-). However this would only affect total Mg prediction, as NMR can only pick up effects of $[Mg^{2+}]_{free}$ on ATP and DPG.

4) Other competitive Hb binding was possible. For example, CO_2 may compete with the binding of phosphocompounds to Hb due to the formation of carbamino-Hb. Again, this has not been included here.

"Although we need to be cautious in equating in vitro and in vivo solutions we must, nevertheless, make the leap to progress our understanding of cell processes. As a deeper understanding of the aqueous phase in cells emerges, refinements of such techniques as described in this paper will occur. In the meantime, comparisons with other techniques are a useful indicator if the various assumptions are realistic within our present understanding. For more precise determination, knowledge of the effect of changes of the ionic strength on the concentration equilibrium constants in the vitro experiments and a better understanding of the aqueous phase within the cell are required."

Golding and Golding, 1995

"If we do not develop models, we do not learn why they are false."

Heinrich and Schuster, 1998

CHAPTER 4

Verification of analysis

4 CHAPTER 4 – VERIFICATION OF ANALYSIS

The method described in Chapter 3 included the effects that haemoglobin, Hb, would have on ^{31}P NMR spectra on binding to metabolites in the red blood cell. It was important that this inclusion could be justified, and results from an investigation of this are presented first in this Chapter. Having developed a new approach to analysing pH and $[Mg^{2+}]_{free}$ in human erythrocytes, it was also important to verify that the technique could give accurate results over a range of conditions. This was achieved by comparing the model's prediction of total cellular magnesium with that measured by a more invasive technique, and also by invasively altering $[Mg^{2+}]_{free}$ in the cell, whilst confirming that the model could determine the changes. Finally, results are presented from an 18-month time course study of a single volunteer, to analyse the reproducibility of measurements.

4.1 EFFECT OF HAEMOGLOBIN ON METABOLITE NMR 'VISIBILITY'

4.1.1 Theory

If the metabolite relaxation times were sufficiently long when bound to Hb, the Hb bound component of the overall observed chemical shift would be broad enough to be lost in spectral noise. If this were the case, then Hb bound metabolites would not contribute to the observed NMR peak, termed 'MR invisible', and could be omitted from the analysis. The equations would then not need the inclusion of such metabolites, their chemical shifts or their association constants. Given the important effect of Hb binding on the analysis of the chemical shifts in determining pH and $[Mg^{2+}]_{free}$ (see Section 3.3.3), the effect of Hb on metabolite NMR 'visibility' was investigated.

Solutions of DPG and ATP were prepared as described in Section 2.7.2, with and without haemoglobin. NMR spectra of these solutions were obtained as described in Section 2.4.

4.1.2 Results and Discussion

The linewidths of DPG and ATP peaks each increased by 60% on addition of Hb (α-LW from 28 to 45 Hz, and β-LW from 50 to 80 Hz). Despite this, when the normalised peak integral was divided by the assayed total metabolite concentration, the values changed by less than 3% on addition of Hb. This indicated that ATP and DPG MR 'visibilities' were not significantly impaired by binding to Hb, in that the Hb bound component of the observed chemical shift was not so broad as to be lost in the noise of the spectrum.

This conclusion was verified by another method, that of estimating the concentration of ATP in normal red blood cells from the relative peak integrals of ATP and DPG in the observed spectra and using the assayed concentration of DPG. There was less than a 10% variation between this value and that of the spectrophotometrically measured ATP concentration. This confirmed that DPG and ATP remain NMR 'visible' when bound to Hb.

As there was no loss in metabolite 'visibility' on binding to Hb, the effect of oxygenated Hb binding had to be included in the determination of $[Mg^{2+}]_{free}$ from metabolite chemical shifts.

4.2 DETERMINATION OF ACCURACY OF THE MODEL

4.2.1 Total intracellular Mg

As discussed in the previous Section, having fully characterised the concentrations of each species of metabolite in the erythrocyte, all those containing Mg can be summed, and an estimate of total intracellular Mg, Mg_T, can be made and compared with a standard assay for Mg_T (described in Section 2.5). Results are presented from the analysis of all cells in this Thesis, for all investigations, as Mg_T varied considerably, from 1.5 to 4.5 mM (Table 4.1). Results are also shown for normal cells, giving the absolute concentrations measured. In Figure 4.1, a plot has been made of assayed Mg_T against the model Mg_T for all cells analysed, with the best-fit line and equation shown. Clearly, a gradient of 1 would indicate that the calculated value was identical to the measurement, and, although this was not quite achieved, there was excellent agreement.

Table 4.1 Comparison of Mg_T as calculated by the model and assayed by standard colorimetric analysis.

Parameter	Value
Average % difference in all cells analysed (n = 220) *	6 %
Calculated Mg_T for normal erythrocytes (n = 33)	3.09 ± 0.30
Assayed Mg_T for normal erythrocytes (n = 33)	3.30 ± 0.39

* It was not possible to obtain an assayed measurement of intracellular Mg_T for those experiments in which erythrocytes were incubated in high levels of extracellular Mg_T (Section 5). This is because the plasma concentration, which is subtracted from the whole blood Mg_T, was so much higher than the intracellular concentration that the error in the measurement became too great.)
These values were previously presented in Table 3.7.

The assayed Mg_T of normal erythrocytes was in exact agreement with the report in previous work of 3.3 mM (Millart et al., 1995). The model's calculation was close to that found experimentally, but was significantly lower ($p < 0.05$ using a

statistical paired t-test). Despite this, the calculated value is considerably greater than previous NMR calculations, which have predicted significantly lower concentrations of around 2.4 mM (Gupta et al., 1978; Ouwerkerk et al., 1989).

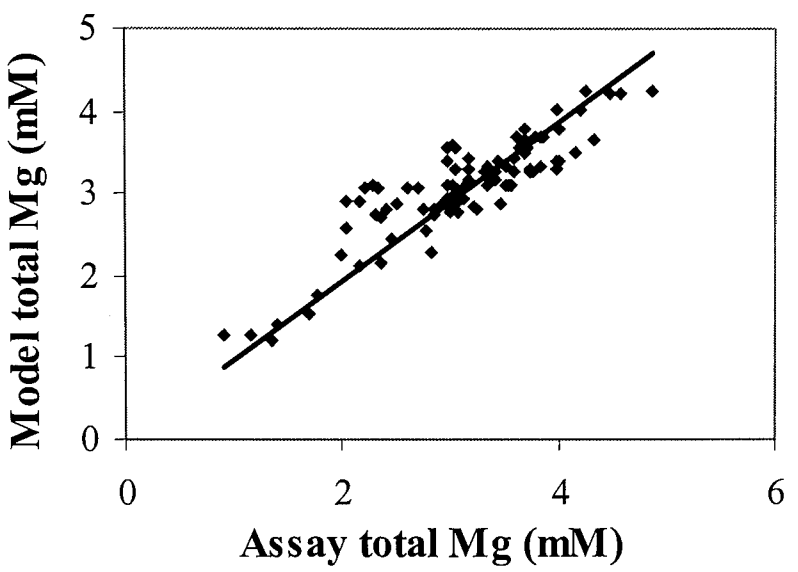


Fig 4.1 Plot of the calculated Mg_T from the model vs. that measured by standard colorimetric assay. Equation of best fit line: $y = 0.967x$, $R^2 = 0.70$.

The fact that the assayed value was identical to that reported was helpful proof of its success as a technique. However it could be seen that the assay's precision was not as great as that of the NMR technique, as shown by the greater standard deviation of the assay measurement. The slightly lower value predicted by the model compared to that of the assay could be explained by considering exactly how the model calculated Mg_T. A summation was made of all the concentrations of Mg-bound metabolites in the equations to give an overall 'total Mg' value. However this could not account for Mg that was bound to other compounds, in particular, intracellular proteins, the membrane and haemoglobin. Haemoglobin may be the most important 'other chelator' of Mg^{2+}, as some reports (Raftos et al., 1999) have suggested it can bind up to 10% of cellular Mg, though the precise extent is unknown. This binding is thought to occur at sites that do not compete with Hb's binding to metabolites, and as

such can be considered as separate to this model. Hence, such additional protein-Mg interactions could account for the 6% difference between assay and model. Rather than suggesting an error with the model, the smaller value it predicts for Mg_T relative to the assay may actually represent further verification.

4.2.2 Using the Ionophore A23187 to alter Mg_T

Theory

Ionophores are small, amphipathic molecules that dissolve in phospholipid bilayers and greatly increase their ionic permeability. The inner part of an ionophore is made of polar groups forming a tetra- or octahedral geometry that fits and encloses the desired ion. Around this polar cage are hydrophobic groups that allow the solubilisation of the charged ion-ionophore complex in apolar solvents or lipid membranes. The ionophore can thereby shield the electric charge as the ion passes through the membrane. Since ionophores are not coupled to energy sources, they only permit net movement of ions down their electrochemical gradients.

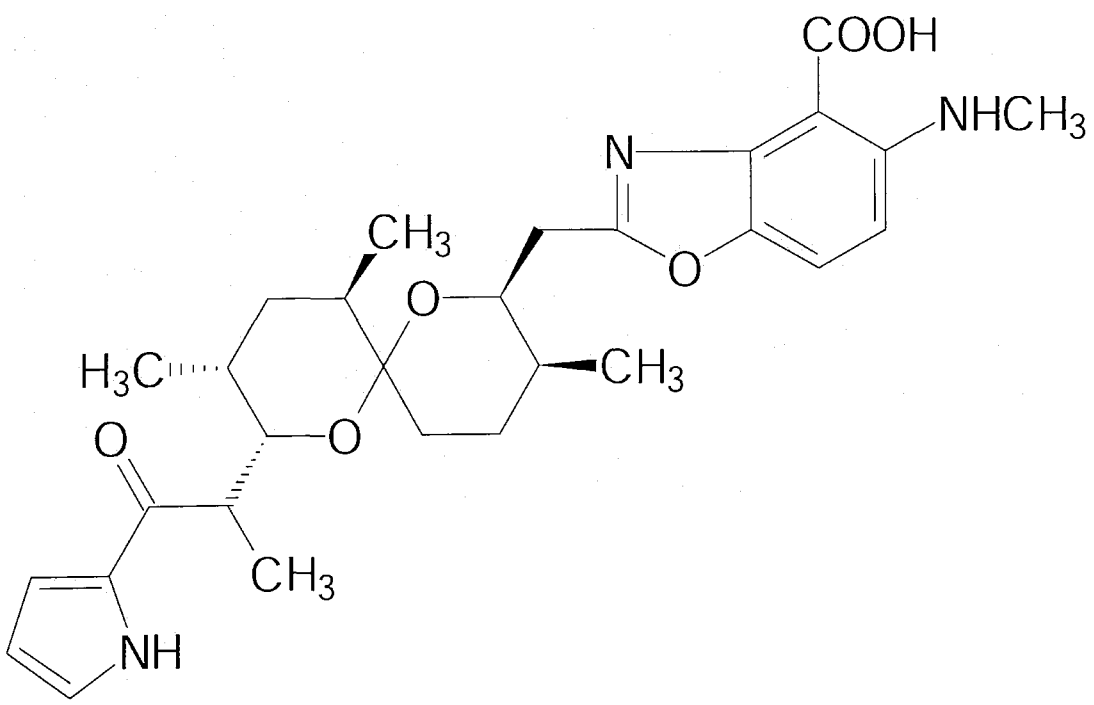


Fig 4.2 Chemical structure of the ionophore A23187.

Ionophores can be either mobile or channel-forming; A23187 is an example of a mobile ion carrier (Fig 4.2). It binds to divalent cations, such as Ca^{2+} or Mg^{2+}, on one side of the membrane, diffuses across the bilayer and releases the cation on the other side. It normally acts as an ion-exchange shuttle, carrying two H^+ across the cell membrane for every divalent cation transported in the opposite direction.

In 1980, Flatman (Flatman, 1980) developed a method for measuring intracellular ionised Mg^{2+}, $[Mg^{2+}]_i$, in intact erythrocytes using the ionophore A23187 (the notation $[Mg^{2+}]_i$ for Mg^{2+} is used to distinguish this value from the intracellular free Mg^{2+} as calculated by the new NMR model, $[Mg^{2+}]_{free}$). It was based on the principle that, in the presence of A23187, the total magnesium content of erythrocytes, Mg_T, could be altered by changing the concentration of ionised magnesium in the external medium, $[Mg^{2+}]_{ex}$. It was possible to find a level of $[Mg^{2+}]_{ex}$ at which the addition of A23187 caused no change in Mg_T. Then, knowing that at electrochemical equilibrium:

$$[Mg^{2+}]_{ex} = r^2 \times [Mg^{2+}]_i$$

where the concentrations are molal concentrations in cell water and medium, and by measuring r^2 from the chloride distribution ratio, $[Mg^{2+}]_i$ can be calculated. This zero-point titration technique has been used frequently and its relative efficacy discussed by many (see Section 1).

It was not intended to use this method to measure the absolute value of $[Mg^{2+}]_{free}$ in erythrocytes as this has been achieved by many other suitably skilled workers. Instead, the method was used to vary the amount of Mg_T, and therefore also $[Mg^{2+}]_{free}$, within normal erythrocytes to determine whether the new NMR technique could successfully identify the direction and magnitude of the Mg changes.

Manipulation of Mg_T

To manipulate Mg_T, the ionophore was added to cell preparations as described above and the external concentration of Mg^{2+} set at approximately 0.1, 0.2 and 0.3 mM, confirmed accurately by chemical assay (Section 2.5). After harvesting, the cells were analysed by NMR and the results are shown in Table 4.2. A plot of the intracellular Mg^{2+} as expected from equilibration of $[Mg^{2+}]_{ex}$ across the cell membrane, $[Mg^{2+}]_i$, against that measured by NMR, $[Mg^{2+}]_{free}$, is shown in Figure 4.3.

Table 4.2 Results from experiments in which Mg_T was altered in normal erythrocytes using the ionophore A23187. $[Mg^{2+}]_i$ indicates the expected intracellular free Mg^{2+} according to the Donnan equilibrium and $[Mg^{2+}]_{free}$ is that calculated by NMR.

	$[Mg^{2+}]_i$	$[Mg^{2+}]_{free}$	r	ATP	DPG	$Mg_{T,model}$	$Mg_{T,assay}$
Exp 1*	0.26	0.21	0.75	0.47	9.17	1.29	1.39
	0.60	0.57	0.72	0.80	8.59	2.25	2.54
	0.21	0.22	0.76	0.58	8.98	1.39	1.40
Exp 2	0.46	0.42	0.76	0.67	8.62	2.25	2.36
	0.58	0.55	0.76	0.79	8.93	2.81	2.82
	0.17	0.18	0.78	0.51	9.16	1.15	1.27
Exp 3	0.39	0.43	0.76	0.65	9.22	2.29	2.39
	0.55	0.67	0.76	0.75	9.33	3.29	3.33

All concentrations are expressed in mM.
*One of the data sets in experiment 1 was abandoned due to error. It was not repeated, as it was not considered vital to the overall conclusions

Discussion

As can be seen, the $[Mg^{2+}]_{free}$ as analysed by NMR was close to that expected from equilibration of Mg^{2+} across the membrane via A23187. In doing so, it could be concluded that the model could accurately follow changes in $[Mg^{2+}]_{free}$ over physiological extremes, from 0.17 to 0.60 mM. This conclusion was further justified

by the fact that again the model's prediction of Mg_T, $Mg_{T,model}$, in each case matched that as measured by chemical analyses, $Mg_{T,assay}$, despite a relatively large variance in absolute concentration.

The only assumptions that may be questioned here was that the ratio r was measured at the time of NMR analysis, and not during the time of incubation with the ionophore. Previous workers have measured this ratio by analysing the distribution of radioactive ^{36}Cl inside and outside the cell membrane (Flatman, 1980; Raftos et al., 1999), which may give a more accurate value. However, the ratio found here was very close to that expected physiologically, and hence could be considered a reasonable approximation. The small increase in r from 0.7 to an average of 0.76, could be attributed to the slightly more acidic extracellular pH, 7.28, at the time of NMR analysis, which has been shown to lead to an increase in r.

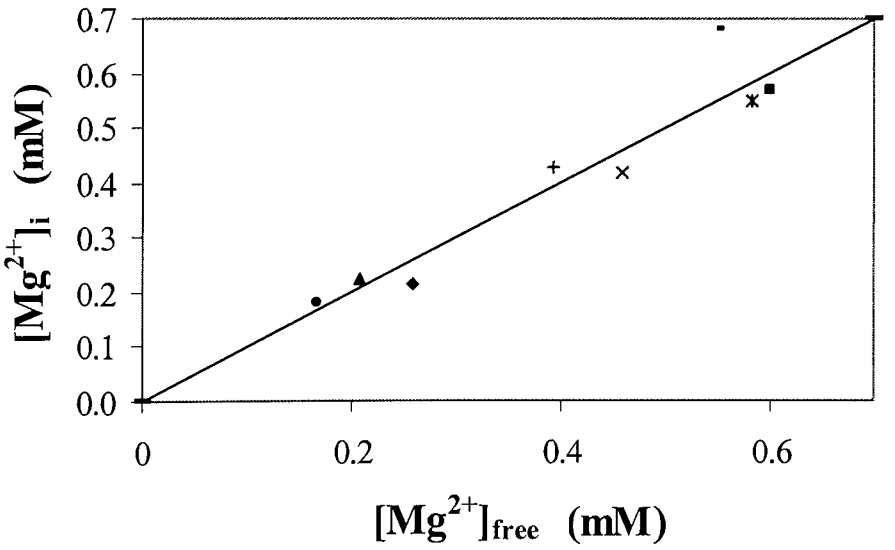


Fig. 4.3 Plot of expected intracellular free Mg^{2+} according to the Donnan equilibrium, $[Mg^{2+}]_i$, vs. the intracellular free Mg^{2+} as calculated by NMR, $[Mg^{2+}]_{free}$. Each point represents a different experiment from Table 4.2. The line representing the equation y=x is drawn.

A second assumption was that the cell water content remained constant in all samples at 70% of total cell volume. Again, previous workers have attempted to monitor changes in this variable, after making an assumption of mean red cell density

equal to 1.094 kg/L (Raftos et al., 1999). However, if there were significant changes in this, it is unlikely that measurements by the model of *both* $[Mg^{2+}]_{free}$ and Mg_T would match independent measurements by assay.

Changes in metabolite concentrations were observed following incubation with the ionophore. The increase in DPG would be expected from the incubation with inosine. However, the significant decrease in ATP was somewhat surprising. It may have been that, despite inclusion of EGTA, elevation of cell Ca^{2+} occurred leading to maximum activation of the Ca^{2+} pump and subsequent rapid decline in cell ATP. But the source of this Ca^{2+} was unknown, given its absence from the incubation medium. Hydrolysis of ATP may have occurred to maintain other ion balances across the membrane, and there was some evidence for this in the NMR spectra with the presence of small ADP peaks. However, these could not be quantified due to noise interference, and any inorganic phosphate produced was not visible, masked by the 2P-DPG peak.

The fact that the final intracellular ATP concentration seemed to depend on the $[Mg^{2+}]_{ex}$ may have suggested that, as Mg^{2+} equilibrated across the membrane with A23187, perhaps ATP did so as well. This might have been possible given the strength of the MgATP complex. The lower the concentration of $[Mg^{2+}]_{ex,}$ the more ATP and Mg^{2+} were lost from the cell (Figure 4.4). If this were true, the fact that ATP loss occurred when $[Mg^{2+}]_{ex}$ was greater than physiological $[Mg^{2+}]_{free}$ suggests that a dynamic equilibrium of Mg and ATP was being set up across the cell membrane using A23187, i.e. MgATP could also be moved across the membrane with A23187.

Support of this hypothesis was provided by evidence of ATP in the incubation medium. However, it was difficult to show that cellular ATP was in the medium. It

was possible that several processes were operating, including loss of ATP when the ionophore was washed out of the cell membrane. The reason for the loss was not important for this verification of the model, as the aim of varying $[Mg^{2+}]_{free}$ was successfully achieved. However, great care clearly needs to be taken with the use of the ionophore when attempting to measure *in vivo* $[Mg^{2+}]_{free}$ to ensure alterations to natural cellular environments do not occur.

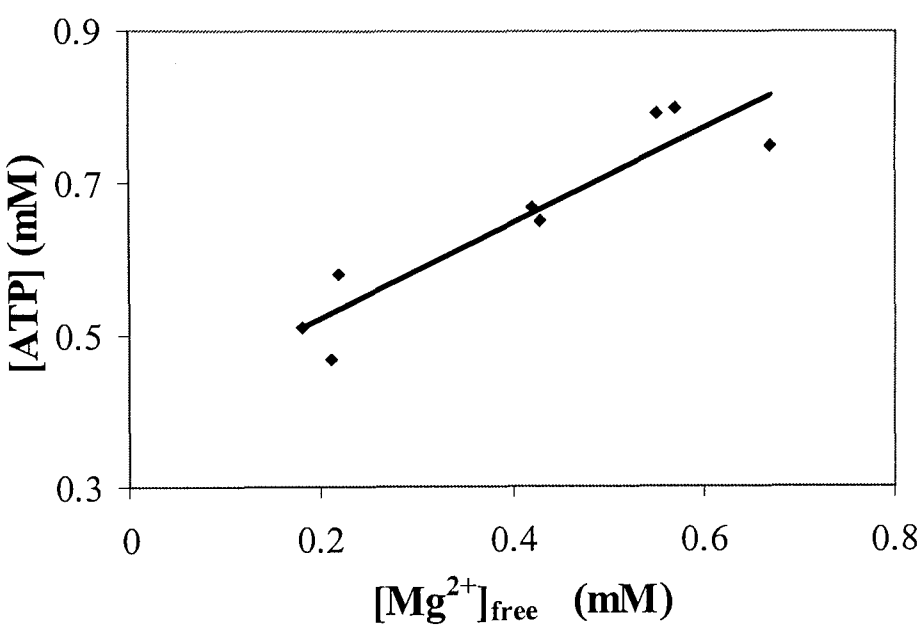


Fig. 4.4 Plot of intracellular ATP vs. $[Mg^{2+}]_{free}$ following incubation with A23187. The line of best fit was described by the equation $y = 0.63xMg + 0.4$, $R^2 = 0.9$.

4.3 MEASUREMENT REPRODUCIBILITY

Plots are shown in Figure 4.5 of all of the measured parameters for a single volunteer over an 18-month time period, with the values displayed in Table 4.3. A total of 17 samples were taken at regular monthly intervals, with the time of phlebotomy randomised between 09:00 and 15:00 hours.

4.3.1 Results and Discussion

It can be seen that there was good reproducibility of the measurements, with all parameters varying by less than 10%. The variation of all parameters was less than that previously presented for normal red blood cells from different subjects (Table 3.6) but particularly for the concentration of ATP, with 15% variation between subjects. As this value was especially important for the calculation of both $[Mg^{2+}]_{free}$ and Mg_T, it was not surprising to see the variation in $[Mg^{2+}]_{free}$ and Mg_T also decrease.

Table 4.3 Variation in parameters for a single subject over 18 months.

Parameter	Value	% Variation
DPG (mM)	7.61 ± 0.38	5%
ATP (mM)	1.79 ± 0.17	9%
$[Mg^{2+}]_{free}$ (mM)	0.41 ± 0.03	7%
Mg_T (mM)	3.03 ± 0.13	4%
pH_i	7.21 ± 0.01	<1%
pH_{ex}	7.39 ± 0.02	<1%
pCO_2 (mmHg)	39 ± 1	3%
Haematocrit (%)	47 ± 2	4%

This highlighted the fact that some of the deviations seen in normal cells could be attributed to natural variation in different subjects, and would necessarily infer insensitivity in the new technique. Also, there was no clear diurnal variance, at least over the range of hours that phlebotomy was performed.

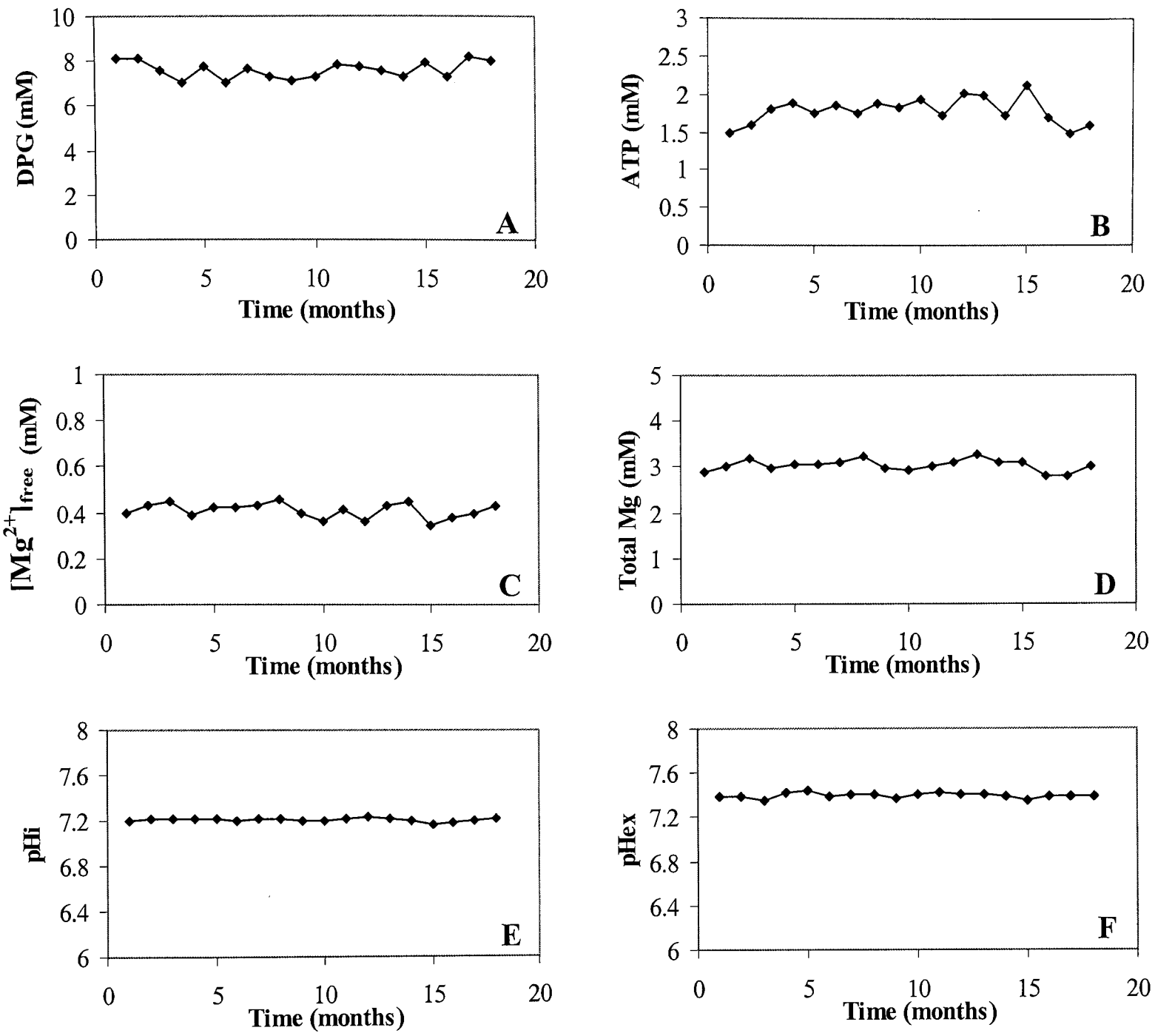


Fig. 4.5 Plots of all the measured parameters for a single volunteer over 18 months. **(A)** Intracellular DPG (DPG), **(B)** intracellular ATP (ATP), **(C)** intracellular free Mg^{2+} ($[Mg^{2+}]_{free}$), **(D)** Total intracellular Mg (Total Mg), **(E)** intracellular pH (pH_i), **(F)** extracellular pH (pH_{ex}).

CHAPTER 5

Effects of hypoxia on erythrocytes

CHAPTER 5 – EFFECTS OF HYPOXIA

The previous Chapters have presented the development of a new analysis of NMR spectra obtained from red blood cells, analysed as arterialised whole blood. It has been shown that this method was both qualitatively and quantitatively accurate. The following Chapters describe applications of this analysis to clinical situations to examine the role of H^+ and $[Mg^{2+}]_{free}$ in erythrocytes. In the present Chapter, erythrocytes were studied whose DPG concentration, one of the principal buffers of $[Mg^{2+}]_{free}$, had been altered *in vivo*.

5.1 INTRODUCTION

5.1.1 Respiration

The relation between the partial pressure of O_2 (pO_2) and the volume of O_2 carried in the blood can be defined as a sigmoidal dissociation curve, in which the blood reaches 90% O_2 saturation (equivalent to carrying ~15 ml O_2 per 100 ml blood) when the pO_2 is greater than 60 mmHg. Air at sea level typically contains a pO_2 of 150 mmHg, and so normal respiration can easily fully saturate the blood with oxygen. Saturation at 50% occurs when pO_2 is 26-28 mmHg, and so any reduction below 60 mmHg causes severe desaturation.

The shape of the O_2 dissociation curve is affected by a number of physiological variables, the most important of which are raised pCO_2, increased H^+, raised temperature and increased concentration of DPG, all of which shift the curve to the right and thereby facilitate the removal of O_2 in the tissues. In chronic hypoxia (anoxic and anaemic) the concentration of DPG is increased to right-shift the curve to counteract the effect of diminished O_2 content in the blood at 100% saturation.

Normal 'stores' of both O_2 and CO_2 contain 1 L and 17 L, respectively. Practically all O_2 is in blood, whereas most CO_2 is in the tissue fluids as bicarbonate. If ventilation is changed, either gas composition or volume, it takes about 2 minutes for the O_2 stores to adjust to a new level. However, the CO_2 stores will change over more than 15 minutes. But a greater length of time than either of these is required before the body metabolism adjusts to the change. Approximately 10.5 hours is required for the important concentration of DPG to reach the new steady-state level (Mulquiney and Kuchel, 1999).

5.1.2 2,3-diphosphoglycerate (D-2,3-dihydroxy-2,3-diphosphopropanoic acid)

In 1925, 2,3-DPG (DPG) was first reported to be in high concentrations in the erythrocytes of humans, dogs and pigs (Greenwald, 1925) (for chemical structure, see Figure 5.1). This was later confirmed to be the case in most mammalian erythrocytes (Rapoport and Guest, 1941) and by around 1950, the pathway of DPG synthesis had been determined by Rapoport and Luebering (Rapoport and Luebering, 1950; Rapoport and Luebering, 1951; Rapoport and Luebering, 1952). However its physiological significance was to remain a mystery for some time. It was not until 1967 that two independent research groups reported almost simultaneously on the importance of DPG in oxygen transport (Benesch and Benesch, 1967; Chanutin and Curnish, 1967). After this discovery, a large amount of effort was devoted to understanding the regulation of DPG metabolism during the past decades, culminating in the work of Mulquiney (Mulquiney et al., 1999).

5.1.3 The role of 2,3-DPG in Oxygen transport

The role of DPG in oxygen transport can be considered in terms of tissue oxygen demand (Meldon, 1985). Tissue oxygen demand (V_{O2}; mol s^{-1}) depends

primarily on three factors; cardiac output (Q; L s^{-1}), haemoglobin concentration ([Hb]; mol L^{-1}), and the O_2 saturation difference (mol O_2 per mol Hb) of Hb between arterial (S_A) and venous blood (S_V) such that:

$$V_{O2} = Q\,[Hb]\,(S_A - S_V) \tag{5.1}$$

DPG is an allosteric effector that decreases the affinity of haemoglobin for O_2 by binding preferentially to deoxygenated-Hb (deoxy-Hb). Thus an increase in DPG concentration causes a rightward shift in the oxygen-Hb dissociation curve and under most conditions causes an increase in the difference between arterial and venous haemoglobin oxygen saturation ($S_A - S_V$). Therefore, a decrease in any one of the three factors of Eqn 5.1 can be offset by an increase in the concentration of DPG.

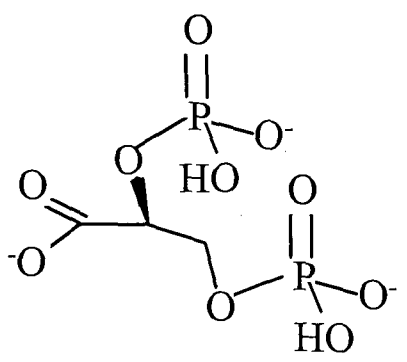


Fig. 5.1 Chemical structure of **D**-2,3-dihydroxypropanoic acid-2,3-phosphate, DPG.

The importance of DPG in oxygen transport becomes apparent in certain disease states and environmental changes (Harken, 1977; Meldon, 1985). In clinical conditions where [Hb], or cardiac output have been compromised, such as anaemia and congenital heart disease, higher concentrations of DPG are evident. Similarly, exposure to hypoxic high altitude, which lowers S_A, causes an increase in the concentration of DPG (Aste-Salazar and Hurtado, 1944; Lenfant et al., 1971; Mairbaurl et al., 1993).

5.1.4 Regulation of 2,3-DPG concentration

The study of disease and environmentally correlated changes in DPG concentration has indicated that blood pH and oxygen tension are important external effectors of DPG metabolism (Harken, 1977; Meldon, 1985). Lenfant et al. (Lenfant et al., 1971) found that subjects initially respond to the hypoxia of high altitude by hyperventilating. This causes respiratory alkalosis (Section 1.3.2). If alkalosis is prevented, the usual accumulation of DPG is prevented. Duhm and Gerlach (Duhm and Gerlach, 1971) reported similar findings in rats exposed to gas mixtures of low oxygen content. The importance of blood pH in regulating DPG concentration is also supported by a number of studies that show that DPG concentration is highly dependent on pH. Increases in pH above the usual physiological value result in DPG accumulation, while decreases result in depletion (Rapoport and Guest, 1939; Asakura et al., 1966a; Astrup et al., 1970; Rapoport et al., 1977). This pH dependence is very dramatic; the steady state concentration of DPG in glycolysing erythrocytes drops from its usual steady-state value of ~7 mM to zero with a decrease of only 0.4 pH units (Rapoport et al., 1977) and a rise in intracellular pH of 0.01 units causes the DPG concentration to increase by ~0.3 mM. However, the fact that elevated levels of DPG are not always associated with respiratory alkalosis (Duhm and Gerlach, 1971), indicates that haemoglobin oxygen saturation itself may also be an important factor controlling DPG concentration, caused by deoxy-Hb binding many phosphorylated glycolytic intermediates with greater affinity than oxy-Hb. Thus a change in oxygen tension can significantly affect the free concentrations of glycolytic intermediates and effectors and thus significantly affect metabolism (Hamasaki and Rose, 1974; Geier et al., 1978a; Geier et al., 1978b; Gupta et al., 1978; Bunn and Forget, 1986; Mulquiney and Kuchel, 1997).

Mulquiney and co-workers successfully modelled the modulation of DPG by both H^+ and O_2 and showed that they exert their strongest effects on DPG concentration via the glycolytic enzymes, hexokinase (HK) and phosphofructokinase (PFK) (Mulquiney et al., 1999). However, it was also demonstrated by the same authors that such modulation occurred without substantially changing the glycolytic rate. 2,3-DPG synthase is very susceptible to changes in [DPG] around the normal *in vivo* steady state due to product inhibition but feedback inhibition of HK and PFK is as important in controlling DPG concentration (Mulquiney and Kuchel, 1999).

5.1.5 Effect of isocapnic hypoxia on [DPG] and $[Mg^{2+}]_{free}$

Hypoxia can be used to metabolically alter erythrocytes *in vivo*. However, as DPG is sensitive to changes in pO_2 but also is one of the main buffers of $[Mg^{2+}]_{free}$, the effects of hypoxia on $[Mg^{2+}]_{free}$ were investigated. As mentioned above, hypoxia initially causes hyperventilation, which correspondingly leads to hypocapnia and subsequent respiratory alkalosis. To remove this effect of changing pH_{ex}, volunteers were exposed to 8 hours hypoxia, where the pO_2 was lowered to 55 mmHg but the expired pCO_2 was held at constant, as described below.

5.1.6 Exposure to isocapnic hypoxia

Initial blood samples (10 ml) were taken from volunteers at rest and the partial pressure of CO_2 in their expired air was measured. The volunteers were then placed in a sealed chamber for 8 hours, in which the partial pressure of O_2 was lowered to 50 mmHg. After every hour of exposure, ventilatory variables were recorded and the pCO_2 in the chamber adjusted to ensure that the pCO_2 of the expired air remained equal to the volunteers' pre-exposure value. After 8 hours exposure to this isocapnic

hypoxia, a further 10 ml blood sample was taken. Both blood samples were prepared and analysed as described in Section 2.4, with all measurements made in duplicate.

All experiments were carried out under medical supervision, with heart rate and breathing rate monitored continuously.

5.2 RESULTS

The time-courses of the parameters that changed significantly for each volunteer are presented in Figure 5.2 with the values of all parameters shown in Table 5.1. There was a 9% increase in the concentration of DPG during the 8 hours hypoxia, and this was matched by a 9% decrease in $[Mg^{2+}]_{free}$. There was no observed change in any of the other parameters measured, except the volunteers' haematocrit, which rose by 3%, representing a 6% relative increase. It was important to statistically analyse the results using paired (matched) tests as otherwise the inherent natural variation in the parameters between subjects would mask experimental changes.

Table 5.1 Results of analysis of blood samples taken from volunteers (n = 6) pre- and post- 8 hrs exposure to isocapnic hypoxia.

	Pre-hypoxia	Post- hypoxia
DPG (mM)	7.56 ± 0.61	8.24 ± 0.47 ✓
ATP (mM)	1.89 ± 0.20	1.89 ± 0.22
pH_i	7.18 ± 0.01	7.17 ± 0.02
pH_{ex}	7.38 ± 0.01	7.36 ± 0.02
$[Mg^{2+}]_{free}$ (mM)	0.41 ±0.02	0.37 ± 0.05 ✓
Mg_T (mM)	3.11 ± 0.22	3.07 ± 0.28
pCO_2 (mmHg)	38 ± 1	39 ± 1
Haematocrit (%)	44 ± 4	47 ± 4 ✓

✓ indicates significance where $p < 0.05$ using a statistical paired t-test.

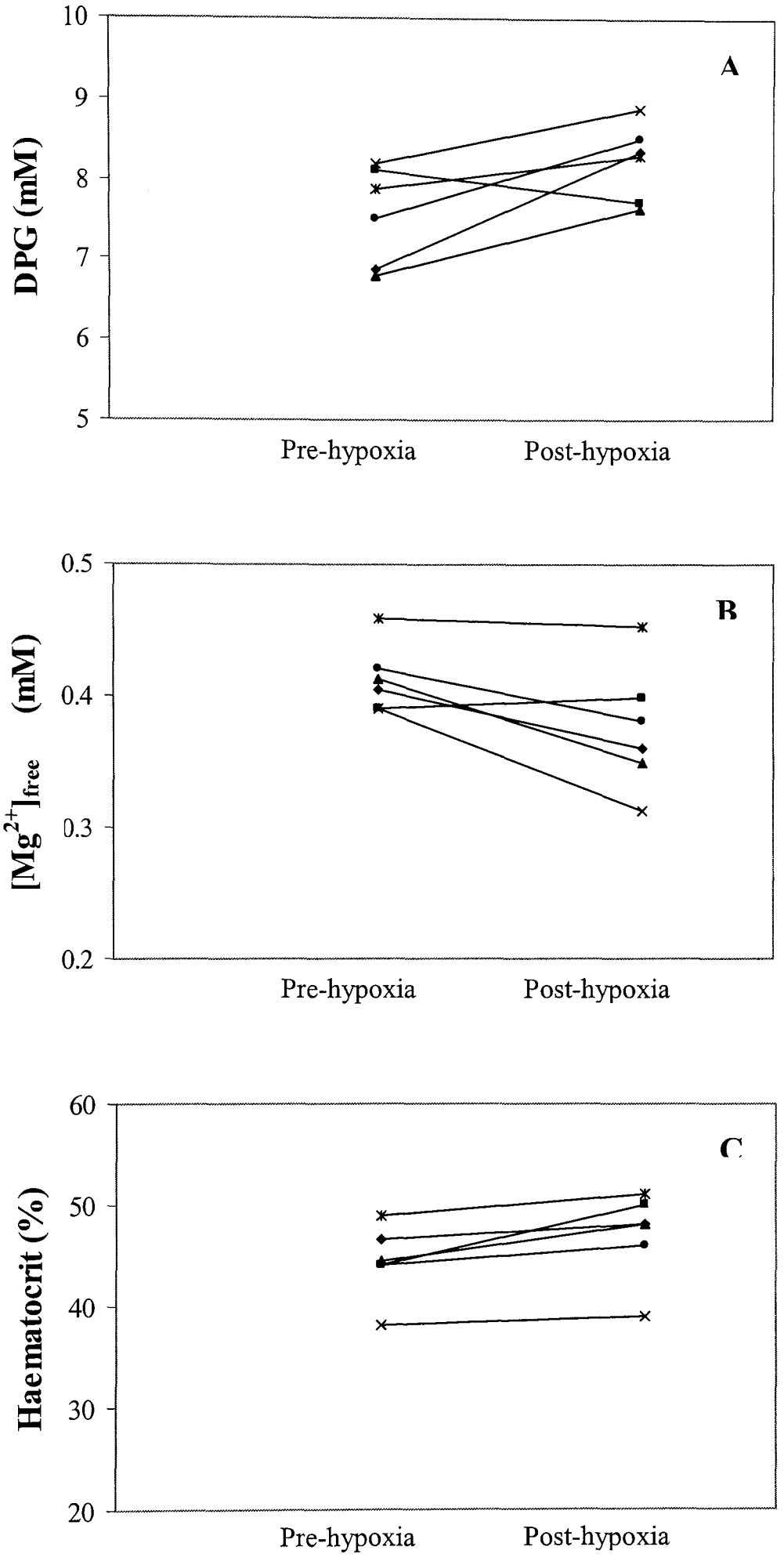


Fig. 5.2 Plots of parameters that changed during 8 hours hypoxia. **(A)** Intracellular DPG, **(B)** intracellular free Mg^{2+}, **(C)** haematocrit.

5.3 DISCUSSION

The isocapnic conditions led to no observable changes in the volunteers' blood pH, either externally in the plasma, or intracellularly in the erythrocytes, despite observed hyperventilation in all subjects. However, the DPG concentrations in the blood did significantly increase, and, as $[H^+]$ was held constant, it can be concluded that the hypoxic conditions were sufficient to lower the other powerful modulator of [DPG], oxy-Hb. This change in DPG was observed despite not allowing enough time to reach true steady-state conditions as calculated theoretically by Mulquiney et al. at 10.5 hours (Mulquiney and Kuchel, 1999) and experimentally determined by Mairbaurl et al. at 4 days (Mairbaurl et al., 1993). On this basis, it can be concluded that the DPG concentrations would have continued to rise for some time. In agreement with Mulquiney, the lack of any observable change in ATP concentrations, suggests that the modulation of DPG did indeed occur *without* any substantial overall change in glycolytic rate. This may have also been caused by the decrease in $[Mg^{2+}]_{free}$, which has been reported to decrease glycolytic rate via modulation of the enzymes HK, PFK and enolase (Mulquiney and Kuchel, 1999).

It might have been expected to see a change in pH_i, due to the fact that as DPG has a high negative charge and does not permeate the cell membrane, the Donnan ratio, r, equal to $[H_{ex}^+]/[H_i^+]$, would have had to decrease as DPG accumulated. This would have caused the pH_i to correspondingly decrease. However, with only a 9% increase in DPG, the change in pH_i would have only been minor. Using the equations developed by Duhm (Duhm, 1971), a 9% increase in DPG would only lower pH_i by 0.01 pH units at constant pH_{ex}. This change is within the error of the measurement, although a decrease by 0.01 pH units was indeed observed.

This analysis has shown that as DPG rose by 9%, the $[Mg^{2+}]_{free}$ fell by an equivalent 9%, as might have been expected in such a buffer system. The fact that Mg_T predicted by the NMR model remained constant concurred with this finding, but also suggested that, over this time period at least, no Mg transport across the cell membrane occurred in an attempt to keep $[Mg^{2+}]_{free}$ constant. This may be either because the steady-state had not yet been reached, or that such Mg transport into the cell was not possible. This inverse relationship between DPG levels and $[Mg^{2+}]_{free}$ has been reported previously (Mairbaurl and Hoffman, 1992), and it was suggested that inter-individual difference in $[Mg^{2+}]_{free}$ were caused primarily by differences in DPG concentrations in an inverse relationship.

These authors also showed that erythrocytes with high DPG, and low $[Mg^{2+}]_{free}$, had a large steady-state cell volume, with the low $[Mg^{2+}]_{free}$ concentrations inhibiting Na/K/2Cl cotransport (with an apparent $K_{0.5}$ for $[Mg^{2+}]_{free}$ of ~0.4 mM for the cotransport rate), and hence cell volume regulation. Therefore, the fact that, in all cases, the subjects' haematocrit rose, it was possible to conclude that their red blood cells might have increased in volume, agreeing with the work of Mairbaurl and Hoffman. However, haematocrit increases may have also been due to the fact that the subjects became dehydrated during the experiment. One further possibility is that the hypoxia was sudden and severe enough to initiate the release of immature red cells into the peripheral blood from the bone marrow. However, whatever the cause, the haematocrit increase was small and was not expected to have significantly affected the above results.

5.3.1 Summary

The steady-state concentration of DPG depends on a balance between its rate of synthesis and its rate of hydrolysis. The activity of DPG synthase is believed to be

limited by the low cellular levels of 1,3-DPG and by the high level of its inhibitory product, DPG (Rose, 1968). Thus the steady-state concentration of DPG depends on the concentrations of some glycolytic intermediates, and an understanding of the regulation of DPG concentrations also requires an understanding of the regulation of glycolysis. As pH and oxygenation state are known to affect glycolysis in a number of interrelated ways (Jacobasch et al., 1974), determining the mechanism by which these effectors influence DPG concentration is a difficult task. In addition, the association of many glycolytic metabolites and effectors with Hb and $[Mg^{2+}]_{free}$ are highly dependent on pH and oxygenation state. In this work, the effect of oxygenation state on DPG concentration has been analysed, whilst removing pH interference. The interaction between $[Mg^{2+}]_{free}$ and changing DPG concentrations *in vivo* has been investigated using the newly developed NMR technique, and has helped to demonstrate the technique's sensitivity.

CHAPTER 6

Sickle Cell Anaemia

CHAPTER 6 – SICKLE CELL ANAEMIA

As well as DPG concentrations responding to changes in oxygen levels to satisfy tissue oxygen demand, it can be seen from Equation 5.1 that changes in haemoglobin concentrations, [Hb], will also exert an effect. Such changes in [Hb] are manifested in clinical conditions that cause anaemia; a common condition caused by genetic mutation is sickle cell anaemia.

6.1 INTRODUCTION

6.1.1 Sickle cell anaemia

Sickle cell anaemia is present in 0.3–1.0% of the West African, Caribbean and North American black population, originating from areas of high malarial incidence (Serjant, 1992). The genetic defect in sickle cell anaemia results in the synthesis of an abnormal β haemoglobin (HbS) subunit, in which a single amino acid substitution is made; glutamate is replaced by valine at position 6 of the β chain. The precise aetiology of sickle cell anaemia is complicated and poorly understood. As a result of the amino acid substitution, HbS is able to form polymers, especially when deoxygenated or when its concentration is increased. This polymerisation produces the characteristic sickle deformation in red blood cells and leads to microcirculatory occlusion and consequent tissue ischaemia during painful episodes of sickle cell crisis. Sickle cells cycle through various reversibly sickled states before finally becoming irreversibly sickled (ISC) and consequently removed from the blood stream. Their average lifespan is considerably diminished, at about 40 days, roughly a third of normal red blood cells. Sickling depends strongly on the intracellular Hb concentration (Noguchi and Schechter, 1981) with the lag time to polymerisation inversely proportional to about the 30^{th} power of [HbS] (Eaton and Hofrichter, 1987).

Because of this, maintenance of normal cell volume is crucial, but importantly, sickle cells are highly susceptible to dehydration.

Therapeutic strategies for patients suffering from sickle cell anaemia aim at reducing HbS polymerisation. Hydroxyurea achieves this effect by increasing the cellular concentration of fetal haemoglobin, HbF, which has been seen to reduce HbS polymerisation in patients (Brugnara et al., 2001; Atweh and Loukopoulos, 2001). An additional strategy is based on reducing the cellular concentration of HbS by preventing cell dehydration.

Sickle cell dehydration is due to loss of K^+, Cl^-, and water: the two major determinant pathways of dehydration of sickle erythrocytes are the Ca^{2+}-activated K^+ channel (IK1 or Gardos channel) and the KCl cotransport (KCC) (see Ellory (Ellory et al., 1998) for reviews) with both probably contributing to overall dehydration. Specific inhibitors of the first of these pathways that have been tested in patients with sickle cell anaemia are clotrimazole and derivatives of clotrimazole metabolites, which have been shown to specifically block the Gardos channel (Brugnara et al., 2001).

It has been shown that both high pH_i and cytosolic free magnesium, $[Mg^{2+}]_{free}$, can inhibit the other dehydration path (Ellory et al., 1998; Brugnara and Tosteson, 1987; Canessa et al., 1987), as $[Mg^{2+}]_{free}$ is thought to regulate the KCl cotransporter (Flatman, 1988; Ellory et al., 1983). As a result, oral supplements have been used as a therapeutic aid in the form of magnesium pidolate, which is believed to increase the total magnesium content in sickle cells, Mg_T, and thereby inhibit KCC (De Franceschi et al., 2000; Brugnara, 1989).

However, whether Mg_T actually can be increased by oral dosing has not been fully confirmed (Weller et al., 1998; de Valk et al., 1998; Desbiens et al., 1992) or

instead if magnesium excretion merely increases in compensation (Basso et al., 2000). Furthermore, sickle cells are heterogeneous with respect to total Mg, DPG content and pH (Kaperonis et al., 1979; Ortiz et al., 1990), which vary with cell density as the cell sickles. Despite hypothesising that low intracellular magnesium results in the pathology of sickle cell anaemia (Adeyefa et al., 1986; Akenami et al., 1999; Olukoga et al., 1990), it is not clear if the important $[Mg^{2+}]_{free}$ is indeed decreased in patients with sickle cell anaemia.

Consequently, measurements of pH_i and $[Mg^{2+}]_{free}$ using the new NMR technique were made on sickle cells, but allowing for the heterogeneity of the cells by first separating them into three fractions using a discontinuous arabinogalactose density gradient. Analyses of unfractionated whole blood were also performed for comparison.

6.1.2 Separation of red blood cells by density

Among the density separation methods, the albumin gradient techniques, either continuous or discontinuous, appear to produce the most valid separations (for reviews see Chapter 8 of Red Cell Metabolism by Beutler (Beutler, 1984)). However, the expense and technical requirements to prepare isomolar albumin solutions are not conducive to widespread utilisation. An alternative, more simple and economical method for producing isopycnic density separations with a non-toxic gradient material was therefore developed by Corash and co-workers (Corash et al., 1974).

Arabinogalactan is a natural polysaccharide that is commercially isolated from the heartwood of the Western Larch tree (Larix occidentalis) and referred to as Stractan II. Chemically, Stractan is a branched polysaccharide with a galactose:arabinose ratio of 6:1. The molecular weights are reported to be 16,000 Da and 100,000 Da (Beutler, 1984). It is readily soluble in aqueous solutions up to a

concentration of 60% and provides a range of densities adequate to perform buoyant density separations of red cells between 1.080 g/ml and 1.160 g/ml. Stractan can be prepared and purified in working solutions using batch procedures so that density fractions can be stored and ready to use at short notice. As well as giving good density separations, separation of red cells on Stractan gradients does not cause any harmful side effects, such as cellular agglutination or disruption of the glycolytic enzyme pathways (Piomelli and Corash, 1976).

The densities for the layers of Stractan solution required to separate sickle cells were chosen so as to give a good separation but at the same time ensuring that enough cells were present in each fraction to allow effective NMR analyses. A description of Stractan solution preparation is given in Section 2.8.

6.2 RESULTS

6.2.1 Fractionation

Fractionation of the 8-10 ml of packed sickle cells acquired from two patients with sickle cell anaemia gave fraction volumes in the approximate ratio 3:5:2 for light:medium:dense fractions. When resuspended in their plasma, the haematocrits were in the range 35-50%. Morphological microscopic examination, as estimated by criteria described by previous workers (Ortiz et al., 1986) (thin, elongated cells with sharp, spike-like ends), revealed that the dense fraction contained between 50-70% irreversibly sickled cells, the medium contained approximately 5%, and the light had fewer than 1%.

6.2.2 Analysis of DPG, ATP, pH, $[Mg^{2+}]_{free}$ and Mg_T

The determined levels of DPG, ATP, pH_i, $[Mg^{2+}]_{free}$, and Mg_T, both predicted using the model and assayed, are presented in Table 6.1 for normal and sickle whole

blood and for each of the separated sickle cell fractions, light, medium and dense. It is worth mentioning here that it was assumed, for ease of comparison, that all cells contained 70% water. It has been suggested that this would be nearer 60% for the ISC's. However, if this did decrease in dense cells, then both Mg_T and all Mg buffers would increase in mM concentration. Therefore, any rise in Mg_T due to a decrease in water, would be combined with the same relative rise in ATP. As the input parameters into the model are the chemical shifts and the total concentrations of ATP, DPG and Hb, absolute changes in these totals due to changes in cellular water content will make little difference to the calculated $[Mg^{2+}]_{free}$.

Table 6.1 ATP, DPG, pCO_2, pH_{ex}, pH_i, $[Mg^{2+}]_{free}$ and total Mg concentrations in normal and sickle red blood cells. All concentrations are given in mM and pCO_2 is measured as mmHg.

	Whole Normal[a]	Fractionated Sickle Cells			Whole Sickle
		Light	Medium	Dense	
DPG	7.7 ± 0.5	10.0 ± 0.9*	10.4 ± 0.8*	8.6 ± 0.2*#	10.2 ± 1.1*
ATP	1.85 ± 0.27	2.03 ± 0.17	1.81 ± 0.18¶	1.94 ± 0.14	1.90 ± 0.38
pCO_2	39.8 ± 1.5	39.2 ± 1.3	38.6 ± 2.9	39.5 ± 1.0	40.8 ± 1.2
pH_{ex}	7.39 ± 0.03	7.16 ± 0.03*	7.16 ± 0.02*	7.15 ± 0.03*	7.32 ± 0.03*
pH_i	7.20 ± 0.02	7.06 ± 0.03*	7.07 ± 0.02*	7.07 ± 0.01*	7.10 ± 0.03*
$[Mg^{2+}]_{free}$	0.41 ± 0.03	0.37 ± 0.03	0.32 ± 0.02*	0.33 ± 0.02*	0.32 ± 0.05*
Model Mg_T	3.10 ± 0.31	3.30 ± 0.07	2.92 ± 0.16¶	2.91 ± 0.18¶	3.02± 0.41
Assay Mg_T	3.30 ± 0.39	3.69 ± 0.17	3.07 ± 0.27	2.86 ± 0.60¶	N/A

Data are presented as means ± SD. Whole normal, n = 33; Fractionated sickle cells, n = 3; Whole sickle, n = 9. *denotes $p < 0.05$ vs. whole normal blood. #denotes $p < 0.05$ vs. light and medium fractions. ¶denotes $p < 0.05$ vs. light fraction.

[a] Whole normal values were previously presented in Table 3.7.

DPG was significantly elevated in all three sickle cell fractions, by a maximum of 2.4 mM in the medium density fraction, compared with normal blood cell DPG of 7.7 mM. However, the DPG concentration of the dense cells was significantly lower than that of the other sickle cells, but not as low as the normal cells. ATP was found to be not statistically different from normal in all the sickle cell samples.

The pO_2 of all samples was never below 150 mmHg and usually was around 300 mmHg, thus ensuring complete oxygenation of the samples. The pCO_2 of whole blood and cell fractions was set to be the same for all the analyses, which gave normal blood a measured pH_{ex} of 7.39 ± 0.03. However, at a pCO_2 of 40 mmHg, the sickle blood was significantly more acidic by 0.08 pH units. In parallel with the more acidic pH_{ex}, the pH_i of all sickle cell samples was significantly more acidic than normal cells, by 0.1 pH units.

$[Mg^{2+}]_{free}$ was significantly decreased in the medium and the dense cells, and in the whole sickle blood, compared with normal cells. In verification, the model predicted lower Mg_T for medium and the dense cells compared with the light cells, and this trend was confirmed by the chemical assay.

6.3 DISCUSSION

6.3.1 Fractionation

The fractionation of the cells was sufficient to highlight any differences between cell types. Though it could have been improved by acquiring a fraction of 100% ISC, unfortunately this is not practical for NMR analysis where a relatively large minimum volume of cells is required.

6.3.2 $[Mg^{2+}]_{free}$, Mg_T, ATP and DPG

For the first time, sickle cells were shown to have a significantly decreased $[Mg^{2+}]_{free}$ compared with normal red cells, whether studying medium and dense cells after fractionation or whole, unfractionated sickle blood. This correlated with decreased Mg_T as the density of the cells increased, coupled with increased DPG, which increased the Mg^{2+} binding capacity in sickle cells. ATP was normal, but there did appear to be a small decrease between the lightest and middle fraction of the sickle cells. This may be as a response to the low $[Mg^{2+}]_{free}$ which is known to inhibit glycolysis within the cell (Mulquiney and Kuchel, 1999).

It has been reported that, despite the decrease in Mg_T as the cell density increases, the decrease in DPG leads to an overall *increase* in $[Mg^{2+}]_{free}$ as the binding capacity of the cell diminishes (Ortiz et al., 1990) thus ultimately causing to efflux of Mg_T from the cell. Using the method of Flatman and Lew to quantify $[Mg^{2+}]_{free}$ (Flatman and Lew, 1977), it was found that $[Mg^{2+}]_{free}$ increased from 0.4 to around 0.7 mM in irreversibly sickled cells (Ortiz et al., 1990). However, even theoretically, this increase would be unlikely. Assuming Mg_T remained constant, a 3 mM decrease in DPG would lead to a $[Mg^{2+}]_{free}$ increase of about 0.3 mM, as only around 10% of DPG is complexed to Mg. However, the 15% free ATP in the cell would bind ~85% of this $[Mg^{2+}]_{free}$, according to the distribution of the equilibria, and therefore the rise in $[Mg^{2+}]_{free}$ would be ~0.06 mM, not 0.3 mM. If a 15% decrease in Mg_T should occur, $[Mg^{2+}]_{free}$ would hardly change. In fact, this is what was found in the current experimental work. For $[Mg^{2+}]_{free}$ to increase to 0.7 mM while DPG decreased and ATP remained constant, Mg_T would have to *rise* to around 4 mM, which has not been observed. Both the model and the assay showed Mg_T to decrease as the density of the

cells increased, with the change being about 15%, in accord with previous reports (Ortiz et al., 1990).

One likely explanation for the previous report of increased $[Mg^{2+}]_{free}$ in the dense fraction of sickle cells (Ortiz et al., 1990) may have been a decrease in ATP concentration. If this major Mg-buffer decreased, then a concomitant rise in $[Mg^{2+}]_{free}$ would have been observed. This hypothesis cannot be verified as no data was given about ATP concentrations in the separated dense fraction, however, a loss in ATP could readily occur unless cells remained constantly at ~0°C and analysed quickly, as was carefully achieved in this work. In fact, only a 15% decrease in ATP could double the observed $[Mg^{2+}]_{free}$.

6.3.3 pH_i and pH_{ex}

This study has also shown that the intracellular pH of sickle cells was 0.10 pH units more acidic than that of normal erythrocytes. Thus the pH_i values of 7.20 and 7.10 for normal and sickle cells, respectively, is in agreement with previous NMR studies of buffer-washed cells (Tehrani et al., 1982). The difference has been explained by the net charge difference on the β chain of sickle haemoglobin. The slightly lower values of pH_i in all the separated fractions of sickle cells compared with the unfractionated may be due to the increased length of time between phlebotomy and NMR analysis (5-7 h vs. 1-2 h). It is known that sickle cells have a high rate of metabolism of organic phosphates (Lam et al., 1979) whilst in storage, and enough extra glycolysis might have occurred during this longer incubation period to account for the slight acidification.

However, in this work, at the same pCO_2, sickle blood pH_{ex} was also significantly more acidic, by 0.07 pH unit, than normal, contradicting the result of

similar pH_{ex}'s found in previous work on whole blood (Lam et al., 1979). The exact cause of this change in pH_{ex} is difficult to ascertain. However, any lower pH_{ex} combined with a higher intracellular DPG concentration would both alter the chloride ratio and contribute to the change in pH_i (Duhm, 1971).

It is interesting to consider that for a given multiple equilibria system containing a fixed concentration of phosphate compounds and magnesium, a decrease in pH naturally would lead to an increase in $[Mg^{2+}]_{free}$ as H^+ competitively inhibits Mg^{2+} binding. Despite the lower pH_i in sickle cells, the $[Mg^{2+}]_{free}$ was still below normal, contrary to expected. In fact, for the same concentrations of metabolites and Mg_T, but raised to pH 7.2, the model predicts that $[Mg^{2+}]_{free}$ of medium density sickle cells would in fact be 0.29 mM, even more decreased than normal.

6.3.4 Summary

Thus, using this new method which considered all significant interactions between the metabolites, H^+, K^+, Mg^{2+} and Hb, 100% oxygenated sickle blood had a significantly lower pH_{ex} and pH_i, and lower $[Mg^{2+}]_{free}$ than normal cells, both in the dense fractions following separation and as unfractionated whole blood.

Low $[Mg^{2+}]_{free}$ and low pH will both activate KCC1 (De Franceschi et al., 2000; Ellory et al., 1998) which may partially explain the high activity of this transporter in sickle cells. Cell shrinkage via overactivity of KCC1 remains the principal hazard in irreversible sickling. For a patient with sickle cell anaemia in a steady state, the percentage of red blood cells that are irreversibly sickled is about 15%. With a haematocrit of 20%, this corresponds to 3% of total blood volume. Therefore, although it is important to analyse the dense cell fraction to understand the mechanisms behind cell sickling, it is clinically important to analyse whole sickle blood, using considerably smaller volumes, to assess the state *in vivo* of the majority of red blood cells in an effort to prevent sickling.

CHAPTER 7

Effect of increased external Mg on stored erythrocytes

CHAPTER 7 – EFFECT OF INCREASED EXTERNAL Mg IN STORED ERYTHROCYTES

The preceding Chapters have assessed how changes in metabolite concentrations can lead to changes in $[Mg^{2+}]_{free}$. For the sickle cell, these changes may, in part, be responsible for the observed pathological state. Given the importance of $[Mg^{2+}]_{free}$ to metabolism and ion transport, it would seem logical that its concentration would be carefully regulated. Two questions arise from this; how dependent is metabolic rate on $[Mg^{2+}]_{free}$ and can the body control intracellular $[Mg^{2+}]_{free}$ by changing Mg_T in the face of an altered metabolic state?

7.1 REGULATORY ROLE OF $[Mg^{2+}]_{FREE}$

The regulatory role for Mg in glycolytic flux in erythrocytes has yet to be confirmed. It was reported in 1996 that glucose utilisation was strongly dependent on $[Mg^{2+}]_{free}$, but with a half maximal flux occurring at 0.03 mM (Laughlin and Thompson, 1996). Given that the physiological concentration of $[Mg^{2+}]_{free}$ is 0.4 mM, it was concluded that $[Mg^{2+}]_{free}$ would have very little role in the regulation of glycolysis *in vivo*. However, the same workers demonstrated in 1998 that the half maximal rate through phosphofructokinase and phosphoglycerate kinase occurred when $[Mg^{2+}]_{free}$ was 0.16 mM and 0.27 mM, respectively (Page et al., 1998). It was therefore concluded that $[Mg^{2+}]_{free}$ regulated ADP phosphorylation and evidence was also presented for its regulation of adenine nucleotide synthesis in human erythrocytes (Page et al., 1998).

More recently, Mulquiney reinvestigated this issue with a complete mathematical model for erythrocyte metabolism, and predicted that the half maximal rate of glycolysis was at a $[Mg^{2+}]_{free}$ of 0.3 mM, very close to the physiological value,

and concluded that $[Mg^{2+}]_{free}$ may play a regulatory role in erythrocyte glycolysis (Mulquiney and Kuchel, 1999b; Mulquiney and Kuchel, 1999a). This model predicted that a decrease in $[Mg^{2+}]_{free}$ by a factor of three would cause a decrease in the glycolytic rate of ~30%. This agreed with experimental evidence that a decline a red cell glycolytic rate had been observed in dogs and rats after their magnesium concentrations had been reduced by a similar amount (Rapoport, 1968).

7.2 Mg TRANSPORT

In order to control $[Mg^{2+}]_{free}$, the cell would need to alter total intracellular Mg as metabolite levels changed. Therefore, an efficient Mg transport system, both in and out of the cell, must exist, and external stores of Mg must be readily available. However, Mg transport has not been fully characterised, although has been more extensively studied in the past decade (Flatman, 1988; Flatman, 1991; Flatman, 1992; Flatman and Smith, 1996; Gunther, 1993; Ebel and Gunther, 1999; Millart et al., 1995; Rasgado and Gonzalez, 2000). The study of Mg transport is problematical as cells incubated in normal, decreased or increased extracellular concentrations of Mg^{2+} do not significantly release or take up Mg^{2+} if they contain normal intracellular levels of $[Mg^{2+}]_{free}$ (Gunther, 1993). Therefore, Mg^{2+} transport has often been measured by using $^{28}Mg^{2+}$. But, not only does this radioactive cation have a very short lifetime (~21 hours) and is difficult to obtain, thus limiting efficiency of transport measurements (Rasgado and Gonzalez, 2000) but also transport of $^{28}Mg^{2+}$ across the cell membrane does not necessarily mean net Mg^{2+} transport (Gunther, 1993). Despite this, some conclusions of Mg^{2+} efflux and influx have been drawn.

7.2.1 Efflux

As efflux of Mg occurs against an electrochemical gradient, there must be an energy-coupled mechanism for the extrusion of Mg. Most efflux measurements are made on erythrocytes pre-loaded with $[Mg^{2+}]_{free}$ using the ionophore A23187 (see Section 4.2.2). Using this method, it has been shown that such cells exhibit a high rate of Mg^{2+} efflux via an electoneutral Na^+/Mg^{2+} antiport, at ~0.1 mM/hr (Mairbaurl and Hoffman, 1992; Flatman, 1988) which continues until the cells return to normal Mg_T levels (Millart et al., 1995). This efflux is strongly temperature dependent, with an activation energy of 55 kJ/mol, is stimulated by hypotonicity (Feray and Garay, 1986) and is not reversible. The driving force of this antiport is thought to be the extra- (140 mM)/intracellular (5 mM) Na^+ gradient as this would explain its ATP and energy dependence (Gunther, 1993). However, Mg^{2+} efflux was also seen from Mg^{2+}-loaded cells suspended in Na-free potassium medium, suggesting the presence of a Na^+-independent mechanism (Gunther, 1993; Millart et al., 1995). This had an associated loss of Cl^- through band 3 for charge compensation.

But whether the properties of Mg^{2+} efflux in Mg^{2+}-loaded and in non-Mg^{2+}-loaded cells are the same is still an open question. It has been shown that rat erythrocytes at a physiological level of $[Mg^{2+}]_{free}$ exhibited Mg^{2+} efflux when incubated in nominally Mg^{2+}-free media. Two types of efflux were shown, Na^+_{ex} dependent, and Na^+_{ex} independent (Ebel and Gunther, 1999), but this has yet to be demonstrated in human erythrocytes.

Overall, it appears that the loss of Mg^{2+} appears to be of the order of 5-10 μM/hr over the cells lifetime, with the corresponding observation that older red blood cells have lower total Mg levels than younger ones (Flatman, 1991; Ouwerkerk et al., 1989).

7.2.2 Exchange

When incubated with $^{28}Mg^{2+}$, cells take up $^{28}Mg^{2+}$ without an alteration in Mg_T and therefore this uptake represents $^{28}Mg^{2+}$-$^{24}Mg^{2+}$ exchange (Gunther, 1993; Millart et al., 1995). Although this rate of exchange is low, approximately 5 μmol/hr, clearly erythrocytes can be considered permeable to Mg^{2+} under steady-state conditions.

7.2.3 Influx

Much less is known about Mg^{2+} influx. Even when erythrocytes were depleted of $[Mg^{2+}]_{free}$, either by incubation with A23187, or by feeding rats Mg^{2+}-deficient diets, reincubation with Mg^{2+} did not lead to significant reuptake of Mg^{2+} (Gunther, 1993).

Mg influx into the cell is thought to mainly occur by diffusion from the slightly higher free concentration in the extracellular space (typically 0.7 over 0.4 mM). This is promoted by the membrane potential, negative on the cytosolic side (Flatman, 1991). However it does not appear to be via the Ca channels as their blockers had no effect (Flatman, 1991). One possibility is electroneutral symport with anions such as hydrogencarbonate as observed in tumor cells and hepatocites (Gunther, 1993).

Thus, Mg^{2+} influx in erythrocytes was investigated. With the most likely influx due to diffusion of the higher extracellular concentration of Mg^{2+}, experiments were performed in which erythrocytes were stored in plasma to which high levels of Mg^{2+} had been added for 24 hours. The new NMR technique was used to accurately measure any changes in $[Mg^{2+}]_{free}$ or Mg_T.

7.3 STORAGE OF BLOOD

7.3.1 Introduction

It is clearly important that blood should be able to be stored for long periods of time without the erythrocytes suffering too much damage. As a result, several investigations have analysed the effects of long-term storage. One of the main problems is a depletion of DPG during storage. This depletion is accelerated by the declining pH caused by the progressive accumulation of lactate and H^+ from glycolysis (Bishop, 1962a; Bishop, 1962b). It is generally considered that the pH-dependence of glycolysis and/or the pH-dependence of the 2,3-DPGSynthase reaction is responsible for the decline (Mulquiney et al., 1999; Rapoport et al., 1977).

However, it has been reported that storage of blood in media containing high levels of Mg^{2+} can offset this decline and preserve DPG levels (Darley, 1979). This may be because the response coefficient of DPG to Mg_T is large (Mulquiney and Kuchel, 1999b), although this does make the assumption that Mg_T can increase when erythrocytes are stored in high Mg^{2+} concentrations. Conversely, storage of cells at 4 °C in media containing no added magnesium for up to a week leads to little change in magnesium content (Flatman, 1988). Furthermore, there is no correlation between the magnesium content of the cells and the time they are stored in a blood bank (up to 6 weeks) (Flatman, 1980; Bock et al., 1985).

Intracellular $[Mg^{2+}]_{free}$ is believed to fall to one-third of its original level after 11-40 days storage at 4 °C in standard citrate preservation media (from 0.241 to 0.081 mM) (Bock et al., 1985) with a corresponding pH decrease from 7.16 to 6.89. The fall in $[Mg^{2+}]_{free}$ occurs despite little change in the Mg_T indicating a change in the buffering characteristics of the cytoplasm. It has been suggested that binding of Mg

to ligands other than ATP and DPG, perhaps influxed citrate, must increase during storage, whilst Mg binding attributable to ATP and DPG declines (Millart et al., 1995).

7.3.2 Method of storage of erythrocytes in plasma with and without high [Mg^{2+}]

In a series of 7 experiments, 45 ml of venous blood was collected from a consenting volunteer and aerated according to Section 2.3.3 to simulate arterial blood. This was then divided into 5 equal aliquots, two of which were analysed using ^{31}P NMR immediately as described above and the measurements averaged. In two of the other three aliquots, sufficient quantities of stock 1 M $MgCl_2$ or 1 M $MgSO_4$ were added to produce a final plasma concentration of Mg^{2+} of 10 mM after the haematocrit had been measured (Section 2.3.4); approximately 40 µl were typically required. No additions were made to the final aliquot. These three aliquots were then placed on ice and left for 24 hours on a mixing plate before finally being analysed using NMR.

7.3.3 Results

The results from storage on ice for 24 hours in incubation media consisting of plasma, plasma with 10 mM $MgCl_2$, or plasma with 10 mM $MgSO_4$ are presented in Table 7.1 and Figures 7.1a & 7.1b. The high concentration of Mg^{2+} was chosen as the same value is used in preservation media for transplanted organs.

In both samples with added Mg^{2+}, the DPG concentrations increased by the same amount and showed a corresponding decrease in pH_{ex}, but there was no significant change in DPG during 24 hours on ice when no addition was made. The ATP concentration decreased in all cases during incubation, although by a smaller amount for those erythrocytes incubated in 10 mM $MgCl_2$.

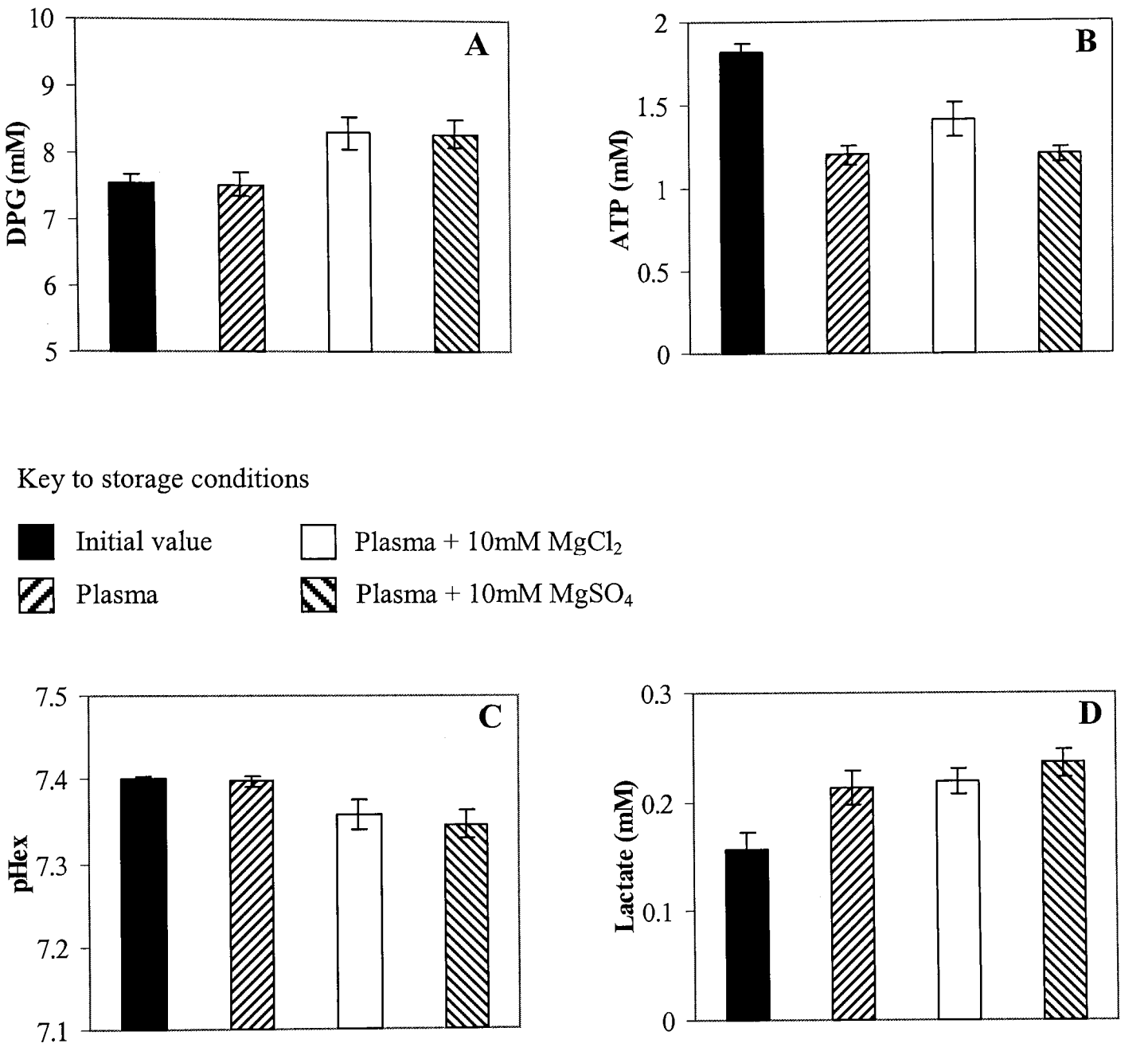


Fig. 7.1a Plots of blood parameters before and after storage on ice for 24 hours. **(A)** Intracellular DPG concentration, **(B)** intracellular ATP concentration, **(C)** extracellular pH, **(D)** extracellular lactate concentration.
Standard error bars are shown.

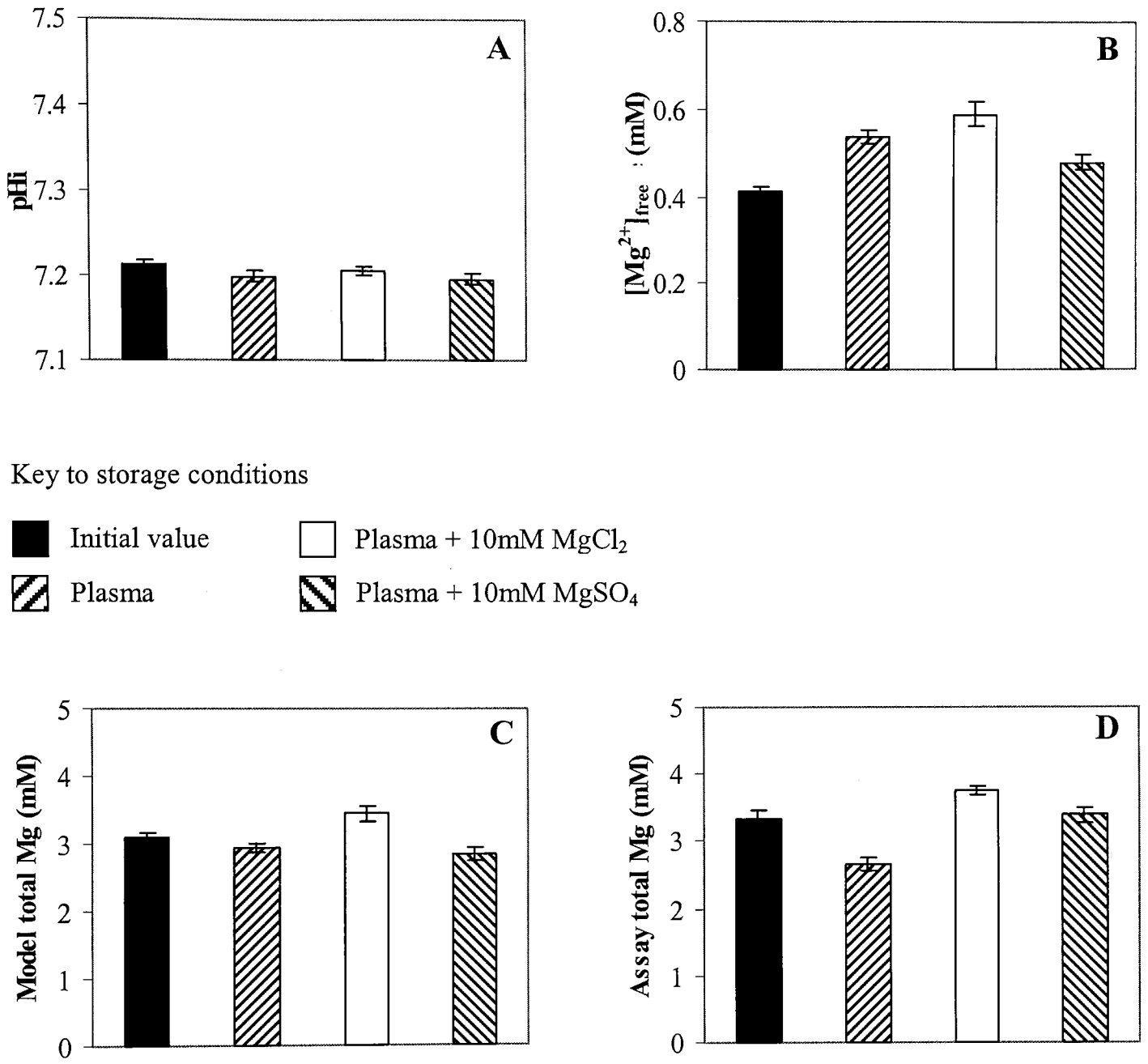


Fig. 7.1b Plots of blood parameters before and after storage on ice for 24 hours. **(A)** Intracellular pH, **(B)** intracellular $[Mg^{2+}]_{free}$ concentration, **(C)** total intracellular Mg, calculated by the model, **(D)** total intracellular Mg, measured by colorimetric assay. Standard error bars are shown.

Table 7.1: Values obtained from erythrocytes incubated in plasma for 24 hours with either no additions or 10 mM $MgCl_2$ or 10 mM $MgSO_4$, compared to their pre-incubation values (n = 7).

	Pre-incubation	No addition	10 mM $MgCl_2$	10 mM $MgSO_4$
DPG (mM)	7.55 ± 0.31	7.53 ± 0.45	8.32 ± 0.64*#	8.29 ± 0.56*#
ATP (mM)	1.82 ± 0.13	1.20 ± 0.16*	1.41 ± 0.28*	1.20 ± 0.11*¶
pH_i	7.21 ± 0.01	7.20 ± 0.02*	7.21 ± 0.01	7.19 ± 0.02*
pH_{ex}	7.40 ± 0.01	7.40 ± 0.02	7.36 ± 0.05*#	7.35 ± 0.04*#
$[Mg^{2+}]_{free}$ (mM)	0.41 ± 0.02	0.54 ± 0.04*	0.59 ± 0.07*	0.48 ± 0.05¶
$Mg_{T,model}$ (mM)	3.11 ± 0.11	2.90 ± 0.16¶	3.44 ± 0.33*#	2.83 ± 0.26¶
$Mg_{T,assay}$	3.33 ± 0.38	2.66 ± 0.26*¶	3.76 ± 0.15	3.39 ± 0.28
Lactate	0.16 ± 0.04	0.21 ± 0.04*	0.22 ± 0.03*	0.24 ± 0.03*
Haematocrit	47 ± 1.5	47 ± 2.9	45 ± 4.6	46 ± 3.5

Data are presented as means ± SD. The following indicate significance using a statistical paired t-test: *denotes $p < 0.05$ vs. Pre-incubation. #denotes $p < 0.05$ vs. No additions. ¶denotes $p < 0.05$ vs. 10 mM $MgCl_2$.

$[Mg^{2+}]_{free}$ increased during incubation on ice when $MgCl_2$ was added, but also when no addition was made. However, only with the addition of Mg^{2+} was any increase in Mg_T observed. The concentration of lactate in the plasma increased in all cases during the 24-hour period.

7.3.4 Discussion

In agreement with Darley (Darley, 1979), the supplementation of magnesium to stored blood produced an increase in DPG concentration. However, only for $MgCl_2$ was there an observed correlated increase in Mg_T. That the pH_i only decreased by a very small amount was responsible for the stability of DPG when no addition was made, as had been expected.

Contrary to previous reports (Bock et al., 1985), $[Mg^{2+}]_{free}$ increased in all cases, which was particularly marked in the case of addition of $MgCl_2$. (It should be mentioned that this study was only over 1 day, whereas the decreases in $[Mg^{2+}]_{free}$ have been observed after 11-40 days storage). This increase was almost certainly due to the decrease in ATP concentration, the major buffer of $[Mg^{2+}]_{free}$ and occurred despite the increase in DPG, the other major buffer, in the Mg-supplemented samples.

Quite why the ATP concentrations were greatest after incubation in $MgCl_2$ was more difficult to explain. If $[Mg^{2+}]_{free}$ were responsible for regulating the rate of glycolysis, and its increase caused a corresponding increase in this rate, then the significant increase in $[Mg^{2+}]_{free}$ might have explained the higher ATP. However one would also expect there to have been a corresponding increase in lactate and H^+ production, above the baseline increase in lactate, but it may have been that this was just not detected. Some evidence for this was that the pH_{ex} was more decreased following incubation with $MgCl_2$ than incubation in normal plasma. The changes in pH_i were only minor, and it was difficult to conclude whether or not these might have been responsible for any of the differences between the groups.

It was also difficult to rationalise why $MgCl_2$ produced a much greater increase in Mg_T than the same external concentration of $MgSO_4$. However, it is possible that the greater negative hydration enthalpy for $MgCl_2$ compared to $MgSO_4$ (-55 $kJmol^{-1}$ vs 38 $kJmol^{-1}$) would cause an effectively higher concentration of $[Mg^{2+}]_{ex}$, which would then produce a greater rate of influx of Mg^{2+}. Indeed, when the osmolality of samples of each plasma were tested, $MgCl_2$ gave a higher value than $MgSO_4$ at 292 and 270 mOsm, respectively, providing some evidence for this hypothesis. Also the addition of 10 mM Cl^- only represents a ~10% increase on that

found physiologically in plasma, whereas any addition of SO_4^{2-} could be regarded as non-physiologic, and this could have caused an adverse effect.

Despite the apparent similarity in final $[Mg^{2+}]_{free}$ and Mg_T concentrations between incubations in normal plasma and incubations with 10 mM $MgSO_4$, clearly there were different processes occurring to produce this. After incubation in normal plasma, a small efflux appeared to occur of the order of 5-10 μM/hr. However, alongside this there was a decrease in ATP at a rate of ~10 μM/hr. The overall effect of this was to produce an increase in $[Mg^{2+}]_{free}$ at a rate of ~5 μM/hr. However, in the case of incubation with $MgSO_4$ the effect of Mg^{2+} produced an increase in DPG concentration at a rate of ~40 μM/hr (along with an expected production of H^+) and because this extra DPG bound Mg^{2+} no increase in $[Mg^{2+}]_{free}$ was observed. However, after incubation with $MgCl_2$, which produced an overall influx of Mg^{2+} at a rate of ~16 μM/hr, despite the same rate of increase in DPG compared with incubation with $MgSO_4$, $[Mg^{2+}]_{free}$ increased as well, at a rate of ~10 μM/hr. This occurred despite the increased binding capacity of the higher ATP concentrations.

Although it is important to study the effect of different media on erythrocytes during storage, there is a compounding problem that the cells are continuing to function, but are in a fixed environment, where energy sources are limited and waste products are not removed. This causes a problem when analysing the slow rates of Mg transport, as the cells must be stored for some time. Although it may be helpful to slow the production of waste products down by lowering the temperature, not only will this slow Mg transport as well, bearing in mind the temperature dependence of the efflux (Feray and Garay, 1986), but may also change the positions of the equilibria that are being analysed.

7.3.5 Summary

It has been shown that increasing the external concentration of Mg^{2+} can cause an increase in Mg_T. Associated with this increase, increases in DPG concentrations were observed, suggesting that Mg^{2+} can indeed modulate the concentration of this metabolite and glycolytic rate. It was possible that changes in $[Mg^{2+}]_{free}$ might have also had an effect on ATP concentration, but this was less clear in these experiments, where changes in ATP were already occurring due to the 24h incubation time in a non-circulating buffer.

CHAPTER 8

Intravenous infusion of $MgSO_4$ in patients suffering sub-arachnoid haemorrhage

CHAPTER 8 – INTRAVENOUS INFUSION OF $MgSO_4$ IN PATIENTS SUFFERING SUB-ARACHNOID HAEMORHAGE

In the previous Chapter, the effects on human erythrocytes of a higher level of external concentration of Mg^{2+} than normal were investigated. However, it was concluded that the effects of storage on ice in a non-circulating buffer complicated the analysis, as well as limiting the length of time that the erythrocytes could be exposed to the high Mg^{2+} levels. To examine such effects more accurately, it would be better to use a constantly refreshed buffer, i.e. removing the accumulation of waste products, and to expose the erythrocytes to high $[Mg^{2+}]_{ex}$ at normal temperature, i.e. 37°C.

This is most easily achieved *in vivo*, with constant infusion of Mg^{2+} to maintain a high $[Mg^{2+}]_{ex}$ and there are some clinical settings where such studies have previously been preformed.

8.1 INTRODUCTION

Injections of Mg^{2+} are generally in the form of $MgSO_4$, and have been used in a wide range of clinical settings, whether it be protection against alcohol-induced, brain injury related haemorrhagic stroke (Altura et al., 1995; Boet and Mee, 2000), decreasing mortality of diabetic patients after myocardial infarction (Rasmussen et al., 1986; De Leeuw et al., 1997; De Leeuw et al., 1998), the treatment of headaches, unresponsive neuropathic pain (Ginder et al., 2000; Crosby et al., 2000), the alleviation of ventricular cardiac arrhythmias (Ceremuzynski et al., 2000), treatment of patients suffering chronic fatigue syndrome (Clague et al., 1992) or therapy for pre-eclampsia (Rudnicki et al., 2000).

8.1.1 Effect of intravenous infusion of Mg^{2+}

Clearly, the primary effect of intravenous (IV) infusion is to immediately increase plasma concentrations of total Mg, with an associated immediate increase in ionised Mg^{2+} in the plasma (De Leeuw et al., 1997; De Leeuw et al., 1998). However, it is not clear whether this will lead to increases in either total intracellular Mg, Mg_T, or free intracellular magnesium, $[Mg^{2+}]_{free}$, in erythrocytes (De Leeuw et al., 1997; Reinhart et al., 1990). Despite this, a slow appearance of ^{28}Mg in red cells has been observed after intravenous injection of the tracer into human volunteers, suggesting that some movement of the ion is possible (Watson et al., 1979). There are two problems frequently associated with these clinical applications. Firstly, the time of infusion is often relatively very short, with typical infusion times of less than 4 hours and at most 48 hours. As discussed in previous Chapters, the time for the cells to reach a steady-state is at least 10 hours, maybe longer, and the Mg-transport timescale may well be considerably longer still. Secondly, infusions of $MgSO_4$ are generally administered merely to produce a particular clinical response, whether that be blocking the N-methyl-D-aspartate (NMDA) receptor (Crosby et al., 2000) or reducing blood-pressure (Rudnicki et al., 2000). Very rarely are such infusions used to examine the effects on erythrocytes, despite these being an excellent source of intracellular tissue. As a result, the full effects on cellular metabolism of long-term increases in plasma Mg concentrations *in vivo* are unknown.

8.1.2 Hypomagnesemia in critically ill patients

Magnesium deficiency, or hypomagnesemia, is a common clinical finding in critically ill patients and can cause, or correlates with hypocalcemia, hypokalemia, hypophosphatemia. Related conditions include hypertension, coronary

vasoconstriction, cardiac arrthythmias and muscle weakness (Polderman et al., 2000; Ryzen et al., 1990). Mg deficiency is thought to result primarily from increased gastrointestinal or urinary loss (Ryzen, 1989), which may be caused by increased levels of trauma-induced circulating endogenous catecholamines (Ryzen et al., 1990). One group of patients at risk from neurologic trauma related hypomagnesemia are those suffering severe head injury (Polderman et al., 2000), demonstrated by deficits in plasma Mg^{2+} (Altura and Altura, 1999).

Sub-arachnoid haemorrhage, SAH, is a common form of severe head injury and has complications that arise from cerebrospinal fluid, CSF, stimulating vascular smooth muscle, causing vasospasm (Cadoux-Hudson et al., 2001; Pyne et al., 2001a). The mechanism of stimulation has been attributed to increases in intracellular Ca^{2+} (Pyne et al., 2001a) or possibly also haemolysis and degradation products of biliverdin or bilirubin in the CFS (Kranc et al., 2000). Furthermore, alterations in cellular metabolism, for example changes in ATP, ADP and PCr, have also been observed following traumatic brain injury (Garnett et al., 2001).

It was seen in 1988 that patients exhibiting vasospasm following SAH had decreased levels of $[Mg^{2+}]$ in the CSF and suggested that *in vitro*, at least, increased levels of Mg^{2+} could depress vascular contraction of cerebral arteries (Miura, 1988). Since then, alcohol-induced haemorrhagic stroke and associated cerebrovasospasm, preceded by a rapid fall in brain $[Mg^{2+}]_{free}$, has been shown to be ameliorated by IV administrations of $MgSO_4$ and even preventable (Altura and Altura, 1999). Data suggests that alcohol-induced cellular loss of $[Mg^{2+}]_{free}$ is associated with cellular Ca^{2+} overload (Altura and Altura, 1999).

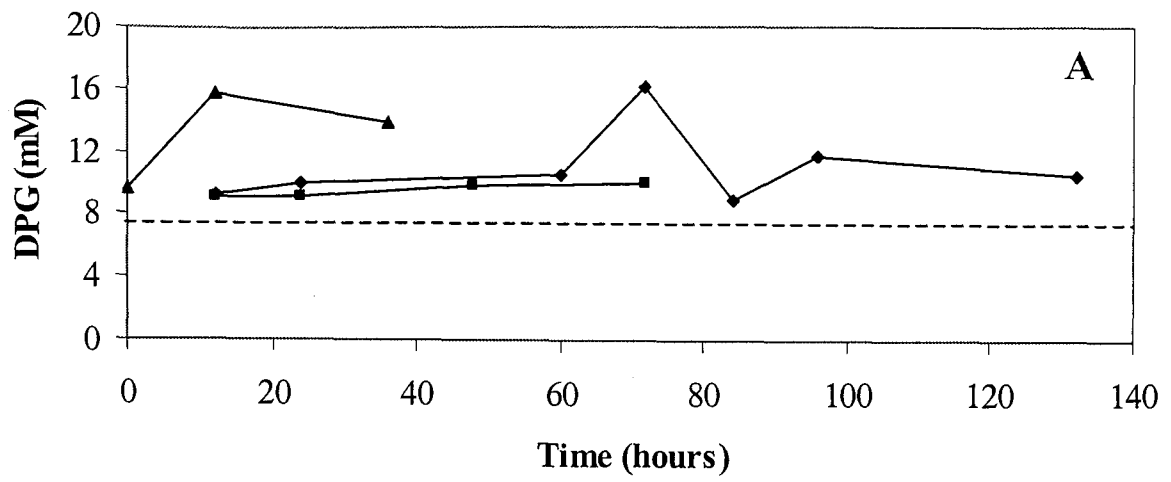
Thus, Mg^{2+} has recently been proposed for the treatment of cerebral vasospasm. *In vitro*, it has been shown that Mg^{2+} caused a dose dependant decrease in

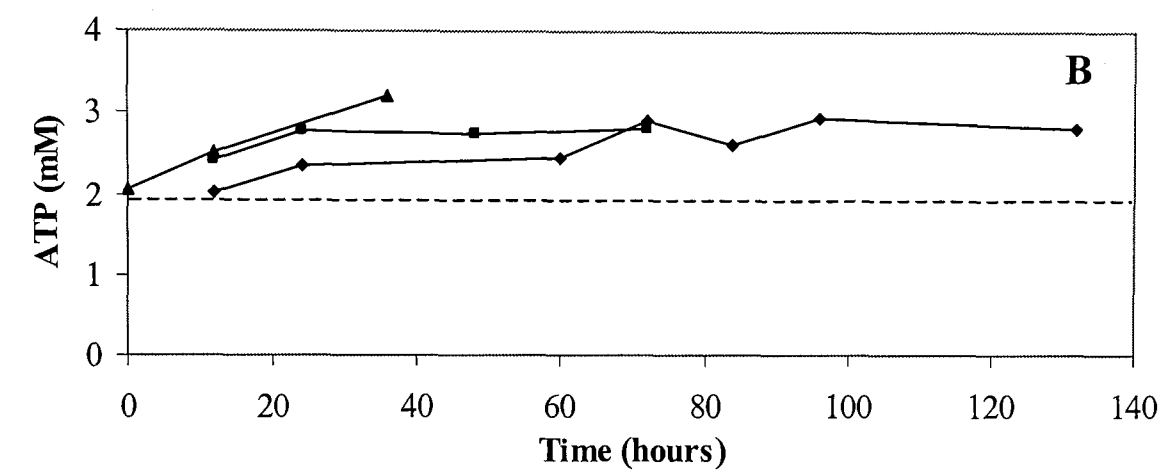
tension of carotid artery following CSF-induced contraction, although 12 mM concentrations of Mg^{2+} were required to produce normalisation of tension (Pyne et al., 2001b). Even so, $MgSO_4$ therapy is currently under active investigation as a neuroprotective agent in pilot trials. Single bolus treatment has not been shown to produce any significant decrease in cerebral blood flow velocity as measured by transcranial Doppler ultrasonography (Brewer et al., 2001). However, an initial bolus followed by constant infusion may provide a better strategy (Boet and Mee, 2000).

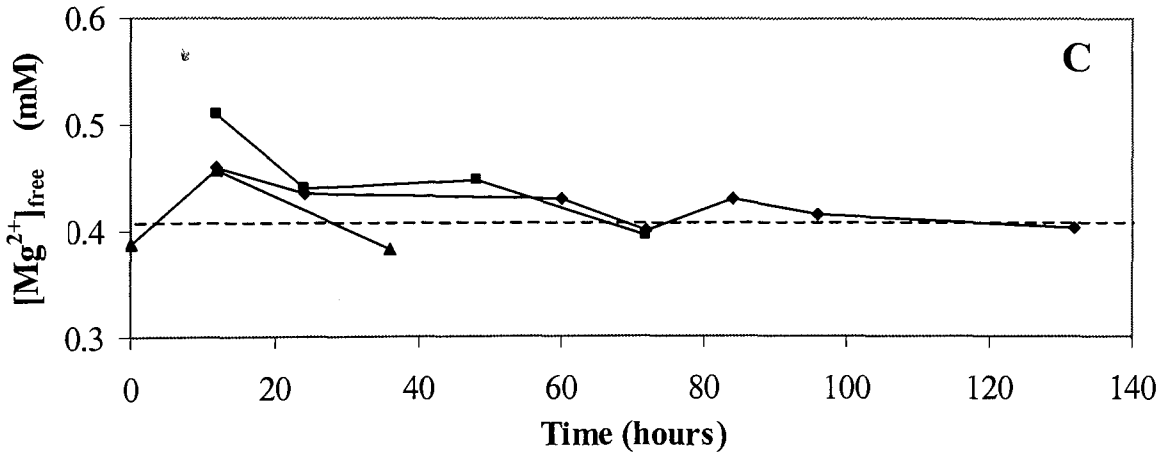
Therefore, in a pilot trial performed at the High Dependency Unit, HDU, in the John Radcliffe hospital in Oxford, patients suffering SAH were treated with $MgSO_4$ as described in Section 2.9.1. The aim was to maintain total blood serum Mg levels at 2-3 times normal, i.e. 2.5 mM for a period of 10 days. Blood samples were taken from these patients to examine the effect of high extracellular concentrations of Mg^{2+} on intracellular total Mg, $[Mg^{2+}]_{free}$ and cellular metabolism in erythrocytes. It was hoped that from examination of this tissue it might be possible to infer intracellular changes in the brain.

8.2 RESULTS

Timecourses of DPG, ATP, $[Mg^{2+}]_{free}$, Mg_T, pH_i, pH_{ex} and haematocrit of three patients suffering with SAH and treated with IV infusion of $MgSO_4$ are shown in Figure 8.1a & b. Infusion began at time = 0 and continued over the full timecourse. Only one patient completed the study. Due to technical complications, only one patient could provide a blood sample for analysis prior to the infusion.







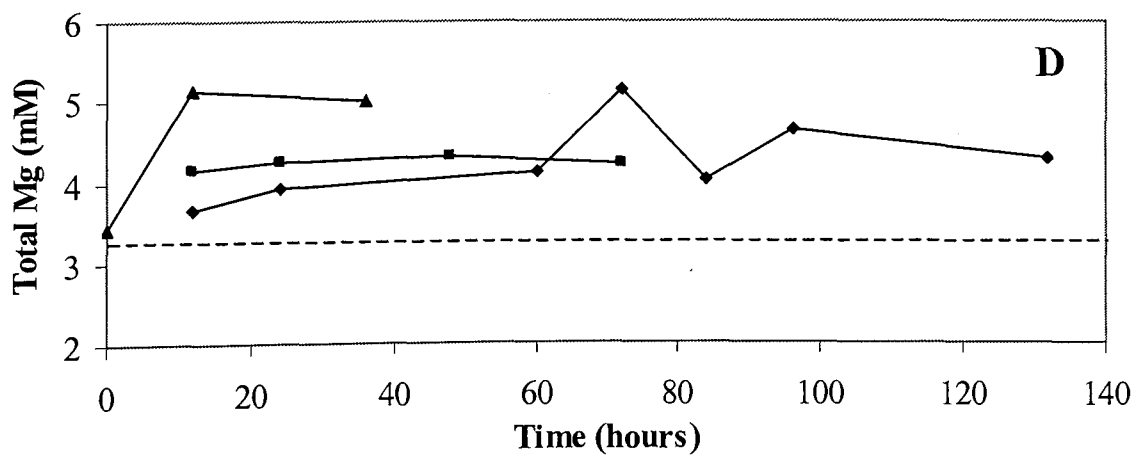


Fig. 8.1a Plots of blood parameters for each of three patients during intravenous infusion with $MgSO_4$. **(A)** Intracellular DPG concentration, **(B)** intracellular ATP concentration, **(C)** intracellular $[Mg^{2+}]_{free}$, **(D)** total intracellular Mg concentration. Mean normal values (Table 3.7) are represented by a dashed line.

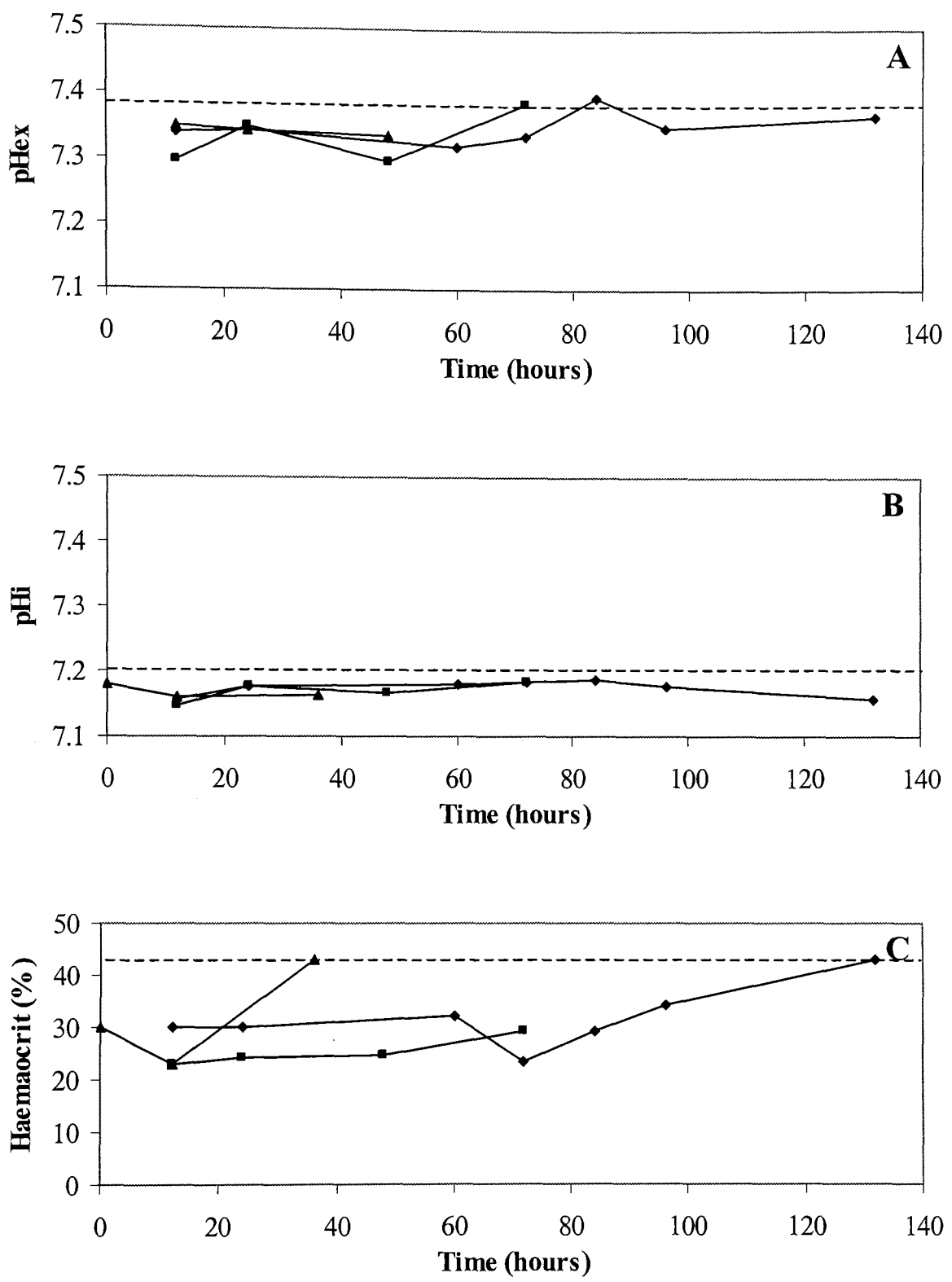


Fig. 8.1b Plots of blood parameters for each of three patients during intravenous infusion with $MgSO_4$. **(A)** Extracellular pH, **(B)** intracellular pH, **(C)** haematocrit. Mean normal values (Table 3.7) are represented by a dashed line.

DPG concentrations of all patients were higher than normal at ~10 mM (cf 7.7 mM in Table 3.6), and were relatively constant during the study. However, in Pt 1 (time = 72 hrs) and Pt 3 (time = 12 hrs), large increases in DPG were observed following significant reductions in haematocrit levels. Subsequent 1L blood infusions then accounted for a return in these values at the next time point.

Total Mg concentrations increased to their maximum of ~4.1 mM within 24 hours of initial bolus and although $[Mg^{2+}]_{free}$ rose in the initial 12 hour period, they subsequently returned to normal levels over the following 2 days. ATP concentrations were seen to gradually increase during the first 40-70 hours of infusion.

Intra- and extracellular pH were relatively constant over the full timecourse, however both were significantly more acidic than normal as measured in fully oxygenated whole blood with a pCO_2 of 40 mmHg.

8.3 DISCUSSION

As expected, following haemorrhage all patients became anaemic which correlated with increased concentrations of DPG in erythrocytes to increase the delivery of oxygen to tissues. It was interesting to note that a sharp fall in haematocrit caused a large rise in DPG in two patients.

The initial bolus and subsequent constant infusion to maintain plasma concentrations of Mg 2-3 times normal at 2.5 mM caused both the intracellular Mg_T and $[Mg^{2+}]_{free}$ to increase by 35% and 25%, respectively, with both reaching maximal values within 24 hours. Although DPG levels only increased slightly in response to this increase, the other main buffer of Mg within the erythrocyte, ATP, was seen to

increase significantly over a 2-3 day timecourse by ~35%. As a result, given that Mg_T did not continue to increase greatly after the initial 24 hour period, $[Mg^{2+}]_{free}$ decreased as ATP concentrations rose.

It can be hypothesised that with DPG levels at a physiological maximum, and yet still with increased $[Mg^{2+}]_{free}$ in the cell, an increase in glycolytic rate occurred, leading to increased ATP. This then caused a decrease in $[Mg^{2+}]_{free}$, generating negative feedback, acting to slow the glycolytic rate, but with metabolite concentrations at a new steady-state, reflecting the permanent change in extracellular Mg. Some justification for this is the similar rise in ATP and Mg_T and the fact that ATP increased sufficiently to return $[Mg^{2+}]_{free}$ levels to normal values (0.41 mM, Table 3.6).

What may be clinically important is that patients treated with $MgSO_4$ infusion following SAH, commonly showed neuronal function improvement in the first 24 hours, however, over the following 2-3 days, function gradually reduced. It may be that the initially rise in $[Mg^{2+}]_{free}$ that occurred in erythrocytes, may be reproduced in brain cells, and could prevent the Ca^{2+} overloads that subsequently cause cerebrovasospasm and decrease vascular muscle tension. However, metabolic changes could then take place to buffer $[Mg^{2+}]_{free}$ to normal levels, thereby removing its protective effect and resulting in the occurrence of damaging vasospasm.

Clearly a larger study would be needed to confirm this, but it can be tentatively concluded that, in erythrocytes at least, long periods of exposure to high levels of Mg^{2+} *in vivo* can first increase Mg_T and $[Mg^{2+}]_{free}$. But metabolic changes then occur to buffer $[Mg^{2+}]_{free}$ back to normal levels, initially by increasing one of the main buffers, DPG, but followed by ATP, over a longer period of time. This suggests that $[Mg^{2+}]_{free}$ has an important regulatory role in glycolysis in the human erythrocyte.

CHAPTER 9

Magnesium in pregnancy and the effects of pre-eclampsia

CHAPTER 9 – MAGNESIUM IN PREGNANCY – EFFECTS OF PRE-ECLAMPSIA.

Eclampsia is thought to be related to cerebral vasospasm (Bearchell et al., 1998). It is preceded by pre-eclampsia, a complication of pregnancy that causes maternal vasoconstriction and hypertension, and can be fatal for mother and child. Treatment with magnesium has been seen to be effective in the treatment of eclamptic seizures and its hypothetical underlying pathological role in the disease is investigated here.

9.1 INTRODUCTION

9.1.1 Hypertension and magnesium

The effect of magnesium on the circulatory system, and in particular its vasodilatory action, has been recognised for well over a century (Laffont, 1879). However, the pathological role of magnesium in cases of increased blood pressure is, at best, controversial. Epidemiological studies have shown an inverse relationship between dietary Mg intake and the level of blood pressure, yet the evidence is inconsistent and many of the clinical studies are methodologically imperfect and based on small study populations (see (Saris et al., 2000) for reviews). The picture is no clearer if one considers the effect of Mg supplementation, as several studies have shown this treatment reduces blood pressure (Widman et al., 1993; Kawano et al., 1998; Yang and Chiu, 1999), but not all (Cappuccio et al., 1985; Ferrara et al., 1992).

The confusion tends to arise from the use of different markers of magnesium status, whether they be serum total magnesium, serum ionised magnesium, plasma total magnesium, plasma ionised magnesium, erythrocyte total magnesium, or erythrocyte ionised magnesium (Sasaki et al., 2000), and whether supplementation is

by injection or orally, and if the latter, the exact quantity and salt form of the supplement.

Resnick et al (Resnick et al., 1987; Resnick et al., 1990) were the first to describe a strong inverse relationship between erythrocyte intracellular free Mg^{2+}, $[Mg^{2+}]_{free}$, and diastolic blood pressure, although even this has been disputed (Woods et al., 1988). Since then, other groups have suggested that $[Mg^{2+}]_{free}$ is usually lower in vascular smooth muscle and circulating blood cells of hypertensive compared to normotensive subjects (Altura and Altura, 1996; Resnick, 1995). It may be safest to conclude that, at present, a major role for magnesium as an antihypertensive factor has not been established but magnesium deficit is an important nephrocardiovascular risk factor and there may be some support for magnesium as an antihypertensive cofactor (Durlach et al., 1992).

The mechanism by which a magnesium deficit might induce hypertension may involve interaction with calcium. By competing with Ca^{2+} membrane-binding sites and by modulating calcium binding and release from the sarcoplasmic reticulum (SR) in muscle and endoplasmic reticulum (ER) membranes in endothelial cells, Mg^{2+} could maintain low resting levels of intracellular free Ca^{2+}, $[Ca^{2+}]_{free}$, and control muscle relaxation (for reviews, see (Altura and Altura, 1996)). Deficits in extracellular Mg^{2+} can results in elevation of $[Ca^{2+}]_{free}$ in cardiac and vascular smooth muscle cell membranes (Fu et al., 1998; Altura and Altura, 1994; Resnick, 1995; Barbagallo et al., 2000) leading to an elevation in the ionised Ca^{2+}/Mg^{2+} ratio in plasma from the normal value of 2.0 (Resnick et al., 1993) and to subsequent peripheral vasoconstriction or spasm and possibly atherogenesis (Altura and Altura, 1996).

An indirect effect of plasma magnesium may involve oxidative stress. Magnesium deficiency in rats led to an increase in production of radical oxygen species and subsequent oxidative stress. In particular, the concentration of nitric oxide markedly increased in the plasma, resulting from activation of inducible nitric oxide synthase, iNOS (Rock et al., 1995). Some evidence for this mechanism has recently been demonstrated, suggesting that the L-arginine/nitric oxide pathway is responsible, in part, for the vasodilatory action of Mg^{2+} on vasculature (Dawes and Ritter, 2000). Furthermore, infusions of the endogenous antioxidant, glutathione, can lower blood pressure (Barbagallo et al., 1999b) and it is unsurprising that such infusions also have been shown to increase total intracellular Mg in human erythrocytes, Mg_T, thus demonstrating a clinical linkage between cellular magnesium and glutathione (Barbagallo et al., 1999a).

9.1.2 Pre-eclampsia and treatment with magnesium sulphate infusions.

Pregnancy may pose an added stress on Mg balance, with disturbances in normal homeostasis of both Mg^{2+} and Ca^{2+} in normal pregnancy and pre-eclampsia (Kisters et al., 1998). Although oral supplementation with magnesium is not particularly effective (Makrides and Crowther, 2000), intra-venous infusions of magnesium sulphate are much more so (Rudnicki et al., 2000), and were used in the prevention of eclamptic seizures as early as 1906 (Saris et al., 2000). Currently, it is becoming widely used as a routine therapy to prevent eclamptic seizures in pregnant women with hypertension (Brewer et al., 2001). The Collaborative Eclampsia Trial provided compelling evidence in favour of the use of $MgSO_4$, rather than diazepam or phenytoin, in the management of eclampsia (Duley, 1998).

The evidence suggests that magnesium metabolism is altered in pregnancy and particularly in disease processes found during pregnancy, but it is unknown how

erythrocytes respond to these alterations. Therefore, the effects of pregnancy on magnesium concentrations in erythrocytes in normal women and patients with pre-eclampsia were investigated.

9.2 RESULTS

Blood samples were taken from normal age-matched non-pregnant female subjects (n = 6), from control normotensive pregnant subjects (n = 6) and from pregnant subjects with high blood pressure (n = 7) and analysed for DPG, ATP, $[Mg^{2+}]_{free}$, Mg_T, pH_i, pH_{ex}, and haematocrit as described in Chapter 2. The results are presented in Table 9.1 and Figure 9.1a & b.

Table 9.1: Values obtained from erythrocytes from normal subjects (n = 6), control pregnant subjects (n = 6) and from pre-eclampsic patients (n = 7).

	Non-pregnant	Pregnant	
	Normal	Control	Pre-eclampsic
DPG (mM)	8.21 ± 0.57	8.95 ± 0.34*	9.29 ± 0.49$^{\#}$
ATP (mM)	1.99 ± 0.34	2.12 ± 0.30	2.07 ± 0.24
pH_i	7.21 ± 0.01	7.18 ± 0.02	7.15 ± 0.01$^{\#\P}$
pH_{ex}	7.40 ± 0.02	7.37 ± 0.03	7.30 ± 0.03$^{\#\P}$
$[Mg^{2+}]_{free}$ (mM)	0.40 ± 0.02	0.39 ± 0.04	0.38 ± 0.04
$Mg_{T,model}$ (mM)	3.14 ± 0.20	3.42 ± 0.35	3.41 ± 0.25
$Mg_{T,aassay}$ (mM)	3.23 ± 0.23	3.49 ± 0.20	3.39 ± 0.31
Haematocrit	42 ± 3	36 ± 3$^{\#}$	37 ± 2$^{\#}$
H^+_{ex}/H^+_i	0.66 ± 0.01	0.67 ± 0.03	0.70 ± 0.04*

Data are presented as means ± SD. The following indicate significance using a statistical paired t-test: *denotes $p < 0.05$ vs. Normal. $^{\#}$denotes $p < 0.01$ vs. Normal. ¶denotes $p < 0.01$ vs. Control.

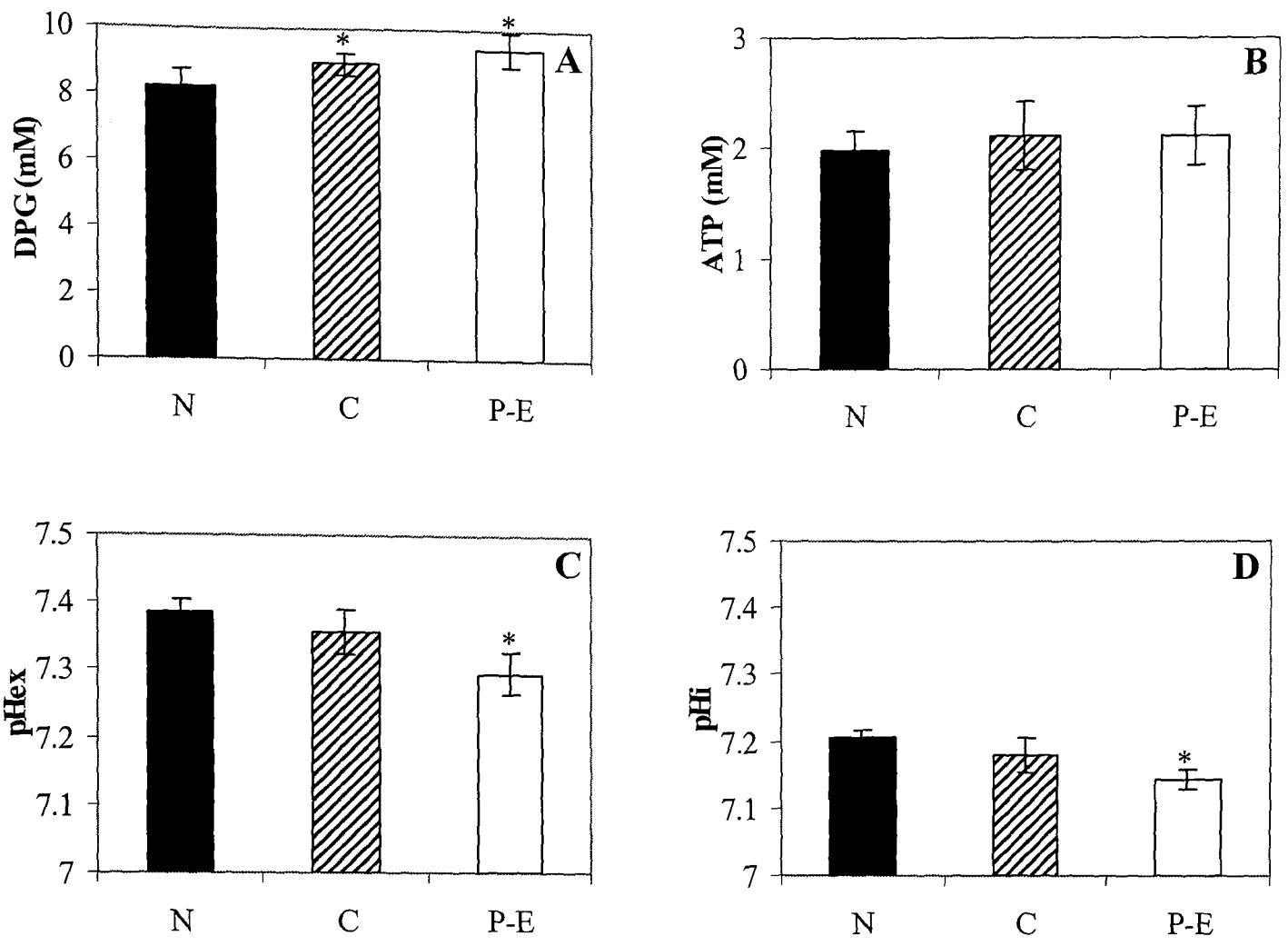


Fig. 9.1 Blood parameters from normal non-pregnant subjects (N), control pregnant subjects (C) and pre-eclamptic subjects (P-E). **(A)** Intracellular DPG, **(B)** intracellular ATP, **(C)** extracellular pH, **(D)** intracellular pH, **(E)** haematocrit, **(F)** intracellular $[Mg^{2+}]_{free}$ **(G)** total intracellular Mg, calculated by the model, **(H)** total intracellular Mg, measured by colorimetric assay.
* denotes significance ($p < 0.05$).

Pregnancy caused a significant increase in erythrocytes in DPG concentrations and a decrease in haematocrit. Patients with pre-eclampsia demonstrated a significantly lower extracellular pH for samples of whole blood aerated at pO_2 of 150 mmHg and a pCO_2 of 40 mmHg, which was coupled with significant acidification of pH_i. No significant difference was seen in ATP, $[Mg^{2+}]_{free}$ or Mg_T, although there was a trend of the latter to increase in pregnant subjects.

9.3 DISCUSSION

An increase in the concentration of DPG would be expected during pregnancy to ensure efficient delivery of oxygen to the developing foetus (MacLennan et al., 1976). In previous Chapters, conditions that increased in DPG, anaemia and hypoxia, also decreased in $[Mg^{2+}]_{free}$ due to the increased buffering capacity of Mg^{2+} as DPG rose. However, in pregnancy, whether or not complicated by high blood pressure, no change in $[Mg^{2+}]_{free}$ was observed. This could have occurred if (i) either the concentration of another Mg-buffer decreased, but no change in ATP, the other main buffer, was seen, or if (ii) the total Mg concentration in the erythrocytes increased. Although not statistically different in this study, there was a clear trend of increased Mg_T and were the data from the normal group studied in Chapter 3 used, then statistical significance would have ben achieved.

The reason behind the lack of a clear difference in Mg_T in this study could be readily understood. Although the rise in DPG during pregnancy was ~1 mM, representing a relative change of ~13%, this would have caused a decrease in erythrocyte $[Mg^{2+}]_{free}$ by ~0.1 mM if Mg_T had remained constant, as only 10% of DPG complexes with Mg. But, as one third of Mg_T is bound to DPG (see Table 3.6), this decrease could have been offset by an increase in Mg_T by 0.3 mM. This predicted change is of the order of the standard deviation of the measurement of Mg_T and more

than 6 control subjects would have been required to produce a statistical difference. Having said this, the trend was for the Mg_T to increase by ~0.3 mM, as predicted by the model, and this suggests that, during pregnancy, erythrocytes increase Mg_T in compensation for the obligatory increase in DPG. This would allow the erythrocytes to maintain constant $[Mg^{2+}]_{free}$ and to continue normal regulation of cellular metabolism.

However, the extra intracellular magnesium must have come primarily from the plasma. As the total magnesium plasma in all groups was 0.89 ± 0.05 mM, the erythrocytes would have required about a 25% of this to maintain $[Mg^{2+}]_{free}$ (allowing for the difference between total plasma volume and total erythrocyte volume), but no change in plasma Mg^{2+} was observed. The lack of any change in plasma total Mg concentrations was in agreement with that found previously (Altura and Altura, 1996), but those workers also found that ionised Mg^{2+} in the plasma of women at term delivery were 20% less than in non-pregnant controls.

This may have important consequences for the development of hypertension during pregnancy, in that the absorption by erythrocytes of plasma Mg^{2+} may be sufficient to lower Mg^{2+} such that $[Ca^{2+}]_{free}$ in other tissues begins to rise. This may be particularly highlighted for individuals already suffering with magnesium deficiency, an increasingly common condition in the western world. Or, it may be that individuals who develop pre-eclampsia have a problem mobilising the body stores of magnesium to cope for the increase in demand by erythrocytes. As mentioned above, it is unlikely that magnesium alterations are entirely responsible, but it may be that they exacerbate an existing dysfunction, ultimately leading to vasoconstriction and a rise in blood pressure.

An identical pathology has been identified in alcohol-induced hypertension and stroke, where ingestion of alcohol, a notorious cause of Mg-wasting, was clearly shown to result in a reduction of Mg from vascular smooth muscle cells with a concomitant increase in cellular Ca (Altura and Altura, 1994). Furthermore, these studies also demonstrated that pretreating animals with Mg prevented ethanol from inducing stroke.

As in pregnancy, a rise in Mg_T in response to alterations in steady-state concentrations of metabolites has been reported for patients with hereditary pyrimidine-5'-nucleotidase deficiency, who had increased levels of UTP and CTP in erythrocytes, which also bind Mg^{2+} (Swanson et al., 1983). As a result, erythrocytes from these patients also contained higher concentrations of Mg_T while $[Mg^{2+}]_{free}$ remained constant.

The decrease in pH_i agreed with previous work that reported a significant and consistent inverse relationship between intracellular pH and blood pressure (Resnick et al., 1987). However, it remains difficult to decide whether the changes in pH_i were primarily involved in the cause of hypertension or were reflective of it. Even if the decreased pH_i were a consequence of the hypertension, it might still, in turn, contribute to the blood pressure. One possible hypothesis is that increased plasma Ca^{2+}, caused by a decrease in Mg^{2+}, may have led to activation of the Ca^{2+}/H^+ exchange pump, which has been identified in erythrocytes. This enables the erythrocytes to maintain low intracellular Ca^{2+} levels, but with a concomitant increase in H^+_i, and therefore a decrease in pH_i. However, there was a complicating factor that at the same pCO_2, whole blood from pre-eclamptic patients was significantly acidic. The reasons for this are unknown, although the observed decreased haematocrit would have changed the overall buffering ability of the blood and a decrease in pH_{ex} would

have been expected. This decrease in pH_{ex} would also play a part in decreasing pH_i through the Donnan ratio, although this too would have been expected to change in response to changing pH_{ex} (Duhm and Gerlach, 1971). Indeed, the ratio, when measured by H^+_{ex}/H^+_i (Table 9.1), did increase with decreasing pH_{ex}, as expected, and helped to confirm the finding of decreased blood pH in patients with pre-eclampsia.

In summary, natural shifts in magnesium stores must occur during pregnancy in response to the change in tissue oxygen requirement to allow normal erythrocyte metabolic function. However, the changing O_2 demand may not be able to be met in certain individuals, either directly or as part of a greater dysfunctional state, and it is possible that the end result in part causes an increase in $[Ca^{2+}]_{free}$ and subsequent vasoconstriction, leading to a rise in blood pressure and possible eclampsia.

CHAPTER 10

Case Studies

CHAPTER 10 – CASE STUDIES

In this Chapter, two clinical settings are discussed following preliminary investigations. However, in both cases, a more detailed study was not possible due to logistical complications.

10.1 CHRONIC FATIGUE SYNDROME

10.1.1 Introduction

Chronic fatigue syndrome, CFS, or benign myalgic encephalomyelitis, ME, (also known as postviral fatigue syndrome, epidemic neuromyasthenia, Iceland disease and Royal Free disease) has been extensively described and investigated over the past decade, but has probably been endemic for centuries (Simpson, 1989; Cox et al., 1991b; Hinds et al., 1994; Moorkens et al., 1997; Manuel y Keenoy et al., 2000) and for reviews see (Werbach, 2000). As long ago as the second century, Galen described a disease complex similar to CFS (Cox et al., 1991b). It is a disabling, often painful, and little-understood disorder of unknown origin and uncertain outcome. Its duration appears to be variable; some people seem to recover completely, or experience periods of remission and relapse; others develop a condition of unresolving physical and/or cognitive disability, while a further small but significant minority deteriorate to a housebound and ultimately immobilised state. As case history research on CFS is still in its infancy, and the disease is chronic, this last category may be considerably larger than has been supposed.

The syndrome is thought to be caused by viral infection associated with defective immunoregulation (Mukherjee et al., 1987). It is characterised by a variety of symptoms but mainly fatigue and myalgia (Shafran, 1991; Komaroff, 1993). Consensus definitions have been published which include the presence of fatigue for

at least six months duration (see Table 10.1). Whilst aetiological factors producing this syndrome remain the subject of debate, patients are considerably debilitated by the symptom of fatigue which is present at rest, made worse by exercise which is then followed by a prolonged period of exhaustion (Gibson et al., 1993).

Table 10.1 International CFS Study Group definition of Chronic Fatigue Syndrome

1. Clinically evaluated, unexplained persistent or relapsing chronic fatigue that: - is of new or definite onset (has not been lifelong. - is not the result of ongoing exertion. - is not substantially alleviated by rest. - results in substantial reduction in previous levels of occupational, educational, social, or personal activities.
2. The concurrent occurrence of four or more of the following symptoms, all of which must have persisted or recurred during six or more consecutive months of illness and must not have predated the fatigue: - self-reported impairment in short-term memory or concentration severe enough to cause substantial reduction in previous levels of occupational, educational, social, or personal activities - sore throat - tender cervical or axillary lymph nodes - muscle pain - multi-joint pain without joint swelling or redness - headaches of a new type, pattern, or severity - unrefreshing sleep - postexertional malaise lasting more than 24 hours.

Data from (Werbach, 2000).

The current interest in attempting to define and treat CFS stems from several studies in the mid-1980's that found elevated levels of antibody to Epstein-Barr virus in people with CFS-like symptoms, most of whom had had a history of infectious mononucleosis a few years earlier (Cox et al., 1991b). It later became apparent that healthy people could also have elevated Epstein-Barr virus antibody titers while some CFS sufferers had normal titers. Despite considerable worldwide efforts, no single aetiology has been found to explain the syndrome. It is considered likely that multiple factors promote its development, sometimes with the same factors both

causing and being caused by the syndrome. Many of these factors constitute specific pathophysiological entities that characterise certain subsets of chronic fatigue patients (Werbach, 2000).

10.1.2 The use of magnesium in the treatment of CFS

The investigation of the role of magnesium in CFS was previously initiated because it had been noted that many of the symptoms of CFS were similar to those of magnesium deficiency; namely nausea, learning disability, personality change, weakness, tiredness and myalgia (Cox et al., 1991b; Durlach et al., 1997) along with palpitations, paraesthesia and depression (Hinds et al., 1994). Cox et al published results in 1991, demonstrating that not only did patients with CFS have low red blood cell magnesium (inferring from this an overall magnesium deficiency), but also that magnesium treatment by intramuscular injections alleviated the symptoms of fatigue and restored magnesium levels in erythrocytes (Cox et al., 1991b).

However, these findings have been disputed and debated (Wessely, 1991; Young and Trimble, 1991; Richmond, 1991; Shepherd, 1991; Cox et al., 1991a; Davies, 1991; Gantz, 1991; Deulofeu et al., 1991; Clague et al., 1992; Howard et al., 1992) with some clinicians finding no evidence of magnesium deficiency in plasma, erythrocyte or whole blood measurements (Clague et al., 1992). A final report on this was published in 1994 after a study of a much larger patient cohort, which concluded that erythrocyte magnesium concentrations in patients with CFS were not significantly altered form normal (Hinds et al., 1994). However, no study has yet assessed whether the physiologically active $[Mg^{2+}]_{free}$ is altered in the erythrocytes of patients with CFS.

10.1.3 Alterations in blood cells in patients with CFS

It was first observed in the late-1980's that patients with CFS exhibited abnormal erythrocyte morphology, with deformations occurring as spherocytes, stomatocytes and unusual forms, best described as dimpled spherocytes (Simpson et al., 1986; Mukherjee et al., 1987; Simpson, 1989). However, the pathology was further complicated by the observation that the deformations were often transient. Even so, it was concluded that such changes could impair microcirculation and exert a profound effect on mass exchange of oxygen and lactate. This would at least give some explanation for the feeling of fatigue experienced by the patients, although clearly the cause of the deformation was still unknown.

The effect of changes to red cell shape on muscular fatigue was further investigated (Simpson, 1991; Simpson, 1992; Simpson et al., 1993) with the continuing conclusion of an association between increased levels of nondiscocytes and greater physiological concept of fatigue as a consequence of inadequate oxygen delivery. Further evidence that CFS is related to circulatory dysfunction and not inherent muscular dysfunction has been put forward as contractile properties of muscle in CFS patients were demonstrated to be normal and recovery of muscle function occurred identically to normal subjects (Gibson et al., 1993). In the same study, patients appeared not to exercise to their full physiological capacity because the exercise was associated with a greater perception of effort.

10.1.4 Methods and Results

Because of the reported changes to erythrocytes in patients with CFS and the possibility of alterations to normal magnesium metabolism, blood samples were taken from consenting volunteers, who had previously been diagnosed with CFS.

Inclusion criteria and sampling of blood

Two volunteers, diagnosed as suffering CFS, defined above in Table 10.1, were admitted on three separate occasions over a two-month period. At each visit, one 15 ml blood sample was taken and analysed in duplicate as described in Chapter 2. The results are presented in Table 10.2 and Figure 10.1.

Table 10.2: Values obtained from erythrocytes from normal subjects (n = 33) and from two volunteers diagnosed with CFS. Three blood samples were obtained over 2 months and analysed in duplicate, generating 6 data points.

Parameter	Normal[a]	CFS 1	CFS 2
DPG (mM)	7.7 ± 0.5	6.29 ± 0.20#	7.03 ± 0.11#
ATP (mM)	1.85 ± 0.27	1.73 ± 0.16	1.85 ± 0.13
pH_i	7.20 ± 0.02	7.18 ± 0.01*	7.19 ± 0.01
pH_{ex}	7.39 ± 0.03	7.36 ± 0.02	7.36 ± 0.02
$[Mg^{2+}]_{free}$ (mM)	0.41 ± 0.03	0.48 ± 0.05#	0.45 ± 0.02*
$Mg_{T,m}$ (mM)	3.10 ± 0.3	3.01 ± 0.24	3.14 ± 0.08

Data are presented as means ± SD. The following indicate significance using a statistical paired t-test:
*denotes $p < 0.05$ vs. Normal. #denotes $p < 0.01$ vs. Normal.
[a]Normal values were previously presented in Table 3.7.

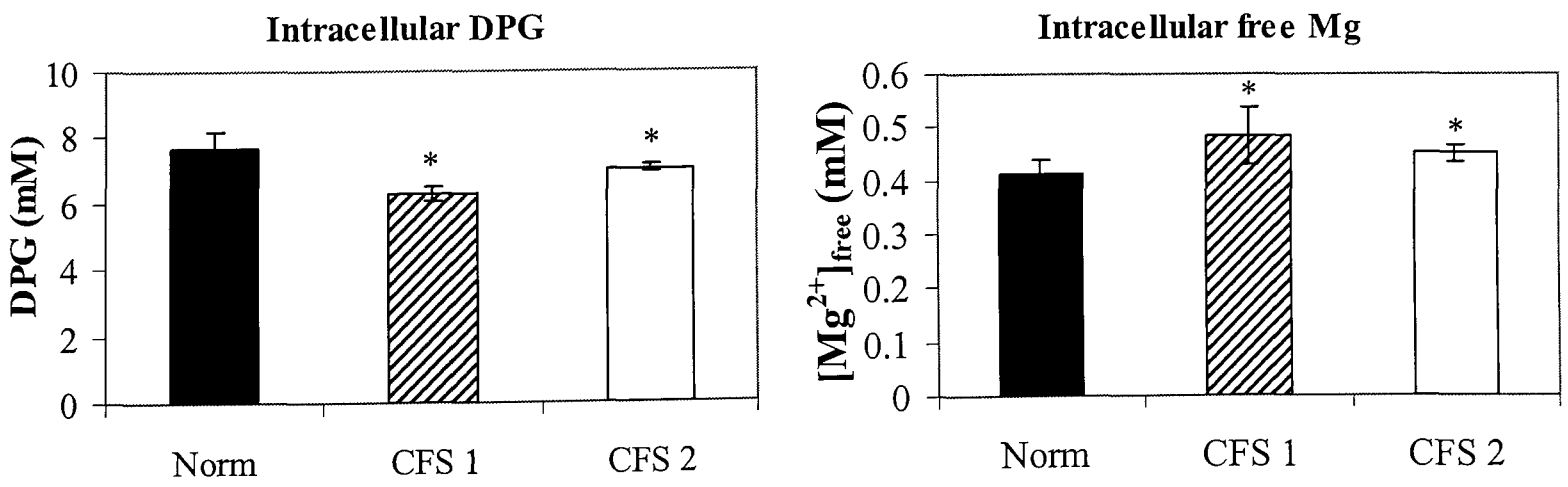


Fig. 10.1 Concentrations of intracellular DPG and $[Mg^{2+}]_{free}$ in patients diagnosed with CFS, compared with normal values. * denotes significance ($p<0.05$).

Both CFS volunteers had significantly lower concentrations of DPG in their red blood cells compared with normal ($p<0.01$) and correspondingly, both $[Mg^{2+}]_{free}$ were increased. No other differences were observed, apart from CFS 1 having a significantly lower intracellular pH, although the absolute difference was small.

10.1.5 Discussion

Although this clearly may only be regarded as a pilot study, the finding of significantly lower DPG concentrations was of considerable interest. By lowering the DPG concentration, the cells' ability to deliver oxygen would be reduced, and this certainly may exacerbate the problem of fatigue, particularly in situations where oxygen utilization by the muscle cells would be high, i.e. during exercise.

It was worth considering what the cause of this decrease in DPG might have been. Previous Chapters have developed the link between $[Mg^{2+}]_{free}$ and DPG concentrations, and in this case, although there was no change in total Mg in the cell, in agreement with the largest study of total magnesium levels in CFS patients (Hinds et al., 1994), $[Mg^{2+}]_{free}$ was seen to be increased as the level of its major buffer decreased. A change in pH was most likely to cause such a decrease, as it has been shown that DPG concentrations are particularly sensitive to pH (Mulquiney and Kuchel, 1999). A possible hypothesis for this might use the observation of altered red cell shape and subsequent impaired microcirculation, to suggest that in reducing overall flow rates of erythrocytes through areas of high oxygen utilisation and high CO_2 production in muscular capillary beds, erythrocytes were exposed to more deoxygenated, acidic conditions. In doing so, erythrocyte pH would decrease, which is known to prevent DPG production, and hence lower its intracellular concentration. As DPG concentration lowered, $[Mg^{2+}]_{free}$ would increase, which in turn would stimulate DPG production and hence re-establish the dynamic equilibrium, but with

altered steady-state conditions, namely reduced DPG. Along with the impaired circulation, this might help cause the observed fatigued state by reducing the cell's ability to efficiently transfer oxygen.

In clinical practice, it has been reported that, aside from whether or not measured levels of magnesium are altered, patients often derive significant relief from their symptoms following treatment with magnesium. It seems possible that such dosing might help increase the level of $[Mg^{2+}]_{free}$ and so also increase the level of DPG, as it has already been shown that increases in $[Mg^{2+}]_{free}$ can cause increases in DPG. Although the effect that this dosing would have on erythrocyte shape, and therefore also circulation, is unknown, at least the erythrocytes' transfer of oxygen would be ameliorated.

It was disappointing that this study could not be progressed further, given promising initial results. This was despite repeated attempts to contact the necessary clinicians. However, as CFS is poorly understood, the clinical department to which patients are directed is in a constant state of flux, with the result of a distinct lack of focus for their care. An apparent lack of cooperation among investigators was reported in 1991 as a disappointing aspect of CFS/ME research (Simpson, 1991), when it was also noted that 'while immunologists, virologists and behavioural scientists continued to retain entrenched positions, those who suffer the problems and frustrations of CFS seemed to have been dismissed to the sidelines rather than retained centre field'. It would appear that 10 years on, these issues still remain, with sufferers, such as the two volunteers who kindly assisted in this study, all too frequently left to fend for themselves.

10.2 IV INFUSION OF $MgSO_4$

A pilot study was undertaken of the effect of continuous intravenous infusion of magnesium sulphate on blood from a normal volunteer.

10.2.1 Treatment protocol

One volunteer received an intra-venous loading dose of 16 mmol $MgSO_4$ in 10 ml 0.9% NaCl over 30 minutes. An intra-venous maintenance dose was administered of 16 mmol $MgSO_4$ in 12 ml 0.9% NaCl over 6 hours. Blood samples were taken from a separate vein immediately prior to infusion, after 2 hours infusion, then after 4 hours, and finally one sample taken 22 hours after the infusion had been stopped. All samples were analysed immediately. Hourly blood pressure and pulse rate were recorded.

10.2.2 Results and Discussion

In the initial sample, all parameters of DPG, ATP, $[Mg^{2+}]_{free}$, Mg_T, pH_i, pH_{ex} were within the normal ranges described in Table 3.7. The parameters that were seen to change during and after infusion are presented in Table 10.3.

Table 10.3: Parameters that varied during $MgSO_4$ IV infusion.

Parameter	Initial	2 hours	4 hours	28 hours
DPG (mM)	7.64	8.00	8.10	6.85
pH_{ex}	7.37	7.34	7.27	7.37
Plasma Mg_T (mM)	0.83	4.15	4.70	0.89
Haematocrit (%)	44	42	38	47

It can be seen that the primary aim of increasing and maintaining high plasma magnesium levels was achieved. However, this high level, approximately 5 times

normal concentration, produced a significant decrease in both external pH and haematocrit over the measured time period. The reasons for this were unknown.

Clinically, a rapid and marked increase in urinary output was noted, presumably as renal function increased. The subsequent obligatory loss in magnesium was sufficient to have returned plasma magnesium concentrations to normal within 22 hours of cessation of infusion. It seemed unlikely that the volunteer dehydrated as sufficient drinking water was available, and dehydration would have been expected to cause an increase in haematocrit. As only small blood samples were taken, one possible explanation for the observed decrease in haematocrit might have been a decrease in size of the red blood cells, suggesting that the cells themselves dehydrated. The mechanism or cause of this was unknown, but possible complications might have been the increase in plasma concentration of SO_4^{2-} ions to an unphysiological level of 8 mM, and the fact that the volunteer experienced feelings of anxiety.

The observed fluctuation in DPG suggested that the infusion did have some effect on intracellular metabolism, but a larger study, preferably with longer infusion times, would be required to confirm this.

CHAPTER 11

General Discussion and Future Directions

CHAPTER 11 – GENERAL DISCUSSION AND FUTURE DIRECTIONS

Magnesium is being increasingly recognised as important for a wide range of cellular functions. However, elucidating the exact role of Mg and the extent to which it can regulate metabolic function has, so far, proved difficult. This is largely due to the problems of measuring an intracellular ion that is strongly bound to other cell constituents and has very low flux across the cell membrane. The problem is compounded by the lack of an experimentally useful radioactive isotope.

The best non-invasive method for measuring intracellular $[Mg^{2+}]_{free}$, ^{31}P NMR spectroscopy, was originally devised in 1978, but since then several problems and errors have been highlighted with the methodology. To address these issues, more detailed and comprehensive analyses of NMR spectroscopic data has been developed in this project, which has been shown to accurately measure intracellular $[Mg^{2+}]_{free}$ and pH in erythrocytes over a wide range of physiological conditions.

The model presented in this work is the first to use explicit association constants and chemical shifts of all the important chelators of Mg^{2+} in the erythrocyte and has demonstrated that the competitive binding effects of Hb must be included for accurate measurements to be made. In doing so, the model was able to account for all Mg within the erythrocyte, which no other NMR-based analysis has previously achieved. A major advantage is that, should future work clarify the association constants or chemical shifts, especially those involving Hb, they can be easily incorporated into the equations.

A novel feature of the model was to include pH and the competitive binding effects of H^+. This allowed the analysis to be applied with greater flexibility, especially for conditions in which changes in pH may be part of the pathological state. Because of this emphasis on application to *in vivo* conditions, analysis was performed

on whole blood that had been prepared to simulate arterial blood. Very few techniques use this approach, despite its obvious significance, choosing instead to treat erythrocytes in *in vitro* conditions. By doing so, erroneous conclusions have been reached. For example, it has been reported that deoxygenation of erythrocytes led to an increase in intracellular pH of 0.07–0.14 pH units due to deoxy-Hb having a higher affinity for protons than oxy-Hb (Duhm, 1971; Labotka, 1984; Mulquiney et al., 1999). Deoxygenation was, in general, achieved by aerating erythrocytes with 100% N_2. The subsequent removal of O_2 from Hb, destabilising the oxy-Hb structure, would indeed cause alkalisation due to an increased association to H^+ as oxy-Hb reconfigured to deoxy-Hb. However, this is the reverse of the *in vivo* situation, where the primary change of acidification around actively respiring muscle cells destabilises oxy-Hb to promote release of O_2. Hence, venous blood is more acidic than arterial blood, and should not be considered fungible with deoxygenated erythrocytes. This is important to consider if certain conditions have acidic arterial blood, for example sickle cell anaemia or hypertension, in which low pH is thought to contribute to the pathology, as the change to venous blood can only exacerbate the problem.

Furthermore, it has been hypothesised that as high pH causes DPG production, increased deoxygenation of erythrocytes in conditions of anaemia or hypoxia, and therefore higher pH, could explain the mechanisms behind increased concentrations of DPG found in such conditions. However, this cannot be the case. Instead, deoxygenated conditions would cause an increase in $[Mg^{2+}]_{free}$ as binding of DPG by Hb would increase, in turn the high $[Mg^{2+}]_{free}$ would modulate DPG production. Coupled with this, it could be hypothesised that partially unsaturated oxygenated blood cannot absorb as much CO_2, and as such the overall circulating blood would be more alkaline. Or, if O_2 levels drop below 60 mmHg, ventilation would increase as

described in Chapter 1, causing respiratory alkalosis. Both hypotheses would cause increases in pH and thus help stimulate DPG production.

It should be remembered that in all preparations of whole blood in this work, the pCO_2 was set at 40 mmHg, as *in vivo*. However, in some conditions, for example sickle cell anaemia, the diminished quantity of erythrocytes may have caused arterial pO_2 to fall below 60 mmHg, causing these patients to increase breathing. As explained above, the satisfaction of oxygen demand can only occur at decreased pCO_2 and such chronic increases in ventilation could cause the medullary chemoreceptors to be adjusted to the lower level of pCO_2. Therefore, when blood from sickle cell patients is analysed, setting the pCO_2 at 40 mmHg may have been higher than exists in the pathological state and would explain the more acidic pH_{ex}.

If this were the case, it would imply that sickle patients do not have quite as acidic pH_i as found in this work, but at a higher pH, Mg binding would increase, and therefore $[Mg^{2+}]_{free}$ would be even more decreased. It would therefore appear that there is a very fine balance in sickle cells between a lower pH_i and maintaining $[Mg^{2+}]_{free}$, which would increase the risk of HbSS polymerisation, or a higher pH_i but with it a lower $[Mg^{2+}]_{free}$, which would stimulate KCl cotransport, dehydrating the cell and once again causing HbSS polymerisation. It may be that sickle cell crises are initiated when this fine balance is disturbed.

One improvement to this technique would be to develop *in vivo* NMR measurements of blood to the same resolution and accuracy as those performed *in vitro*, thus removing the necessity of simulating arterial conditions (by leaving the blood cells *in situ*. Recent advances in localised spectroscopy to small volumes may aid this development, and it may not be long before measurements of arterial blood may be taken directly from arteries or cardiac ventricles, helping introduce this

analysis of $[Mg^{2+}]_{free}$ and pH_i to standard clinical practice. Such progress can only help in the understanding of this important ion.

Although some previous studies have aerated blood with 95% O_2 and 5% CO_2, few have then confirmed that the level of pCO_2 was 40 mmHg. In fact, aerating in this manner causes pCO_2 to be about double this value, causing very acidic pH_{ex} (Figure 11.1). This can be explained by the buffering capacity of haemoglobin, and the dissociation of carbonic acid, coupled with the exchange of HCO_3^- for Cl^-. Thus if whole blood were exposed to a high CO_2 tension, these factors would enable the plasma to take up more CO_2 than it would have done if the cells and plasma were previously separated at a low pCO_2 and the plasma then exposed to the high pCO_2 by itself. This was confirmed by the fact that sickle cell whole blood, with lower total haemoglobin content, exhibited a different buffering capacity to normal whole blood (Figure 11.1). Failure to take such buffering considerations into account would lead to inaccurate pH measurements.

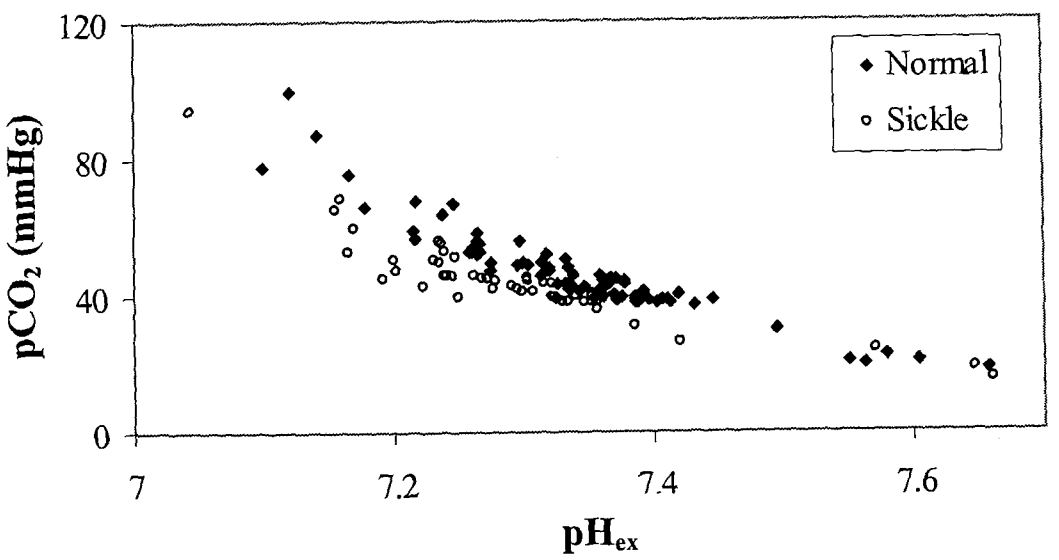


Fig. 11.1 Plot of pCO_2 vs. external pH in fully oxygenated whole blood.

Given that DPG plays an important role in defining the Donnan equilibrium (Duhm, 1971; Raftos et al., 1986), it is interesting to compare the chloride ratio, *r*,

between all the clinical conditions evaluated in this project. From a normal value of 0.65 ± 0.03, two conditions demonstrated significant changes in *r*; sickle cells had a significantly lower value at 0.61 ± 0.05, and erythrocytes from pre-eclamptic patients were significantly increased at 0.70 ± 0.04. Both conditions had decreased pH_{ex}, which is known to increase *r*, but the fact that it decreased in sickle cells may well be due to change in charge caused by the mutation of Hb.

Table 11.1 A summary of pH_{ex}, pH_i and $[Mg^{2+}]_{free}$ for all conditions analysed in this work.

Condition	Extracellular pH	Intracellular pH	$[Mg^{2+}]_{free}$ (mM)
Normal	7.39 ± 0.03	7.20 ± 0.02	0.41 ± 0.03
Hypoxia	7.36 ± 0.01	7.17 ± 0.01	0.37 ± 0.05
Sickle Cell Anaemia	7.32 ± 0.03	7.10 ± 0.03	0.32 ± 0.05
Sub-arachnoid hmg	7.35 ± 0.04	7.17 ± 0.02	0.38 to 0.52
Pre-eclampsia	7.30 ± 0.03	7.15 ± 0.01	0.38 ± 0.04
Chronic Fatigue	7.36 ± 0.02	7.19 ± 0.01	0.47 ± 0.04

This work has focused on intracellular Mg^{2+}, but until this measurement can be performed routinely, serum/plasma Mg levels will continue to be the standard clinical test of body magnesium status. However, it remains difficult to assess the exact relationship between intra- and extracellular levels. It seems likely that once steady state conditions are achieved, the plasma levels will be normal (through renal function) although intracellular Mg may be held at a different level from normal, depending on circumstance (through metabolic control). But if the conditions were in a state of flux, it would be possible that changes in plasma would correlate to changes in intracellular Mg. This would explain how some workers have found correlations in

loading studies (Reinhart et al., 1990) but no correlations have been seen over longer time periods (de Valk et al., 1998). For example, it has been shown that erythrocyte Mg correlated with serum Mg concentration when normal subjects, given a low Mg diet for 4 weeks, demonstrated a progressive fall in both the serum Mg concentration and erythrocyte Mg during Mg depletion (Ryzen, 1989). Conversely, over a longer period of time, dosing with water at 110 mM Mg^{2+} increased erythrocyte Mg but not plasma Mg (Thomas et al., 2000). But before this relationship can be fully defined, further studies are required on Mg transport and the absorption of Mg into the bloodstream from the gut.

This work, primarily, has provided an accurate and reliable method for measuring Mg^{2+} in erythrocytes. However, the explicit formulations of multiple equilibria involving Mg and metabolites could be used in a much wider setting, from measuring $[Mg^{2+}]_{free}$ in other tissues to the setting of precise $[Mg^{2+}]_{free}$ levels in standard buffers and solutions. By improving the accuracy of measuring and controlling $[Mg^{2+}]_{free}$ it is hoped that the role of Mg^{2+} can be investigated further. This will become increasingly important if the western world continues to decrease its intake of Mg, but simultaneously increase the body's demand for the ion. The range of pathologies associated with Mg deficiency is staggering, and those presented in this Thesis represent a fraction of the total. It is highly regrettable that the deficiency of such an inexpensive, low-toxicity nutrient results in diseases that cause incalculable suffering and expense across the world. Hopefully, improved measurements will lead to greater understanding of the potential for therapeutic use of magnesium.

APPENDICES

APPENDIX I - MATHEMATICA PROGRAMME TO PRODUCE THE STANDARD TITRATION SOLUTIONS

```
Off[General::"spell", General::"spell1"]

TotATP=0.005;
TotPi=0.005;
TotDPG=0.005;
TotKCl=0.17834;

pH=8.5;
H=10^-pH;
TotMg=0.0101222;

KaHPO4=0.0000001698;
KbMgHPO4=74.8;
KaATP=0.000000313;
KaHATP=0.000191;
KbMgATP=15723;
KbMgHATP=108;
KaDPG=0.000000009846;
KaHDPG=0.0000003392;
KbMgDPG=2925;
KbMgHDPG=243.7;

Mg=.;HPO4=.;ATP=.;H2PO4=.;MgHPO4=.;HATP=.;H2ATP=.;MgATP=.;
MgHATP=.;DPG=.;HDPG=.;H2DPG=.;MgDPG=.;MgHDPG=.;sHPi=.;

unknowns={Mg,HPO4,ATP,DPG,H2PO4,MgHPO4,HATP,H2ATP,MgATP,
      MgHATP,HDPG,H2DPG,MgDPG,MgHDPG,sHPi}
soln=
   Solve[{KaHPO4*H2PO4== =H*HPO4,KbMgHPO4*Mg*HPO4== =MgPO4,
      KaATP*HATP== =H*ATP,KaHATP*H2ATP== =H*HATP,
      KbMgATP*Mg*ATP== =MgATP,KbMgHATP*Mg*HATP== =MgHATP,
      KaDPG*HDPG== =H*DPG,KaHDPG*H2DPG== =H*HDPG,
      KbMgDPG*Mg*DPG== =MgDPG,KbMgHDPG*Mg*HDPG== =MgHDPG,
      TotMg== =Mg+MgHPO4+MgATP+MgHATP+MgDPG+MgHDPG,
      TotPi== =HPO4+H2PO4+MgHPO4,
      TotATP== =ATP+HATP+H2ATP+MgATP+MgHATP,
      TotDPG== =DPG+HDPG+H2DPG+MgDPG+MgHDPG,
      sHPi== =HPO4+MgHPO4},unknowns]

{Mg,HPO4,ATP,DPG,H2PO4,MgHPO4,HATP,H2ATP,MgATP,
   MgHATP,HDPG,H2DPG,MgDPG,MgHDPG,sHPi}=Part[unknowns/.soln,3]

vH=1;
vMg=2;
vCl=1;
vNa=1;
```

vDPG=5;
vHDPG=4;
vH2DPG=3;
vMgDPG=3;
vMgHDPG=2;
vK=1;
vHPO4=2;
vH2PO4=1;
vATP=4;
vHATP=3;
vH2ATP=2;
vMgATP=2;
vMgHATP=1;

Cl=(2*TotMg)+TotKCl;
Na=5*TotDPG;
K=TotPi+(2*TotATP)+TotKCl;

iH=H*vH^2/2;
iMg=Mg*vMg^2/2;
iCl=Cl*vCl^2/2;
iNa=Na*vNa^2/2;
iK=K*vK^2/2;
iHPO4=HPO4*vHPO4^2/2;
iH2PO4=H2PO4*vH2PO4^2/2;
iATP=ATP*vATP^2/2;
iHATP=HATP*vHATP^2/2;
iH2ATP=H2ATP*vH2ATP^2/2;
iMgATP=MgATP*vMgATP^2/2;
iMgHATP=MgHATP*vMgHATP^2/2;
iDPG=DPG*vDPG^2/2;
iHDPG=HDPG*vHDPG^2/2;
iH2DPG=H2DPG*vH2DPG^2/2;
iMgDPG=MgDPG*vMgDPG^2/2;
iMgHDPG=MgHDPG*vMgHDPG^2/2;
SumIonicStr=iH+iMg+iCl+iNa+iK+iHPO4+iH2PO4+iATP+iHATP+iH2ATP+
iMgATP+iMgHATP+iDPG+iHDPG+iH2DPG+iMgDPG+iMgHDPG
TotMg
pH
Mg
K

APPENDIX II - MATHEMATICA PROGRAMME TO DETERMINE PH AND $[MG^{2+}]_{FREE}$ FROM CHEMICAL SHIFTS

```
(***Define Module to calculate Chemical Shifts from Mgf and pH***)

SCalcds[Mgf_,pH_] := Module[{},

    TotATP=0.0018;
    TotDPG=0.007;
    TotHb=0.00702;
    TotK=0.160;

    KaATP=0.0000002;
    KaHATP=0.000191;
    KaDPG=0.000000078;
    KaHDPG=0.000000446;
    KbMgHATP=5500;
    KbMgHDPG=378;

    KbKATP=12;
    KbKHATP=4;
    KbKDPG=12.9;
    KbKHDPG=10.84;

    KbHbDPG=250;
    KbHbATP=180;
    KbHbMgATP=30;
    KbHbKATP=70;
    KbHbKHATP=30;
    KbHbHATP=70;
    KbHbMg=0.25;

    KbMgATP=75000;
    KbMgDPG=2400;

H=10^-pH;DPGf=.;ATPf=.;Hbf=.;Kf=.;
HbDPG=.;HbATP=.;HbMgATP=.;HbKATP=.;HbKHATP=.;HbHATP=.;HbMg=.;
MgATP=.;MgDPG=.;HATP=.;H2ATP=.;MgHATP=.;
HDPG=.;H2DPG=.;MgHDPG=.;KATP=.;KHATP=.;KDPG=.;KHDPG=.;
dbATP=.;
d3PDPG=.;

dATP=19.1;
dMgATP=15.69;
dHATP=20.6;
dH2ATP=21.5;
dMgHATP=17.4;
dKATP=18.36;
dKHATP=19.6;
```

```
dDPG=6.901;
dMgDPG=6.346;
dHDPG=5.69;
dH2DPG=3.1316;
dMgHDPG=3.789;
dKDPG=6.735;
dKHDPG=4.737;

hbksoln=
    Solve[
        {-DPGf= =-TotDPG+MgDPG+HbDPG+HDPG+MgHDPG+H2DPG+KDPG+
        KHDPG, -ATPf= =-TotATP+MgATP+HbATP+HbMgATP+HATP+
        MgHATP+H2ATP+KATP+KHATP+HbKATP+HbKHATP, -Hbf= =-TotHb+
        HbATP+HbDPG+HbMgATP+HbKATP+HbKHATP+HbMg+HbHATP,
        Hbf= =HbDPG/KbHbDPG*DPGf, Hbf= =HbATP/KbHbATP*ATPf,
        Hbf= =HbMgATP/KbHbMgATP*MgATP,
        Hbf= =HbMg/KbHbMg*Mgf, ATPf= =MgATP/Mgf*KbMgATP,
        ATPf= =KATP/Kf*KbKATP, DPGf= =MgDPG/Mgf*KbMgDPG,
        DPGf= =KDPG/Kf*KbKDPG, HATP= =H*ATPf/KaATP,
        KaHATP= =H*HATP/H2ATP, HATP= =MgHATP/Mgf*KbMgHATP,
        HATP= =KHATP/Kf*KbKHATP, KaDPG= =H*DPGf/HDPG,
        KaHDPG= =H*HDPG/H2DPG, HDPG= =MgHDPG/Mgf*KbMgHDPG,
        HDPG= =KHDPG/Kf*KbKHDPG,
        dbATP= =(((MgATP+HbMgATP)/TotATP)*dMgATP)+
        (((ATPf+HbATP)/TotATP)*dATP)+(((HATP+HbHATP)/TotATP)*dHATP)
        +(((MgHATP/TotATP)*dMgHATP)+(((KATP+HbKATP)/TotATP)*dKATP)
        +(((KHATP+HbKHATP)/TotATP)*dKHATP)+
        (((H2ATP/TotATP)*dH2ATP),
        d3PDPG= =(((MgDPG)/TotDPG)*dMgDPG)+
        (((DPGf+HbDPG)/TotDPG)*dDPG)+(((HDPG)/TotDPG)*dHDPG)
        +(((MgHDPG/TotDPG)*dMgHDPG)+(((KDPG)/TotDPG)*dKDPG)
        +(((KHDPG)/TotDPG)*dKHDPG)+
        (((H2DPG/TotDPG)*dH2DPG)}];
Results={dbATP,3PDPG}/.hbksoln;
Do[
        If[Results[[i,1]]≥15.7&&Results[[i,1]]≤18.5,
        {dbATP,d3PDPG}=Results[[i]];Break[]],{i,Length[Results]}];
]

(***Define Module to calculate next estimate of Mgf and pH***)

SNextEst[ResdbATP_,Resd3PDPG_] :=Module[{},
    Estsoln=
      Solve[{ResdbATP= =(nextdMgf*dbATPdiffdMg)+(nextdpH*dbATPdiffpH),
      Resd3PDPG= =(nextdMgf*d3PDPGdiffdMg)+(nextdpH*d3PDPGdiffpH)}];
    EstResults=Part[{nextdMgf,nextdpH}/.Estsoln,1];
    {nextdMgf,nextdpH}=EstResults;]

(***Observed Chemical Shifts of bATP and 3PDPG***)
dbATPobs=16.35;
```

```
d3PDPGobs=6.15;
(***Initial estimates of pH and Mgf***)
pH[1]=7.2;
Mgf[1]=0.0004;

Do[
    pHest=pH[i];
    Mgfest=Mgf[i];

ΔMgf=0.000001;
ΔpH=0.0001;

dMgf=.;dpH=.;dbATPSlopedMg=.;d3PDPGSlopedMg=.;dbATPSlopedpH=.;
d3PDPGSlopedpH=.;dbATPdiffdMg=.; dbATPdiffdpH=.;d3PDPGdiffdMg=.;
d3PDPGdiffdpH=.;ResdbATP=.;Resd3PDPG=.;ResdbATP=.;nextdMgf=.;nextdpH=.;


(***Calculate first chemical shift estimates***)

SCalcds[Mgfest,pHest];
dbATP1=dbATP;d3PDPG1=d3PDPG;

(***Calculate Differential of d's wrt Mgf and pH***)

dMgf=Mg[i]+ ΔMgf;dpH=pH[i]+ ΔpH;
SCalcds[dMgf,pH[i]];
DbATPSlopedMg=dbATP;d3PDPGSlopedMg=d3PDPG;
SCalcds[Mgf[i],dpH];
dbATPSlopedpH=dbATP;d3PDPGSlopedpH=d3PDPG;

dbATPdiffdMg=(dbATP1-dbATPSlopedMg)/ ΔMgf;
dbATPdiffdpH=(dbATP1-dbATPSlopedpH)/ ΔpH;
d3PDPGdiffdMg=(d3PDPGSlopedMg-d3PDPG1)/ ΔMgf;
d3PDPGdiffdpH=(d3PDPGSlopedpH-d3PDPG1)/ ΔpH;

ResdbATP=dbATP1-dbATPobs;
Resd3PDPG=d3PDPGobs-d3PDPG1;
SNextEst[ResdbATP,Resd3PDPG];

Mgf[i+1]=nextdMgf+Mgf[i];
pH[i+1]=nextdpH+pH[i];
Print[{Mgf[i+1],pH[i+1]}];
If[Abs[(Mgf[i+1]-Mgf[i])/Mgf[i]]<0.0001&&Abs[(pH[i+1]-
pH[i])/pH[i]]<0.0001,Break[]],{i,25}]
```

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Mg^{2+} and H^+ but not high-energy phosphates as observed by in vivo ^{31}P-NMR. *Biochim. Biophys. Acta,* **1358,** 1-5.

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