The Oxidation of Glutathione by Nitroprusside: Changes in Glutathione in Intact Erythrocytes during Incubation with Sodium Nitroprusside as detected by ^{1}H Spin Echo NMR Spectroscopy

ANTHONY R. BUTLER, ADRIANNE M. CALSY-HARRISON, CHRISTOPHER GLIDEWELL*, IAN L. JOHNSON
Department of Chemistry, University of St Andrews, St Andrews, Fife KY16 9ST, U.K.
JOHN REGLINSKI and W. EWEN SMITH
Department of Pure and Applied Chemistry, Strathclyde University, Cathedral Street, Glasgow G1 1XL, U.K.
(Received September 25, 1987)

Abstract

Spin echo ^{1}H NMR spectroscopy showed that when the hypotensive agent sodium nitroprusside, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$, was incubated with intact erythrocytes in ^2H_2O saline, glutathione in the erythrocytes was oxidised to diglutathione. This was confirmed by ^{1}H FT NMR for the *in vitro* reaction. ^{13}C FT NMR showed that the stoichiometry of the glutathione–nitroprusside reaction was 1:1; the inorganic products were nitric oxide and hexacyanoferrate(II), $[Fe(CN)_6]^{4-}$. At no stage was free cyanide liberated. The reaction of nitroprusside with glutathione, which occurs after the nitroprusside has crossed the erythrocyte membrane, is compared with the reaction of nitroprusside with haemoglobin. In neither of these reactions with major erythrocyte components was any free cyanide liberated by sodium nitroprusside.

Introduction

Sodium nitroprusside, $Na_2[Fe(CN)_5NO]\cdot 2H_2O$, is widely used as a hypotensive agent in vascular surgery, in chronic hypertension and in the management of acute myocardial infarction [1–4]. However, it has been reported [5] that the nitroprusside ion $[Fe(CN)_5NO]^{2-}$ interacts with haemoglobin to liberate four moles of free cyanide per mole of nitroprusside and this has been held to be the origin of the widely reported cyanide poisoning occurring during administration of sodium nitroprusside [6–8]. We have challenged this view [9, 10] and have shown that the normal analytical technique [11] employed for the determination of blood cyanide cannot distinguish between cyanide liberated by metabolism of nitroprusside and cyanide liberated as an artifact of the analytical procedure from the ion $[Fe(CN)_5H_2O]^{2-}$, which is very readily formed [12] from nitroprusside upon exposure to visible light.

We have further shown by use of ^{13}C NMR and highly ^{13}C-enriched $Na_2[Fe(^{13}CN)_5NO]\cdot 2H_2O$ that no free cyanide can be detected after incubation of nitroprusside with whole blood [13]. With isolated purified haemoglobin, nitroprusside is unreactive towards oxygenated haemoglobin but interacts with deoxygenated haemoglobin to yield, not free cyanide as previously reported [5], but hexacyanoferrate(II), $[Fe(CN)_6]^{4-}$, and nitrosylhaemoglobin [14].

In this paper we present the results of an ^{1}H spin echo NMR investigation of the interaction of nitroprusside with intact erythrocytes. The advantages of NMR for the study of molecules *in vivo* lie in its sensitivity and its non-invasive nature, allowing monitoring of the target molecules with time in viable cells. Although the normal ^{1}H NMR spectrum of the intact erythrocyte is dominated by resonances from haemoglobin and membrane, a multiple pulse spin echo technique [15] makes it possible to tune out the signals from the large molecules and thus greatly simplify the spectrum [16]; this method has been employed with success in the study of erythrocyte metabolism [16–25].

Experimental

Glutathione was obtained from Sigma Chemical Co., sodium chloride, sodium acetate and deuterium oxide (gold label) from Aldrich Chemical Co., and sodium nitroprusside (AnalaR) from BDH Chemicals. All operations involving sodium nitroprusside were conducted in the absence of light. Spin echo NMR spectra were obtained using a 90-τ-180-τ pulse sequence, with τ either 40 ms or 60 ms. A Bruker 250 MHz spectrometer fitted with an Aspect 2000 computer was used to record the ^{1}H spectra. Samples were maintained at 20 °C during data collection and the data from 2000 complete pulse sequences were

*Author to whom correspondence should be addressed.

0020-1693/88/$3.50

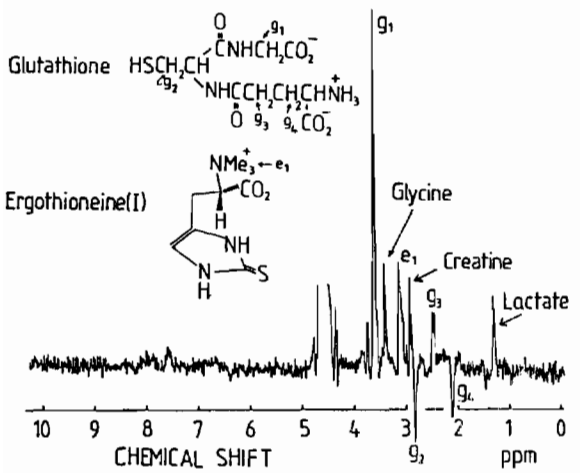

Fig. 1. The ^1H spin echo NMR spectrum of the intact human erythrocyte ($\tau = 60$ ms). The assignments of the major resonances from each species are as defined in refs. 15–25. A small presaturation pulse was applied to the water resonance prior to accumulation.

accumulated for each Fourier transform. Conventional FT NMR spectra were recorded using a Varian CFT-20 spectrometer. EPR spectra were measured in 1-mm quartz capillaries using a Bruker ER 200D spectrometer; d-t-butyl nitroxide was used as the standard for the measurement of the line positions. Optical and infrared spectra were measured using Pye 8150 and Perkin-Elmer 1330 instruments, respectively.

The erythrocytes were obtained from venous blood, collected in heparinised tubes. The blood was centrifuged at 3000 rpm for 5 min at 4 °C and the plasma was drawn off; the packed cells were twice washed with ^2H_2O saline (0.154 M in NaCl). Packed erythrocytes (0.4 ml) were placed in a 5-mm NMR tube with 0.1 ml sodium acetate (1.0 mg/ml) as internal reference (δ 1.764), with the total sample volume being 0.5 ml.

The assignments of resonances due to glutathione (reduced GSH, or oxidised GSSG), ergothioneine, glycine, creatine and lactate follow those of previous studies [19–25].

Results and Discussion

The In Vivo *Reaction of Glutathione and Nitroprusside*

The spin echo NMR spectrum of the human erythrocyte employing a delay time (τ) of 60 ms in the pulse sequence is shown in Fig. 1. This is dominated by resonances from a number of readily identified small molecules [19–25]. Amongst these is glutathione, which as a thiol has a major role to play in the defence against redox changes in the cell. Of particular importance to this study is the

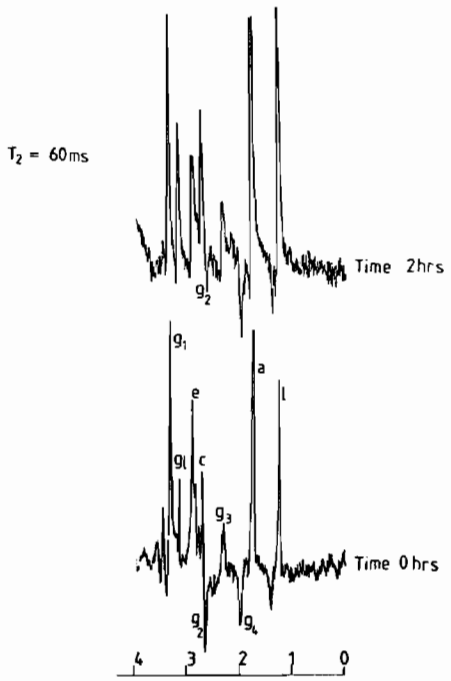

Fig. 2. The effect of 0.5 mg of sodium nitroprusside on the ^1H spin echo NMR of the human erythrocyte ($\tau = 60$ ms): l, lactate; a, acetate (external reference); gl, glycine; c, creatine; e, ergothioneine; g_1–g_4, glutathione; the resonance assignments of glutathione and ergothioneine are shown in Fig. 1. The lower spectrum consists of 2000 scans, whereas the upper required 4000 scans (due to paramagnetism). A small presaturation pulse was applied to the water resonance.

β-methylene residue of the cysteinyl group (g_2, Fig. 1). The proximity of this group to the reactive thiol function produces specific changes in this spectral region during reactions targeted at the thiol function.

With delay times of 60 ms, the g_2 resonance has considerable negative intensity when glutathione is in the reduced form. On oxidation of the thiol group, the resonance intensity becomes more positive. In initial experiments employing a delay time of 60 ms, addition of 0.5 mg (1.68×10^{-3} mmol) of sodium nitroprusside to the cells showed clear evidence (Fig. 2) of a reaction at the β-methylene residue of glutathione. Normally this behaviour would be considered as conclusive evidence of the oxidation of the cytosolic glutathione. However, the process produces a low concentration of a transient paramagnetic species which renders difficult a clear distinction between oxidation of glutathione [16, 23] and simple ligation to glutathione [20]. The latter process has been shown to null the g_2 resonance in the molecule. To confirm further that glutathione was indeed being oxidised, the experiments were repeated using a lower nitroprusside concentration (0.1 mg, 3.36×10^{-4} mmol), thus reducing the

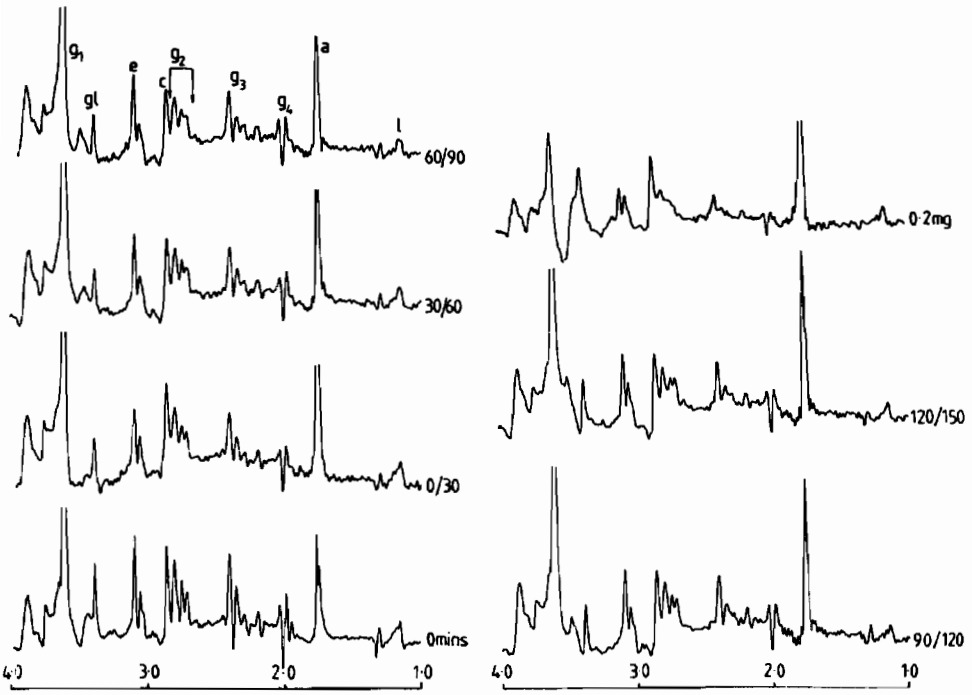

Fig. 3. The time course of the spin echo NMR spectrum of intact erythrocytes (τ = 40 ms) over 30-min intervals after administration of 0.1 mg of sodium nitroprusside. After 150 min a further 0.1 mg was added. Resonance assignments follow Fig. 2. Each spectrum consists of 2000 scans.

paramagnetic effects. A different delay time (40 ms) was employed to emphasise the redox changes observed in the β-methylene residue. At this delay time oxidised glutathione has a null point for the g_2 resonance and subsequently does not contribute to the spectrum in this region [25]. The signal intensity observed at the g_2 position in the spin echo NMR spectrum arises solely from reduced glutathione.

After the addition of the nitroprusside to the red cell suspension, spectra were recorded in 30-min periods up to 150 min, when a further 0.1 mg of nitroprusside was added. The results of these experiments are shown in Fig. 3. The signal intensities in the important region (g_2) were referenced against both creatine and the g_3 glutamyl residue of glutathione. Although both sets of data provide similar plots, the latter (Fig. 4) is more significant as it provides the internal calibration for the oxidation of glutathione by nitroprusside with time (g_2 = reduced glutathione concentration, g_3 = total (reduced + oxidised) glutathione concentration).

By a time 150 min after the addition of nitroprusside, the reduced glutathione concentration had fallen to approximately one half of its initial concentration (Figs. 3 and 4). When a further 0.1 mg aliquot was added, complete oxidation of the glutathione occurred. A significant increase in paramagnetic broadening was also observed.

The poor signal-to-noise ratio generated in Fig. 2 and the broadening observed in Fig. 3 are

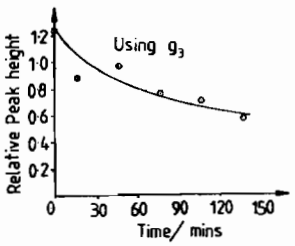

Fig. 4. The time course of the intensity of the g_2 peak of glutathione, referenced against g_3, with τ = 40 ms.

generated by the paramagnetism from the transient paramagnetic iron species within the cell. As the paramagnetism is being observed intracellularly it is clear that the nitroprusside has crossed the cell membrane [24]. This behaviour is directly analogous (but in reverse) to the practice of adding paramagnetic species to the extracellular fluid to remove resonances which do not arise from inside the cell [24].

The In Vitro *Reaction of Glutathione and Nitroprusside*

The oxidation of glutathione (GSH) to diglutathione (GSSG) deduced from the *in vivo* study described above is consistent with the previously reported reactions of simple thiols (RSH), including cysteine, with nitroprusside to give the corresponding disulphide RSSR [26, 27]. In order to confirm the

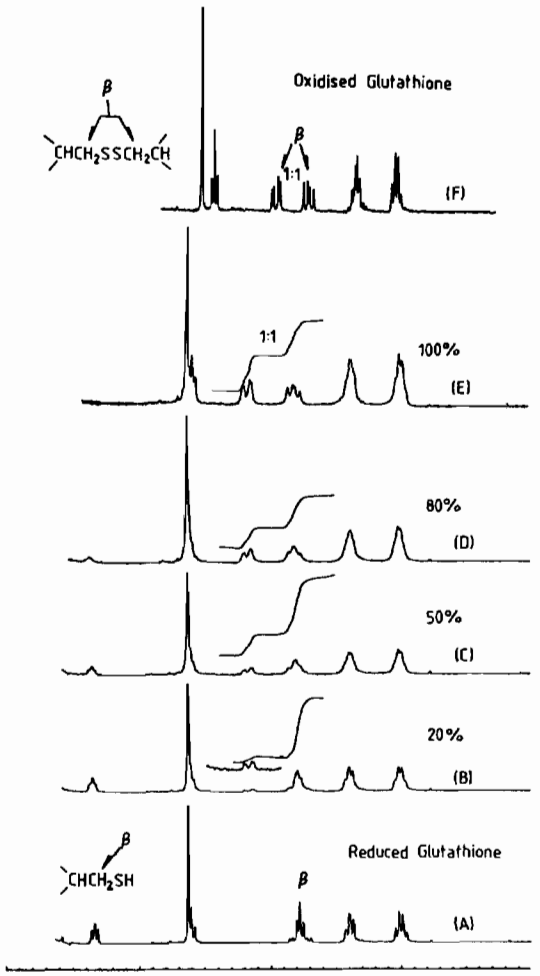

Fig. 5. The titration of reduced glutathione (14.6 mg; 48.6 μmol in 0.4 ml) with sodium nitroprusside (19.4 mg; 64.6 μmol in 0.2 ml) in aqueous solution (^2H_2O/NaCl (0.154 M) Na_2HPO_4 (0.125 M), pH 7.6). (A) reduced glutathione; (B) +10 μl of nitroprusside solution; (C) +20 μl; (D) +30 μl; (E) +40 μl; (F) oxidised glutathione in ^2H_2O. A line-narrowing function was applied to spectra (B)–(E) during Fourier transformation. Spectra (B)–(E) are plotted at double the intensity of (A). Beta indicates the line patterns from the β-cysteinyl function of glutathione in its reduced and oxidised forms.

oxidation of GSH in intact erythrocytes by nitroprusside and to establish both the other reaction products and the reaction stoichiometry, the reaction between GSH and nitroprusside has been studied *in vitro* in phosphate buffer, pH 7.6, using both ^1H and ^{13}C FT NMR.

The results of a typical ^1H NMR titration of GSH against nitroprusside are shown in Fig. 5, and indicate clearly the conversion of GSH to GSSG as the sole hydrogen-containing product. There is, however, significant line-broadening with loss of signal intensity. This we associate with the transient formation of paramagnetic species (see below); similar effects are apparent in Figs. 2 and 3. While the *in vivo* reaction between GSH and nitroprusside is slow (Figs. 3 and 4), the *in vitro* reaction is fast [28], as is also the reaction of cysteine and nitroprusside [27, 28]. This large difference in rates possibly arises from the slow transport of nitroprusside across the erythrocyte membrane.

Since it is known [27] that the observed stoichiometry of reactions between nitroprusside and simple thiols such as cysteine is strongly influenced by molecular oxygen, the stoichiometry of the *in vitro* reaction between glutathione and nitroprusside was accordingly determined under strictly anaerobic conditions. A thoroughly degassed solution, precisely 0.50 molar in each of reduced glutathione and nitroprusside, in phosphate buffer at pH 7.6, rapidly turned magenta (λ_{max} 520 nm); this is characteristic [26–28] of an adduct of the type $[Fe(CN)_5N(O)SR]^{3-}$ between nitroprusside and the thiolate anion. The ^{13}C NMR spectrum accumulated over the following 3 h in the normal FT NMR mode showed the presence of GSSG and $[Fe(CN)_6]^{4-}$ as the sole products of reaction. Thus a clean redox reaction of 1:1 stoichiometry has occurred. The study by EPR spectroscopy of an identical solution showed the presence of the paramagnetic anion $[Fe(CN)_4NO]^{2-}$ [28], while nitric oxide (NO) was detected as a reaction product by infrared gas analysis.

The observation of the intermediates $[Fe(CN)_5N(O)SG]^{3-}$ and $[Fe(CN)_4NO]^{2-}$, together with the determination of the reaction stoichiometry as 1:1 and the identification of GSSG and $[Fe(CN)_6]^{4-}$ as the final products, establishes the following scheme, eqns. (1)–(5) [29], for the reaction of GSH with nitroprusside:

$$GSH + [Fe(CN)_5NO]^{2-} \xrightarrow{fast} [Fe(CN)_5N(O)SG]^{3-} + H^+ \quad (1)$$

$$[Fe(CN)_5N(O)SG]^{3-} \longrightarrow 1/2GSSG + [Fe(CN)_5NO]^{3-} \quad (2)$$

$$[Fe(CN)_5NO]^{3-} \xrightarrow{v.\ fast} [Fe(CN)_4NO]^{2-} + CN^- \quad (3)$$

$$[Fe(CN)_4NO]^{2-} \longrightarrow NO + [Fe(CN)_4]^{2-} \quad (4)$$

$$6[Fe(CN)_4]^{2-} + 6CN^- \longrightarrow 5[Fe(CN)_6]^{4-} + Fe^{2+} \quad (5)$$

No free CN^- was observed at any stage, nor was $[Fe(CN)_4]^{2-}$ detected. Provided only that reaction (5) is the fastest step, this scheme encompasses all our observations. The net redox reaction involves only the reduction of the nitrosyl ligands in nitroprusside, eqn. (6).

$$2GSH + 2NO^+ \longrightarrow GSSG + 2NO + 2H^+ \quad (6)$$

The reaction of nitroprusside with haemoglobin is also a redox reaction [14] in which the final products are nitrosylhaemoglobin and $[Fe(CN)_6]^{4-}$; this reaction also proceeds via the intermediate $[Fe(CN)_4NO]^{2-}$, detected by EPR spectroscopy (*cf.* eqn. (3) above) but without free cyanide being detectable. The net reaction, eqn. (6), of precise 1:1 stoichiometry, is strictly applicable only to the rigorously anaerobic *in vitro* systems. ^1H NMR titrations of glutathione against nitroprusside under aerobic conditions provided reaction stoichiometries of nitroprusside:glutathione in the range 1:2 to 1:4. This apparent difference arises because of the known [27] reformation of $[Fe(CN)_5NO]^{2-}$ from $[Fe(CN)_5NO]^{3-}$ or from $[Fe(CN)_4NO]^{2-}$ and cyanide in the presence of air, eqns. (7) and (8).

$$[Fe(CN)_5NO]^{3-} \xrightarrow{O_2} [Fe(CN)_5NO]^{2-} \quad (7)$$

$$[Fe(CN)_4NO]^{2-} + CN^- \xrightarrow{O_2} [Fe(CN)_5NO]^{2-} \quad (8)$$

Thus in the presence of air, as well as reactions (3) and (4) forming $[Fe(CN)_6]^{4-}$, a competitive pathway, reactions (7) and (8), operates to recycle some of the reduced intermediate back to $[Fe(CN)_5NO]^{2-}$ so that one mole of nitroprusside eventually oxidises more than one mole of glutathione. Indeed under appropriate conditions, the oxidation of cysteine to cystine in air requires only a catalytic quantity of nitroprusside because of this recycling [27]. An effective stoichiometry of nitroprusside: glutathione in the range 1:2 to 1:4 is consistent with the apparent *in vivo* reductive capacity of the intact erythrocyte under aerobic conditions where there is, in the absence of glucose, residual enzyme activity, principally glutathione reductase. After 0.2 mg of nitroprusside had been added, assuming that this was evenly distributed throughout the 0.5 ml of the cell suspension, the overall concentration of nitroprusside (1.3 mM) corresponded to the saturation of the reductive capacity of the cell. The glutathione concentration in intact erythrocytes has recently been measured by spin echo NMR [25] as 2.8 mM, although older estimates cover the range 1.9–2.8 mM [25]. Such a concentration gives a nitroprusside:glutathione ratio of 1:2.1, nicely in the range observed under aerobic *in vitro* conditions. However, it is envisaged that the aerobic catalysis pathway will be reduced *in vivo* compared with the *in vitro* experiments due to poor oxygen diffusion in the biological matrix. This will be offset by the activity of glutathione reductase and catalase, which will be stimulated by glutathione depletion, so that this latter process will provide a certain amount of hidden reductive capacity within the cell.

At high nitroprusside levels (0.5 mg) there is some evidence (Fig. 1) which suggests that on complete oxidation of the glutathione stores, the nitroprusside attacks ergothioneine (which exists as a thione ⇌ thiol tautomer) to give an unknown product. This type of sequential reactive behaviour for the thiol sites (glutathione > ergothioneine) within the red cell has been discussed previously for heavy metal binding [30] and is a simple measure of the reactivity of the thiol function in these molecules. The reaction of nitroprusside with ergothioneine *in vivo* is viewed at this stage purely as a chemical reaction of no immediate biological significance. Any biochemical process which has exhausted the reducing power of both glutathione and haemoglobin will probably also have resulted in cell death; the observation of an ergothioneine reaction occurring after this point is thus considered simply as a chemical process of no biochemical significance.

The reactions of nitroprusside with the two major reactive components of the erythrocyte (glutathione and haemoglobin) are chemically very similar. Each involves the reduction of the nitrosyl ligand in nitroprusside from NO^+ to $NO^\bullet$, with simultaneous oxidation of the erythrocyte component, either of haemoglobin to nitrosylhaemoglobin or of GSH to GSSG, together with formation of $[Fe(CN)_6]^{4-}$ as the final cyano complex. Neither liberates any free cyanide. Contrary to earlier reports [5–8] free cyanide is not to be expected since the equilibrium constant for eqn. (5) can be estimated as *ca.* 10^{12} [31].

Acknowledgements

We thank the Scottish Home and Health Department for financial support for J.R., and the Ministry of Agriculture, Fisheries and Food (U.K.) for financial for I.L.J.

References

1 D. J. Ahearn and C. E. Grim, *Arch. Intern. Med., 133*, 187 (1974).
2 K. Chatterjee, H. J. C. Swan, V. S. Kaishik, G. Jobin, P. Magnusson and J. S. Forrester, *Circulation, 53*, 797 (1976).
3 T. H. Taylor, M. Styles and A. J. Lamming, *Br. J. Anaesth., 42*, 859 (1970).
4 T. H. Tinker and J. D. Michenfelder, *Anaesthesiology, 45*, 340 (1976).
5 R. P. Smith and H. Kruszyna, *J. Pharmacol. Exp. Ther., 191*, 557 (1974).
6 C. J. Vesey, P. V. Cole, J. C. Linnell and J. Wilson, *Br. Med. J., 2*, 140 (1974).
7 C. J. Vesey, P. V. Cole and P. J. Simpson, *Br. J. Anaesth., 48*, 651 (1976).
8 M. Bogusz, J. Moroz, J. Karski, J. Gierz, A. Regieli, R. Witkowska and A. Gołabek, *Clin. Chem., 25*, 60 (1979).
9 W. I. K. Bisset, A. R. Butler, C. Glidewell and J. Reglinski, *Br. J. Anaesth., 53*, 1015 (1981).

10 W. I. K. Bisset, M. G. Burdon, A. R. Butler, C. Glidewell and J. Reglinski, *J. Chem. Res., (S)* 299, *(M)* 3501 (1981).
11 G. E. Boxer and J. C. Rickards, *Arch. Biochem. Biophys., 30*, 372 (1951).
12 S. K. Wolfe and J. H. Swinehart, *Inorg. Chem., 14*, 1049 (1975).
13 A. R. Butler, C. Glidewell, J. McGinnis and W. I. K. Bisset, *Clin. Chem., 33*, 490 (1987).
14 A. R. Butler, C. Glidewell, I. L. Johnson and A. S. McIntosh, *Inorg. Chim. Acta, 138*, 159 (1987).
15 I. D. Campbell, C. M. Dobson, R. J. P. Williams and P. E. Wright, *FEBS Lett., 57*, 96 (1975).
16 F. F. Brown, I. D. Campbell, P. W. Kuchel and D. L. Rabenstein, *FEBS Lett., 82*, 12 (1977).
17 K. M. Brindle, F. F. Brown, I. D. Campbell, C. Grathwohl and P. W. Kuchel, *Biochem. J., 180*, 37 (1979).
18 R. J. Simpson, K. M. Brindle, F. F. Brown, I. D. Campbell and D. L. Foxall, *Biochem. J., 202*, 573 (1982).
19 D. L. Rabenstein and A. A. Isab, *FEBS Lett., 121*, 61 (1980).
20 D. L. Rabenstein, A. A. Isab and R. S. Reid, *Biochem. Biophys. Acta, 720*, 53 (1982).
21 D. L. Rabenstein, S. J. Backs and A. A. Isab, *J. Am. Chem. Soc., 103*, 2836 (1981).
22 G. Otiko, M. T. Razi, P. J. Sadler, A. A. Isab and D. L. Rabenstein, *J. Inorg. Biochem., 19*, 227 (1983).
23 C. N. N. McKay, D. H. Brown, J. Reglinski, W. E. Smith, H. A. Capell and R. D. Sturrock, *Biochem. Biophys. Acta, 888*, 30 (1986).
24 F. F. Brown and I. D. Campbell, *Phil. Trans. R. Soc. London, B289*, 395 (1986).
25 D. L. Rabenstein, D. W. Brown and C. J. McNeil, *Anal. Chem., 57*, 2294 (1985).
26 D. Mulvey and W. A. Waters, *J. Chem. Soc., Dalton Trans.*, 951 (1975).
27 P. J. Morando, E. B. Borghi, L. M. de Schteingart and M. A. Blesa, *J. Chem. Soc., Dalton Trans.*, 435 (1981).
28 M. D. Johnson and R. G. Wilkins, *Inorg. Chem., 23*, 231 (1984)
29 C. Glidewell and I. L. Johnson, *Inorg. Chim. Acta, 132*, 145 (1987).
30 R. S. Reid and D. L. Rabenstein, *J. Am. Chem. Soc., 104*, 6733 (1982).
31 J. J. Christensen and R. M. Izatt, 'Handbook of Metal Ligand Heats', Marcel Dekker, New York, 1970.