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# Study by Carbon-13 NMR and by EPR of the Reactions between the Nitroprusside Ion and Haemoglobins

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## Abstract

High frequency $^{13}C$ NMR spectroscopy using nitroprusside 90% enriched in $^{13}C$ shows that the nitroprusside ion forms a 1:1 complex with deoxyhaemoglobin, but does not interact with either oxyhaemoglobin or methaemoglobin: additionally hexacyanoferrate(II) is formed in the deoxyhaemoglobin reaction only, but does not bind to haemoglobins. EPR spectroscopy shows that deoxyhaemoglobin, but not oxyhaemoglobin or methaemoglobin, reduces nitroprusside to the intermediate $[Fe(CN)_4NO]^{2-}$. A reaction scheme is suggested to rationalise the conversion of $[Fe(CN)_5NO]^{2-}$ to $[Fe(CN)_6]^{4-}$ in these reactions.

## Introduction

We have recently shown [1] by $^{13}C$ NMR, using isotopically enriched (90% $^{13}C$) cyanoferrates, that nitroprusside $[Fe(CN)_5NO]^{2-}$ forms 1:1 and 2:1 complexes with aquocobalamin, vitamin $B_{12a}$. In the 1:1 complexes with the cobaloxime $CH_3Co(dmg)_2$ axial cyano ligand, forming a Fe–C–N–Co bridge: in the 2:1 complex the nitroprusside is bound to two cobalt corrins via a *trans* pair of equatorial cyano ligands. Similar complexes, which appear to be fluxional in solution, are formed between hexacyanoferrate(II) $[Fe(CN)_6]^{4-}$, and aquocobalamin. Both these cyanoferrates form weaker [1, 2] 1:1 complexes with the cobaloxime $CH_3Co(dmg)_2$-$H_2O$ (dmg = mono-anion of dimethylglyoxime, $CH_3$-$C(=NO^-)C(=NOH)CH_3$). The formation of complexes with aquocobalamin significantly influences [3] the pharmacokinetics of the hypotensive [4] action of nitroprusside, so that aquocobalamin cannot be acting simply as an antidote [5] to potential cyanide liberation [6] from nitroprusside during its clinical use.

The nitroprusside anion has been reported [7] to react with haemoglobin, and it was postulated [7] that this reaction causes liberation of four moles of free cyanide per mole of nitroprusside, the fifth cyano ligand being captured in the conversion of haemoglobin to cyanomethaemoglobin. This has been widely regarded as the origin of the reported cyanide liberation from nitroprusside. However the experiments upon which these conclusions were based appear to have been conducted [7] without proper regard to the photo-lability of nitroprusside [8], where the substitution-inert $d^6$ ion $[Fe(CN)_5NO]^{2-}$ is readily converted to the substitution-labile $d^5$ ion $[Fe(CN)_5H_2O]^{2-}$. The reported [7] liberation of four moles of free cyanide is most surprising in view of the very high [9] formation constants of cyanoferrate complexes, (*ca.* $10^{30}$ for pentacyanoferrate(II) species [9]), as well as the kinetic inertness of iron(II) cyanoferrates.

These doubts, together with our earlier finding [1] of specific complex formation between $[Fe(CN)_5NO]^{2-}$ and aquocobalamin, have prompted us to undertake further study of the interactions between nitroprusside and haemoglobins. We show that not only is no free cyanide liberated but that, as might be expected from a consideration of formation constants [9], an important reaction product is hexacyanoferrate(II), $[Fe(CN)_6]^{4-}$, whose formation constant in aqueous media is *ca.* $10^{35}$ [9].

## Experimental

Twice recrystallised, dialysed and lyophilised bovine methaemoglobin was purchased from Sigma: oxyhaemoglobin and deoxyhaemoglobin were prepared from methaemoglobin by reduction with dithionite in isotonic buffer at pH 7.2: the conversions were monitored by observation of the Söret bands in the UV spectra, and the products were shown to be free of dithionite by EPR spectroscopy and to be free of sulphite by the non-occurrence of a Boedeker reaction with nitroprusside [10]. Sodium dithionite, L-cysteine hydrochloride, *N*-acetyl-L-cysteine, and sodium nitroprusside and sodium hexacyanoferrate(II) of normal isotopic composition were all of AnalaR quality: samples of $Na_2[Fe(^{13}CN)_5$-

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$NO]2H_2O$ and $Na_4[Fe(^{13}CN)_6]\cdot 10H_2O$ enriched to 90% in $^{13}C$ were prepared as previously described [1].

NMR spectra were recorded using the Brüker WH-360 spectrometer at the Science and Engineering Research Council regional NMR facility at the University of Edinburgh, using recording conditions as previously described [1]. EPR spectra were measured in 1 mm quartz capillaries using a Brüker ER200D spectrometer: di-t-butyl nitroxide was used as the standard for the measurement of line positions.

## Results

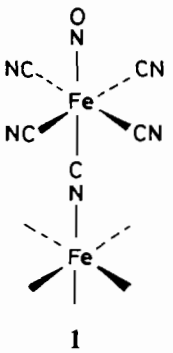
We have previously shown [1, 11], that a wide range of diamagnetic cyanoferrates can readily be distinguished in solution by use of $^{13}C$ NMR spectroscopy and high isotopic enrichment: furthermore the $^{13}C$ spectra were entirely unperturbed by the presence in the solution of paramagnetic cyanoferrates. Hence it has proved convenient in the present study to observe the diamagnetic reaction products by use of $^{13}C$ NMR spectroscopy, while the paramagnetic complexes were detected primarily using EPR spectroscopy.

### *NMR Spectroscopy*

As in previous work [1, 11], we have used $Na_2[Fe(CN)_5NO]$ enriched to 90% in $^{13}C$: although the $^{13}C$ spectrum is dominated by two isotopic species [1], we shall for the sake of convenience restrict our attention to $[Fe(^{13}CN)_5NO]^{2-}$.

The characteristic [1] spectrum of $[Fe(^{13}CN)_5NO]^{2-}$ was entirely unaltered by incubation with either oxyhaemoglobin or methaemoglobin. However with deoxyhaemoglobin the $[Fe(^{13}CN)_5NO]^{2-}$ spectrum was rapidly replaced by that of a second $AX_4$ species having $\delta_A = 138.9$, $\delta_X = 139.7$, and $J = 18.7$ Hz, indicative of an intact diamagnetic pentacyanoferrate (species **1**) together with a sharp singlet $\delta = +177.9$, characteristic [11] of $[Fe(^{13}CN)_6]^{4-}$, (**2**). Control experiments showed that the singlet resonance of preformed $[Fe(^{13}CN)_6]^{4-}$ was unperturbed by deoxyhaemoglobin, and that in the absence of deoxyhaemoglobin but under otherwise identical conditions, no $[Fe(^{13}CN)_6]^{4-}$ was formed from $[Fe(^{13}CN)_5NO]^{2-}$.

The $^{13}C$ chemical shifts in the new $AX_4$ system (species **1**) show that the nitrosyl ligand is still present, and by analogy with the complexes formed [1, 2] by $[Fe(CN)_5NO]^{2-}$ with aquocobalamin, vitamin $B_{12a}$, we assign to species **1** the constitution shown, that of a 1:1 complex formed by complexation of the axial cyano ligand of the $[Fe(CN)_5NO]^{2-}$ to the iron of the haemoglobin:




**1**

Binding via the axial cyanide ligand, known [2] to be most nucleophilic of the ligands, rather than one of the equatorial cyanide ligands is demonstrated by the $AX_4$ $^{13}C$ spectrum of **1**: the nitrosyl ligand in nitroprusside is very much less nucleophilic than any of the cyanides [2]. We observed no 2:1 complexes between $[Fe(CN)_5NO]^{2-}$ and haemoglobin, although such 2:1 complexes are readily formed [1] in the reactions between $[Fe(CN)_5NO]^{2-}$ and aquocobalamin.

Although deoxyhaemoglobin contains high-spin iron(II), with $S = 2$, coordination of any moderately strong field ligand ot the sixth (axial) site will cause spin pairing, yielding a diamagnetic $S = 0$ product [12]: in systems containing six-coordinate iron, even such weakly $\pi$-bonding ligands as piperidine or tetrahydrothiophen, as well as t-butylisonitrile or methylimidazole cause spin-pairing in iron(II) porphines [12]. Thus there can be no doubt that nitroprusside, coordinated via the axial cyanide ligand will cause spin pairing in haem with consequent observation of an $AX_4$ type $^{13}C$ NMR spectrum typical of a diamagnetic complex.

The major difference observed by $^{13}C$ NMR spectroscopy between the nitroprusside/haemoglobin system on the one hand and the nitroprusside/cobalamin system on the other is the clear evidence in the haemoglobin system of ligand reorganisation, in the cyanoferrate complex, with formation of hexacyanoferrate(II). This anion does not show any evidence of binding to the iron(II) of haemoglobin, although it readily binds [1] to the cobalt(III) of cobalamin.

Apart from bound $[Fe(^{13}CN)_5NO]^{2-}$ and unbound $[Fe(^{13}CN)_6]^{4-}$, no other $^{13}C$-labelled diamagnetic species were detected by $^{13}C$ NMR: in particular neither free $[H^{13}CN]$ nor free $[^{13}CN]^-$ was detected in any of the spectra.

### *EPR Spectroscopy*

The formation of nitrosylhaemoglobin **3** from nitroprusside and deoxyhaemoglobin, (although not from oxyhaemoglobin) is well established [7, 13]. We have now observed the formation from $[Fe(CN)_5NO]^{2-}$ and deoxyhaemoglobin of a persistent para-

magnetic mononitrosyl complex **4**, characterised by $g = 2.028$, $A(^{14}N) = 15.2$ G. Such a complex was obtained by Mulvey and Waters [14] from $[Fe(CN)_5NO]^{2-}$ by electrochemical reduction, and by chemical reduction using $NaBH_4$, $Na_2S_2O_4$, or ascorbic acid, and was assigned by them as $[Fe(CN)_5NO]^{3-}$. We have obtained the identical complex **4** by reduction of $[Fe(CN)_5NO]^{2-}$ with $Na_2S_2O_4$, as previously reported [14], and also by reduction with deoxyhaemoglobin and with each of L-cysteine, *N*-acetyl-L-cysteine, and reduced glutathione; by use of isotopically labelled $[Fe(^{13}CN)_5NO]^{2-}$ we have shown [15] that this paramagnetic species is, in fact, the five-coordinate $[Fe(CN)_4NO]^{2-}$, which decomposes to yield, as final products, $[Fe(CN)_6]^{4-}$ and NO. Partial reduction of $[Fe(CN)_5NO]^{2-}$ by $Na_2S_2O_4$ yielded solutions containing both $[Fe(CN)_4NO]^{2-}$, characterised by EPR spectroscopy, and unchanged $[Fe(CN)_5NO]^{2-}$, whose $^{13}C$ NMR spectrum was unperturbed: this confirms the independence from paramagnetic co-solutes of the $^{13}C$ NMR spectra of diamagnetic cyanoferrate complexes.

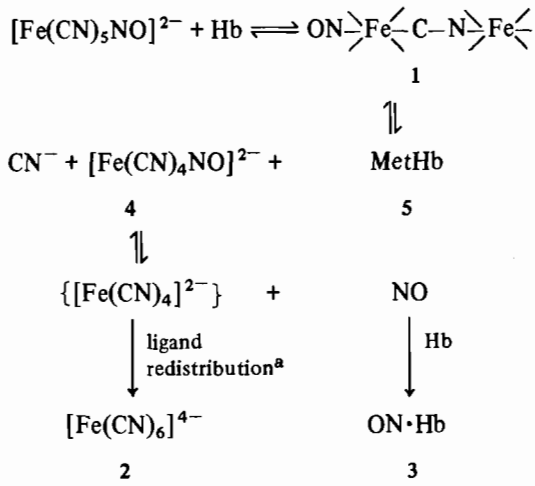
*Optical Spectroscopy*

We have observed, using UV spectroscopy, the formation of **3** and of the iron(III) methaemoglobin **5** in the reaction between $[Fe(CN)_5NO]^{2-}$ and deoxyhaemoglobin: only after photolysis by visible light, did we observe cyanomethaemoglobin, indicating that the formation [7] of this material is simply an artifact of photolysis [8].

## Discussion

Whereas the reaction between $[Fe(CN)_5NO]^{2-}$ and aquocobalamin involves [1] simply a ligand substitution at cobalt, with neither substitution at iron nor any redox changes, the corresponding reaction between $[Fe(CN)_5NO]^{2-}$ is much more complex, involving substitution reactions at both metal centres and a redox reaction in which the cationic nitrosyl ligand ($NO^+$) in $[Fe(CN)_5NO]^{2-}$ oxidises the iron of haemoglobin in a one-electron transfer process. Interaction of the nitroprusside at the metal centre in both vitamin $B_{12a}$ and haemoglobin is demonstrated by the fact that if the sixth ligand site in these substrates is blocked, by cyanide in vitamin $B_{12a}$ [1] or by dioxygen in haemoglobin, then the $^{13}C$ NMR spectrum of nitroprusside in the same solution is entirely unperturbed from the usual $AX_4$ spectrum.

The reaction products from the interaction of $[Fe(CN)_5NO]^{2-}$ with deoxyhaemoglobin are summarised in Scheme 1. This Scheme accommodates all of the reaction products identifiable by $^{13}C$ NMR, EPR, or UV spectroscopy: in addition it also




Scheme 1. Hb, MetHb, and ONHb represent deoxy-, met-, and nitrosyl-haemoglobins respectively. [a]Ref. 15.

accounts, by means of a ligand redistribution step, eqn. (1) [15], converting the labile reactive intermediate $[Fe(CN)_4]^{2-}$ into the observed $[Fe(CN)_6]^{4-}$, for the complete absence of any cyanide found both with whole blood [16] and, in the present work, using isolated haemoglobin.

$$6[Fe(CN)_4]^{2-} + 6CN^- \longrightarrow 5[Fe(CN)_6]^{4-} + Fe^{2+} \quad (1)$$

We conclude that any cyanomethaemoglobin observed in earlier work [7] resulted from the photolytic formation of the labile aqua-ion $[Fe(CN)_5H_2O]^{2-}$.

The fact that complexes **1** and **4** are both formed using deoxyhaemoglobin, but not oxyhaemoglobin suggests that complex **1** is the primary intermediate for the formation of **4**: the conversion of **1** to **4** thus requires an inner-sphere electron transfer across the Fe–C–N–Fe bridge. Hence we have observed both the bridged intermediate **1** and the primary products **4** and **5** of this inner-sphere redox reaction; the net redox reaction is summarised in eqn. (2).

$$Hb(Fe^{II}) + [Fe^{II}(CN)_5(NO^+)]^{2-} \longrightarrow$$

$$MetHb(Fe^{III}) + CN^- + [Fe^{II}(CN)_4(NO^\cdot)]^{2-} \quad (2)$$

The formation of **4** in reactions of $[Fe(CN)_5NO]^{2-}$ with thiolates $RS^-$ follows from the intermediate $[Fe(CN)_5N(O)SR]^{3-}$ formed as usual [17–20] by attack of the nucleophile at the nitrogen of the nitrosyl ligand, followed by loss of $RS^\cdot$ (or of $\frac{1}{2}R_2S_2$), and transient loss of $CN^-$, eqns. (3) and (4).

$$[Fe(CN)_5NO]^{2-} + RS^- \longrightarrow [Fe(CN)_5N(O)SR]^{3-} \quad (3)$$

$$[Fe(CN)_5N(O)SR]^{3-} \longrightarrow$$

$$\tfrac{1}{2}R_2SSR + CN^- + [Fe(CN)_4NO]^{2-} \qquad (4)$$

The complex **4** is established as a precursor of **3**, nitrosylhaemoglobin, which is itself a potent activator [13, 21] of the enzyme guanylate cyclase (EC 4.6.1.2), whose primary product cyclic-GMP is implicated [22] in vascular smooth muscle relaxation, as induced by hypotensive agents, such as nitroprusside [4]. The very ready formation, demonstrated in this work, of **4** through reduction of $[Fe(CN)_5NO]^{2-}$ by a wide range of molecular species present in normal mammalian biochemistry (cysteine, glutathione, haemoglobin) may provide the mechanistic key to the very rapid hypotensive activity of $[Fe(CN)_5NO]^{2-}$.

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## References

1 A. R. Butler, C. Glidewell, A. S. McIntosh, D. Reed and I. H. Sadler, *Inorg. Chem., 25,* 970 (1986).
2 A. F. Cuthbertson, C. Glidewell, A. R. Butler and A. S. McIntosh, *Inorg. Chim. Acta, 135,* 93 (1987).
3 D. S. Hewick, A. R. Butler, C. Glidewell and A. S. McIntosh, *J. Pharm. Pharmacol., 39,* 113 (1987).
4 (a) D. J. Ahearn and C. E. Grim, *Arch. Intern. Med., 133,* 187 (1974); (b) K. Chatterjee, H. J. C. Swan, V. S. Kaushik, G. Jobin, P. Magnusson and J. S. Forrester, *Circulation, 53,* 797 (1976); (c) T. H. Taylor, M. Styles and A. J. Lamming, *Br. J. Anaesth., 42,* 859 (1970); (d) J. H. Tinker and J. D. Michenfelder, *Anesthesiology, 45,* 340 (1976).
5 M. A. Posner, R. E. Tobey and H. McElroy, *Anesthesiology, 44,* 157 (1976).
6 (a) A. J. Merrifield and M. D. Blundell, *Br. J. Anaesth., 46,* 324 (1974); (b) D. G. McDowall, N. P. Keaney, J. M. Turner, J. R. Lane and Y. Okuda, *Br. J. Anaesth., 46,* 327 (1974); (c) L. Greis, N. A. G. Tremblay and D. W. Davies, *Can. Anaesth., Soc. J., 23,* 480 (1976); (d) S. Nakamura, T. Shin, Y. Hirokata and A. Shigematsu, *Br. J. Anaesth., 49,* 1239 (1977); (e) J. E. Cottrell, P. Casthely, J. D. Brodie, K. Patel, A. Klein and H. Turndorf, *N. Engl. J. Med., 298,* 809 (1978).
7 R. P. Smith and H. Kruszyna, *J. Pharmacol. Exp. Ther., 191,* 557 (1974).
8 (a) S. K. Wolfe and J. H. Swinehart, *Inorg. Chem., 14,* 1049 (1975); (b) W. I. K. Bisset, M. G. Burdon, A. R. Butler, C. Glidewell and J. Reglinski, *J. Chem. Res.,* (S) 299, (M) 3501 (1981).
9 J. J. Christensen and R. M. Izatt, 'Handbook of Metal Ligand Heats', Marcel Dekker, New York, 1970.
10 W. Moser, R. A. Chalmers and A. G. Fogg, *J. Inorg. Nucl. Chem., 27,* 831 (1965).
11 A. R. Butler, C. Glidewell, A. R. Hyde and J. McGinnis, *Inorg. Chem., 24,* 2931 (1985).
12 W. R. Scheidt and C. A. Reed, *Chem. Rev., 81,* 543 (1981).
13 P. A. Craven and F. R. DeRubertis, *Biochim. Biophys. Acta, 745,* 310 (1983).
14 D. Mulvey and W. A. Waters, *J. Chem. Soc., Dalton Trans.,* 951 (1975).
15 C. Glidewell and I. L. Johnson, *Inorg. Chim. Acta, 132,* 145 (1987).
16 A. R. Butler, C. Glidewell, J. McGinnis and W. I. K Bisset, *Clinical Chem., 33,* 490 (1987).
17 A. R. Butler, C. Glidewell, J. Reglinski and A. Waddon, *J. Chem. Res.,* (S) 279, (M) 2768 (1984).
18 A. R. Butler, C. Glidewell, V. Chaipanich and J. McGinnis, *J. Chem. Soc., Perkin Trans., 2,* 7 (1986).
19 P. J. Morando, E. B. Borghi, L. M. de Schteingart and M. A. Blesa, *J. Chem. Soc., Dalton Trans.,* 435 (1981).
20 F. Bottomley, W. V. F. Brooks, D. E. Paéz, P. S. White and M. Mukaida, *J. Chem. Soc., Dalton Trans.,* 2465 (1983).
21 (a) P. A. Craven and F. R. DeRubertis, *J. Biol. Chem., 253,* 8433 (1978); (b) L. J.Ignarro, J. N. Degnan, W. H. Baricos, P. J. Kadowitz and M. S. Wolin, *Biochim. Biophys. Acta, 718,* 49 (1982); (c) R. Gerzer, F. Hofmann and G. Schultz, *Eur. J. Biochem., 116,* 479 (1982).
22 C. A. Gruetter, D. Y. Gruetter, P. J. Kadowitz and L. J. Ignarro, *Fed. Proc., 40,* 688 (1981).