The Chemistry of Vitamin B_{12}. Part XVI.† Binding of Thiols to the Cobalt(II) Corrins

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The binding of axial ligands to cobalt(II) forms of cobalamins and cobinamides has been studied by e.s.r. spectroscopy. The measurements show that sulphides (thioethers), thiolate anions, and thiols as well as many nitrogen bases bind to the cobalt. The solvent in which the spectra are recorded has a considerable influence upon the resolution observed in the spectra.

PREVIOUS work [1] has shown, in detail, the way in which thiols interact with the cobalt of vitamin B_{12a}, a cobalt(III) complex. Here we examine the binding of thiols to the cobalt(II) forms of the vitamin and its derivatives. The main experimental tools are spectrophotometry and electron paramagnetic resonance.

† Part XV, *J. Chem. Soc.* (*A*), 1971, 1859.

[1] H. A. O. Hill, J. M. Pratt, R. G. Thorp, B. Ward, and R. J. P. Williams, *Biochem. J.*, 1970, **120**, 263.

PROCEDURE AND RESULTS

The preparation of cobalt(II) cobinamides and cobalamins by controlled-potential reduction (c.p.r.) has been described earlier.[2] Here the cobalt(III) complexes, 0·005—0·01M, were reduced (by c.p.r.) in aqueous 0·1M-KCl. Samples of 0·15 ml were transferred, using a gas-tight syringe, to e.p.r. tubes containing the required reagents in a volume of 0·15 ml. After rapid mixing the e.s.r. tubes were put

[2] P. K. Das, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *J. Chem. Soc.* (*A*), 1968, 1261.

in liquid nitrogen and frozen solutions were measured. The transfer operations were conducted in a nitrogen atmosphere until the solutions were frozen.

Table 1 contains a set of e.s.r. data for a range of cobalamin B_{12r} complexes at high pH and Table 2 gives the corresponding data at low pH. The notes to the Tables give details of solution conditions. In acid solution pH $\leqslant$2·0 it is known that the benzimidazole base of the cobalamin of cobalt(II) forms of the vitamin is not co-ordinated. Corresponding data for Factor B_{12r}, the cobinamide, are given in Table 5 (see later). Details of the spectra and the methods of evaluation of the spectral parameters, in the Tables, are given for each of the series of complexes. Less detailed spectra of some of these complexes have been published previously.[3,4]

TABLE 1

E.s.r. data for cobalamin(II), base co-ordinated: effect of medium [a]

Reagent	pH	$g_{\|}$	$g_{\perp}$	$A_{\|}^{Co}$	$A_{\|}^{N}$ 10^4 cm^{-1}	$A_{\perp}^{Co}$
None	6—10	2·004	2·320	100		27
Ethanol	7·5	2·005	2·315	99	17·3	27
Propane-1,2-diol [b]	6—8	2·004	2·320	100	17·3	27
Propane-1,2-diol [c]	7·0	2·004	g_1 2·28 g_2 2·23	98		
1-Aminopropanol	7·5	2·004	2·315	100	17·4	27
Acetate	7·0	2·005	2·315	101	17·5	27
Ascorbate	7·5	2·003	2·325	103	17·3	27
NN-Dimethyl-formamide	7·5	2·004	2·315	99	17·2	27
Pyridine	9·5	2·004	2·315	100	17·2	27
Cysteine [d]	6—8·5	2·004	2·320	103	17·1	27
Glutathione [d]	6—8·5	2·004	2·320	103	17·1	27
1,3-Dimethyl-glutarate [e]	7·3	2·006		103	17·8	
Enzyme [f]	7·3	2·01		103	16·9	

[a] Unless otherwise specified, B_{12r} was prepared by catalytic or electrolytic reduction (c.p.r.). Samples contained B_{12r} 0·0025—0·003M, reagent 1·0M, and $NaKPO_4$ 0·05M. Error limits are $g_{\|} \pm 0·001$, $g_{\perp} \pm 0·005$, $A_{\|}^{Ca} \pm 1$, $A_{\|}^{N} \pm 0·2$, $A_{\perp}^{Co} \pm 5$. [b] Reagent concentration was 0·15—5·50M (1—40% v/v). [c] Measured at *Q*-band. Reagent concentration was 2·25M (20% v/v). Error limits are $g_{\|} \pm 0·001$, g_1 and $g_2 \pm 0·002$, $A_{\|}^{Co} \pm 2$. [d] Reagents were also used as reductants, concentration 0·8M. pH was adjusted with KOH. [e] Ref. 5, B_{12} was prepared by reduction of B_{12a} with CO or HCO_2K. Samples contained B_{12r} 0·01—0·001M, reagent 0·16M, and $NaPO_4$ 0·066M. [f] Ref. 7, system contained B_{12} coenzyme, dGTP, dihydrolipoate, ribonucleotide reductase, and $NaPO_4$.

TABLE 2

E.s.r. data for cobalamin(II), base un-co-ordinated: effect of medium [a]

Reagent	pH	$g_{\|}$	$g_{\perp}$	$A_{\|}^{Co}$ 10^4 cm^{-1}	$A_{\perp}^{Co}$
None	0·5	2·007	2·57	139	
Propane-1,2-diol [b]	0·5	2·005	2·59	134	
Propane-1,2-diol [b,c]	0·5	2·006	2·40	135	85
Ascorbate	1·0	2·008	2·61	141	
Cysteine [d]	1·0	2·005	2·49	129	
Cysteine [c,d]	0·5	2·005	2·35	130	85
Glutathione	0·5	2·005	2·46	128	

[a] Unless otherwise specified, B_{12r} was prepared by catalytic reduction or c.p.r. Samples contained B_{12r} 0·0025M and reagent 1·0M, acidified with HCl. Error limits are $g_{\|} \pm 0·01$, $A_{\|}^{Co} \pm 1$. [b] Reagent concentration was 2·25M (20% v/v). [c] Measured at *Q* band. B_{12r} concentration was 0·01M. Error limits are $g_{\|} \pm 0·001$, $g_{\perp} \pm 0·02$, $A_{\|}^{Co} \pm 2$, $A_{\perp}^{Co} \pm 10$. [d] Reagents were also used as reductants, concentration 0·8M.

Cobalamins with Co-ordinated Base.—A typical *X*-band spectrum, of cobalamin(II), in 1 : 4 (v/v) propanediol–water is given in Figure 1. It is independent of pH in the range 6—8. The spectrum is consistent with an axially symmetric *g* tensor. The six equally spaced peaks toward high field were assigned to the $g_{\|}$ cobalt hyperfine multiplet. Assuming $A_{\|}$ cobalt to be positive, which is stated to be the case for cobalt(II) phthalocyanine, the series numbers from $M_I = -7/2$ at the high-field end, with the final members $M_I = +5/2$, $+7/2$ largely obscured by the intense 'derivative' signal. $g_{\|}$ was measured at the bisector of the lines $M_I = \pm 1/2$ on an expanded trace. $A_{\|}^{Co}$ was obtained from the separation of these lines, which was equal to the average splitting over the whole set within experimental error. The uniform triplets, resolved on the middle members $M_I = \pm 1/2$, $\pm 3/2$, were attributed to hyperfine coupling from the bound benzimidazole, N(3) nucleus ($I^N = 1$). $A_{\|}^{N}$ was derived as an average from expanded spectra, but no experimentally significant variation with M_I^{Co} was detected. All results were the mean of measurements on at least two equivalent samples. The above procedure was applied to all subsequent spectra.

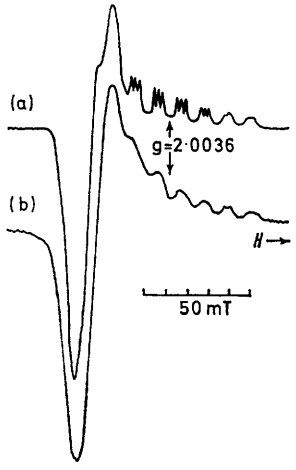

FIGURE 1 The *X*-band e.s.r. spectra of the cobalt(II) form of aquocobalamin in water (b) and propanediol–water (a)

The lack of resolution in the large low-field resonance precludes any accurate measurement of $g_{\perp}$ and $A_{\perp}$. The minimum position, labelled g_1, serves as an approximation to $g_{\perp}$; $A_{\perp}$ was estimated from the peak-to-peak line-width of the 'derivative'. Bayston *et al.* have reported preliminary curve-fitting experiments which promise a more exact analysis of the $g_{\perp}$ region.[5]

A similar sample was recorded at *Q* band. The increased separation in *g* values allows all eight members of the $g_{\|}$ multiplet to be clearly observed, although signal-to-noise ratio and instrument resolving power were not sufficiently good to permit detection of superhyperfine structure. The data for $g_{\|}$ and $A_{\|}^{Co}$ agreed closely with the *X* band results (Table 1), thus supporting the use of peak maxima in the determination of spin Hamiltonian parameters. New

[3] G. N. Schrauzer and L.-P. Lee, *J. Amer. Chem. Soc.*, 1968, **90**, 6541.

[4] S. A. Cockle, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Biochim. Biophys. Acta*, 1969, **177**, 686.

[5] J. H. Bayston, F. D. Looney, J. R. Pilbrow, and M. E. Winfield, *Biochemistry*, 1970, **9**, 2164.

structure was present in the low-field Q-band spectra which was attributed to a small rhombic distortion, unresolved at X band. The two approximate g values, g_1 and g_2, permit a better estimate of the average $g_\perp$ than from the X-band spectrum.

Effect of Solvent on Spectral Resolution.—It was observed that the quality of vitamin B_{12r} e.s.r. spectra was very dependent on the solvent used. Samples prepared in water and measured in 0·05M-$NaKPO_4$ buffer over the pH range 6—10, were poorly resolved and showed no superhyperfine splitting. Introduction of a 1·0M concentration of polar organic reagents containing such groups as OH, NH_2, SH, and CO_2^-, caused appreciable improvement in all cases, with triplets appearing on the central hyperfine peaks. Dimethylformamide and propane-1,2-diol gave the best resolved spectra. To discover optimum conditions, propanediol–water solvents comprising 0·15—5·50M diol (1—40% v/v) were tested within the pH range 6—8. The resolution increased from 0 to 5% but no further improvement was found up to 40% of the diol. A solvent of composition 1 : 4 propanediol–water was frequently employed in subsequent work. The spectral parameters in the presence of different added reagents are given in Table 1.

Cobalamin(II) *with Unco-ordinated Base.*—Displacement of the nucleotide base by acidification leads to gross changes in the e.s.r. spectrum. The X-band spectrum of 0·0025M-cobalamin(II) in 1 : 4 propanediol–water at pH 0·5 is illustrated in Figure 2. Owing to the considerable complexity, it is not evident whether approximate axial

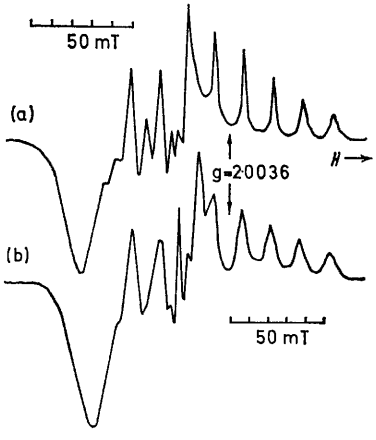

FIGURE 2 The X-band e.s.r. spectrum of the cobalt(II) form of aquocobinamide in water (b) and propanediol–water (a)

symmetry is maintained. A series of eight prominent and equally spaced peaks was identified as the $g_\parallel$ multiplet, with splitting *ca.* 35% larger than in the ' base-on ' form. Pronounced line-broadening occurred towards high field, but the central members were particularly sharp, and showed no evidence of ligand hyperfine structure. The intense resonance at low field, referred to as g_1, should serve as a useful feature for distinguishing ' base-off ' from ' base-on ' forms using very dilute samples, *e.g.* in enzymes, since it is somewhat removed from the analogous peak in ' base-on ' cobalamin(II).

At Q band the spectrum was much simplified: Table 2, for example, gives the spectrum of cobalamin(II) in propanediol–water (1 : 4) at pH 0·5. The $g_\parallel$ multiplet was completely isolated, and the measured parameters agreed with those obtained at X band (Table 2). The broad, low-field signal did not reveal hyperfine structure, but verified that there was no appreciable departure from axial symmetry.

Further Axial Co-ordination in Cobalamin(II).—E.s.r. spectra of cobalamin(II) in neutral solution were recorded in the presence of a variety of organic reagents which were potential ligands. The variations in g values, $A_\parallel^{Co}$, and $A_\parallel^{N}$, are given in Table 3. Pyridine and other heterocyclic

TABLE 3

E.s.r. spectra of cobalamin(II): co-ordination of thiols and pyridine

(a) Thiols [a]

Thiol	pH	$g_\parallel$	$g_\perp$	$A_\parallel^{Co}$ 10^4 cm^{-1}
Cysteine [b]	1·0	2·005	2·49	129
Glutathione [b]	0·5	2·005	2·46	128
Cysteine [c]	12	2·001	2·300	97
Glutathione [c]	11	2·001	2·300	96

(b) Pyridine [a]

Concentration	$g_\parallel$	$g_\perp$	$A_\parallel^{Co}$	$A_\parallel^{N}$
			10^4 cm^{-1}	
8% (1·0M) [d]	2·004	2·315	100	17·2
50% [e]	2·004	2·320	103	17·4
92% [f]	2·010	g_1 2·305 g_2 2·210	101	13·1 [g]

[a] B_{12r} concentration was 0·0025—0·003M. Error limits as Tables 1 and 2. [b] Table 2, reduction by thiol, 0·8M; acidified with HCl. [c] Reduction by thiol, 0·8M; pH adjusted with KOH. [d] Table 1, electrolytic reduction. Sample contained 0·05M $NaKPO_4$, pH 9·5. [e] Electrolytic reduction. [f] Solvent reduction in presence of mercury. [g] Quintet splitting.

bases exert a negligible effect on the spectrum. In view of the profound changes resulting from removal of the nucleotide base, it is concluded that only weak interaction can take place at the sixth co-ordination site under these conditions. By contrast, the decrease in $A_\parallel^{Co}$ caused by cysteine or glutathione suggests co-ordination of the polarisable sulphur group. Since the thiolate anion should form a stronger complex, samples were investigated over the pH range 6—13; cysteine has pK_1 8·14, and pK_2 10·34. In neutral solution the usual spectrum of ' base-on ' vitamin B_{12r} was obtained. Around pH 9—10 formation of a new species was shown by a distortion of the $g_\parallel$ hyperfine lines and their associated triplets. Beyond pH 10·5, corresponding to complete dissociation of the thiol reagents, well-resolved spectra were again recorded, in which the $g_\parallel$ components were sharp singlets, although $A_\parallel^{Co}$ was little changed. The nucleotide base must have become detached, and replaced by the thiolate anion of the amino-acid.

In an effort to produce an identifiable six-co-ordinate complex of vitamin B_{12r}, samples were prepared in the presence of very high concentrations of pyridine. When the proportion of pyridine in the medium was increased to 50%, small but reproducible changes in $A_\parallel^{Co}$ and $A_\parallel^{N}$ were noted (see Table 3). In view of the effect of thiolate in alkaline solution, a possible explanation is that benzimidazole was replaced by pyridine, as proposed by Bayston *et al.*[5] Owing to solubility problems, it was not possible to reduce the proportion of water below 5%. When the pyridine contained 8—10% water, a highly modified spectrum of cobalamin(II) was obtained. The doublet low-field structure was consistent with marked rhombic

distortion, and two g values were estimated on this basis; a Q band spectrum would be necessary to confirm this analysis. The $g_{\|}$ hyperfine component $M_I = +1/2$ was split into a well-developed quintet, with intensities approximately in the ratio 1 : 2 : 3 : 2 : 1. This provides definite proof for the interaction of two essentially equivalent nitrogens (total $I^N = 2$).

Cobalamin(II) *Analogues.*—Cobalt(II) derivatives of five additional cyanocobamides were prepared by catalytic hydrogenation in neutral buffered solution, containing 20% propanediol as resolution enhancer. These vitamin B_{12} analogues incorporated either benzimidazole or adenine bases as listed in Table 4. All spectra were very similar

TABLE 4

E.s.r. spectra of cobalt(II) cobamides [a]

Nucleotide base	pH	$g_{\|}$	$g_{\perp}$	$A_{\|}^{Co}$	$A_{\perp}^{Co}$
				10^4 cm^{-1}	
5,6-Dimethylbenzimidazole [b,c]	7·0	2·004	2·320	100	17·3	27
5-Hydroxybenzimidazole [c]	7·5	2·004	2·320	101	17·1	27
5-Methoxybenzimidazole [c]	7·5	2·004	2·325	101	17·1	27
Adenine [d]	7·5	2·004	2·340	106	16·5	30
2-Methyladenine [d]	7·5	2·004	2·335	105	16·7	30
2-Methylthioadenine [d]	7·0	2·004	2·335	104	16·8	30

[a] Samples were prepared by catalytic reduction, and contained cobalt(II) cobamide 0·002—0·0025M, propanediol 2·25M (20%), and $NaKPO_4$ buffer. Error limits as Table 1. [b] Result from Table 1. [c] $NaKPO_4$ concentration was 0·05M. [d] $NaKPO_4$ concentration was 0·5M.

to that of 'base-on' cobalamin(II), the most extreme change being shown by adeninylcobamide(II). The experimental data illustrate an apparent inverse relationship between $A_{\|}^{Co}$ and $A_{\|}^{N}$, probably related to changes in Co–N bond strength. Thus weakening of the bond should cause an increase in $A_{\|}^{Co}$ but a decrease in $A_{\|}^{N}$. This observation might prove useful in detecting alterations in the cobalt–nucleotide bond length in enzymes.

E.s.r. Spectra of Cobalt(II) *Cobinamides.*—The absence of a 'built-in' axial ligand in cobinamide(II) facilitates the investigation of complex formation with free co-ordinating agents. Experimental procedures were similar to those described in the previous sections. Cobinamide(II) was prepared by controlled potential reduction or catalytic hydrogenation of aquocyanocobinamide, and occasionally by c.p.r. of diaquocobinamide. The mixed solvent 1 : 4 propanediol–water was employed throughout, except in the case of pyridine-rich media. Electrolytic reduction of aquocyanocobinamide in 9 : 1 pyridine–water, with lithium chloride as electrolyte, was required to obtain samples in high concentrations of pyridine.

Aquocobinamide.—*X*-Band spectra of cobinamide(II) in 0·05M-phosphate were measured in the pH range 4—10. The spectrum was almost identical with that of aquocobalamin(II) in acid. Samples prepared between pH 2 and 3 (in the absence of buffer) gave a totally different signal, consisting mainly or entirely of a derivative-like line at $g = 2·08$, Table 5. The origin of this resonance is unknown; a similar signal with greater relative intensity has been observed by Bayston *et al.*[5] who quote a g value of 2·15. The equivalence of aquocobinamide(II) and 'base-off' cobalamin(II) was further demonstrated by Q-band measurements on a sample in neutral buffered solution; the agreement in parameters was again excellent.

Sulphur Complexes of Cobinamide(II).—For some of the thiols a pH-dependent equilibrium was observed between the complex of the undissociated thiol, RSH, in the acid

TABLE 5

E.s.r. spectra of cobinamide(II) aquo- and sulphur-complexes [a]

Sulphur reagent	pH	$g_{\|}$	$g_{\perp}$	$A_{\|}^{Co}$	$A_{\perp}^{Co}$
				10^4 cm^{-1}	
None [b]	4—10	2·005	2·58	133	
None [c]	8	2·005	2·40	134	95
2-Mercaptoethanol [d]	4·0	2·004	2·43	124	
2-Mercaptoethanol [e]	8—10	2·001	2·275	93	23
Dimethylsulphide [f]	6·5	2·004	2·355	111	
Thiourea [g]		2·003		111	
Thiocyanate [g]		2·007		122	

[a] Samples were prepared by catalytic or electrolytic reduction, and contained 2·25M (20%) propanediol. Error limits as Tables 1 and 2. [b] Sample composition was cobalt(II) 0·0025M, $NaKPO_4$ 0·05M. [c] Measured at Q band. Sample composition was cobalt(II) 0·01M, $NaKPO_4$ 0·05M. [d] Sample composition was cobalt(II) 0·001M, thiol 1·0M, NaOAc 0·2M. [e] Sample composition was cobalt(II) 0·001M, thiol 0·2M, KPO_4 0·3M. [f] Sample composition was cobalt(II) 0·001M, sulphide 0·2M, KPO_4 0·3M. [g] Ref. 5, samples were prepared by reduction with HCO_2K in methanol.

region and the complex of the thiolate anion, RS^-, in the alkaline region. This equilibrium was studied over a wide pH range, *e.g.* see Figure 3 and analysed by e.s.r.,

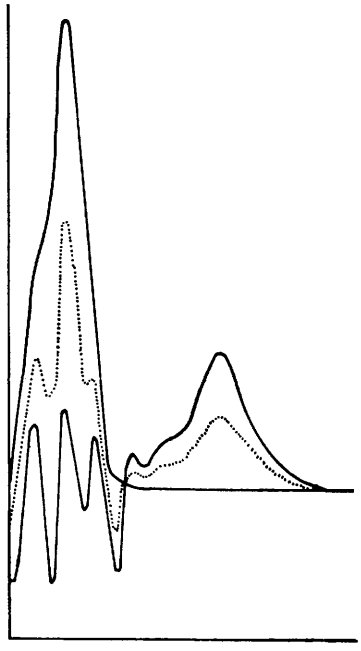

FIGURE 3 The e.s.r. peaks in the g_1 region representing different proportions of $Co^{II}(RS^-)$ and the equilibrium mixture of $Co^{II}(RSH)$ and $Co^{II}(H_2O)$ in 0·2M-mercaptoethanol. The large left-hand peak is that of $Co^{II}(RS^-)$

which is much more sensitive than spectrophotometry for cobalt(II) corrin complexes. In each case the solutions were frozen very rapidly after standing at 20 °C and we assume that the equilibria are those of temperatures near 20 °C.

The equilibria studied are

$$\begin{aligned} Co + RSH &\rightleftharpoons Co(RSH) & K_1 \\ Co + RS^- &\rightleftharpoons Co(RS) & K_2 \\ H^+ + RS^- &\rightleftharpoons RSH & K_a \end{aligned}$$

In a typical case, the study of the complex of 2-mercaptoethanol in propanediol–water (1 : 4) as solvent, 0·3M-K_2HPO_4 buffer, and the pH adjusted with HCl and NaOH, we observed that below pH 6·5 only the e.s.r. signals of cobalamin Co^{II} and cobalamin Co(RSH) were observed. Using the intensity of $g_\perp$ it was found that these two species were in constant proportion independent of pH over the range 4·9—7·7. The amounts of Co(RSH) were strictly dependent on the RSH concentration at the low pH and so K_1 could be determined. Knowing the e.s.r. spectra of two species, we could determine that of the third at high pH. At any pH above 6·5 the following ratio

$$R = \frac{[CoRS]}{[Co] + [Co\ RSH]}$$

could be evaluated from the well-resolved e.s.r. signals. Now clearly

$$R = \frac{T_{Co}K_3}{1 + [H^+]/K_a}$$

where T_{Co} = total cobalt, $K_3 = K_2/(1 + TK_1)$, and T = total thiol. Knowing K_1 and several R values it was then possible to determine K_2. The values were, for $K_a = 10^{-9\cdot3}$, $K_1 = 11{\cdot}5 \pm 1$ mol l^{-1}, $K_2 = 2500 \pm 150$ mol l^{-1}. Similar complexes were formed with other thiols. The individual complexes were also studied by absorption spectrophotometry and Table 6 (see Figure 4) gives a list of their spectra.

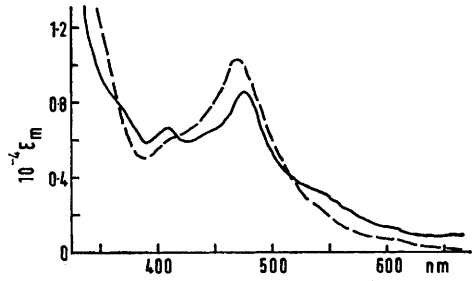

FIGURE 4 The absorption spectra of cobalt(II) cobalamin in water with base co-ordinated (full line) and base un-co-ordinated (broken line)

TABLE 6

The absorption spectra of cobalt(II) corrin complexes below 600 nm

Compound	Absorption maxima (nm)
Cobalamin, base-on	407 (6700), 475 (8600), 535 (3500)
Cobalamin, base-off	415 (6200), 471 (10,400), 537 (3000)
Cobinamide	407 (6500), 473 (8500), 535 (3000)

Molar extinction coefficients are given in parentheses. There are weaker absorption bands due to *d–d* transitions of cobalt(II) at wavelengths beyond 600 nm.[8]

Interaction between cobinamide(II) and dimethyl sulphide was also found to occur over a wide pH range. Table 5 shows that this species is intermediate in character between the two kinds of thiol complex. Bayston *et al.*[5] have reported two further cobinamide(II)–sulphur complexes, in which the ligands are thiourea and thiocyanate; and their spin Hamiltonian parameters are similar to those found here.

Pyridine Complexes of Cobinamide(II).—Samples of cobinamide(II) were prepared containing an excess of pyridine in the concentration range 0·1—95% by volume (0·01—11·8M); propanediol was used as resolution enhancer when the pyridine content was 5% (0·6M) or less. All spectra were similar in appearance to that of ' base-on ' cobalamin(II), showing that only one molecule of pyridine was firmly bound. Throughout the complete range, no variation in magnetic parameters could be detected within experimental error. The coupling constants $A_{\|}^{Co}$ and $A_{\|}^{N}$ were indistinguishable from those of cobalamin(II) in 1 : 1 pyridine–water, a result consistent with displacement of benzimidazole in the latter case. When the medium comprised 50% or more of pyridine, a second low-field peak emerged, attributed to the bis-pyridine complex; however, it was not possible to attain as high a degree of formation as in the cobalamin system. On this evidence, the six-co-ordinate vitamin B_{12r} species is thus concluded to be the ' base-on ' mono-pyridine complex.

Complexes with Other Heterocyclic Bases.—To observe the effect on e.s.r. parameters of changes in ligand basicity, a series of pyridine derivatives was employed, substituted mainly in the 4-position to avoid steric interactions. Full details of sample composition are included in Table 7.

TABLE 7

E.s.r. spectra of cobinamide(II) complexes with heterocyclic bases [a]

Ligand	$g_{\|}$	$g_\perp$	$A_{\|}^{Co}$	$A_{\|}^{N}$	$A_\perp^{Co}$
				10^4 cm^{-1}	
Pyridine [b]	2·004	2·320	103	17·5	27
2-Methylpyridine [c]	2·004	2·320	102	17·7	27
4-Methylpyridine	2·004	2·320	102	17·7	27
2,6-Dimethylpyridine [d]	2·004	2·315	101	17·8	25
4-Ethylpyridine	2·004	2·320	102	17·7	27
4-Cyanopyridine	2·004	2·330	106	17·0	30
4-Chloropyridine	2·004	2·330	104	17·4	27
4-Aminopyridine	2·004	2·315	99	18·3	25
4-Hydroxypyridine	2·004	2·310	98	18·5	25
Benzimidazole	2·004	2·320	102	17·2	27
Imidazole [e]	2·005		104	18·7	
Benzimidazole [e]	2·008		106	17·3	
5,6-Dimethylbenzimidazole [e]	2·01		106	17·4	
Adenine [e]	2·006		109	17·8	
Nitrite ($-NO_2^-$) [e]	2·001		101	24·3	
Azide	2·005		121	12·2	

[a] Unless otherwise specified, samples were prepared by electrolytic reduction, and contained cobalt(II) 0·0025M, base 0·12M, and propanediol 2·25M (20%). Error limits as Table 1. [b] Base concentration was 0·01—11·8M (0·1—95% volume). [c] Base concentration was 5·0M (50%); no propanediol present. [d] Base concentration was 4·4M (50%); no propanediol present. [e] Ref. 5, samples were prepared by reduction with HCO_2K in methanol.

In general respects, the e.s.r. spectra were closely similar to that of pyridinecobinamide(II), showing co-ordination of one base molecule, with well-resolved triplets on the central g- hyperfine components. Some complexes, notably those of 4-cyano-, 4-amino-, and 4-hydroxy-pyridines, exhibited partially resolved structure in the low-field region, but the $g_{\|}$ hyperfine multiplet gave no evidence that more than one species might be present. These features probably arose from the $M_I = +7/2$ peak, and/or from g-hyperfine splitting and small rhombic distortions.

Steric factors hinder the formation of complexes with 2-methyl- and 2,6-dimethyl-pyridines, but surprisingly, no weakening in cobalt–nitrogen bond strength was indicated by the hyperfine parameters.

The results in Table 7 illustrate very clearly the inverse correlation between $A_{\parallel}^{Co}$ and $A_{\parallel}^{N}$ mentioned earlier, which evidently relates to the base strength or σ-donor power of the axial ligand. The interaction of cobinamide(II) with pyridine and various other heterocyclic bases has also been examined by Bayston *et al.*, who point out the same general trend; [5] some of their data are recorded in the Tables. The most extreme examples among nitrogen-bonded complexes are provided by nitro- and azido-cobinamide(II), which give rise respectively to the largest and smallest values of $A_{\parallel}^{N}$ yet observed. Complexes with weakly donating ligands such as water and methanol have larger values of $A_{\parallel}^{Co}$, although there is no associated superhyperfine coupling.

DISCUSSION

The present work has demonstrated the ability of cobalt(II) corrinoids to co-ordinate readily with nitrogen bases and sulphur compounds. Many similar examples of cobinamide(II) complexes have been given by Bayston *et al.*,[5] including those already mentioned and others in which NH_2OH, PPh_3, CN^-, and I^- are ligands. In none of these cases is there evidence that more than one molecule of reagent binds to cobalt, except for the formation of a bis-pyridine complex under extreme conditions. It is evident that corrinoid(II) complexes should be regarded as essentially five-co-ordinated under most circumstances.

Owing to the wide range of organic materials that promote resolution improvement in the e.s.r. spectra, it is unlikely that a single mechanism is operative. In a frozen aqueous solution, variations in the packing of solute and solvent molecules and inhomogeneities in solute concentration are to be expected. The former effect gives rise to a variable microcrystalline environment for each paramagnetic centre, and therefore to variation in spin Hamiltonian parameters and broadening of spectral lines. Local high concentrations of paramagnetic species have an adverse influence on spin-relaxation processes. In classical terms, each neighbouring dipole exerts an increment to the total magnetic field at a given molecule, *i.e.* $H_{total} = H_0 + H_{local}$, where H_0 is the external applied field. If H_{local} varies for different paramagnetic centres, then resonance occurs over a range of field strengths centred on H_0, and line-broadening results.

Polyfunctional molecules such as ascorbate and cysteine should undergo extensive hydrogen-bonding interactions with corrinoid side-chains. This would tend not only to moderate the environment of each B_{12r} molecule, but also to discourage production of localised regions of high concentration. Moreover, these substances should also exert a pronounced structure-forming effect on solvent water, increasing the probability of obtaining a vitreous rather than a polycrystalline medium on rapid freezing. Since a more uniform distribution of solute molecules is expected in a glassy solid, line-broadening by spin–spin relaxation should be reduced. This is probably the most important factor responsible for the effects noted herein; it is well known that aqueous solutions containing hydroxylic or other polar solutes can be vitrified by cooling quickly.[6]

It is significant that none of the spectra obtained by the author or by Bayston *et al.*[5] could match the degree of resolution achieved when cobalamin(II) was generated from DAcobalamin attached at the active site of ribonucleotide reductase.[7] In this example, well-defined triplets appeared on all seven of the separately distinguished cobalt hyperfine peaks. Presumably the enveloping protein constrains all corrinoid molecules in precisely the same conformation.

When the nucleotide is not attached, two axial positions are free, so coupling constants are expected to depend more on the nature of the medium. The effect of introducing propanediol into an aqueous solution could arise from its co-ordination, but is probably due to variations in the packing of solvent molecules above and below the corrinoid plane. Either in the presence or absence of propanediol, a colour change from brown to red took place on cooling, which may indicate formation of a diaquo-complex at low temperatures. In the other examples listed in Table 6, there was no change of colour on cooling, and substitution of bound water by reagent is more likely. The e.s.r. experiments cannot themselves distinguish between five- and six-co-ordination under these conditions.

The demonstration that cobalt(II) B_{12} complexes can bind to thiols and to sulphides requires a closer inspection of the reaction of the methyl-transferring enzymes in which thiols are known to be important. The absorption spectra in Table 6 show that the differences between thiol-bound cobalt(II) and methylcobalamin(III) are very small. In an enzyme these two could be readily confused. The e.s.r. spectrum of a thiol-bound cobalt(II) is however quite distinctive. On the other hand in those cases where cobalt(II) e.s.r. signals from B_{12} coenzymes have been seen in enzymes they are superimposed upon other radical signals.[8] In these cases the absorption spectra of the cobalt(II) shows clearly that the vitamin B_{12} is in the base-on form.[8,9] The significance of the observations on the enzymes will be discussed in a later publication.

[1/1510 *Received, August 19th*, 1971]

[6] G. O. Jones, ' Glass,' Methuen, London, 1956, p. 25.
[7] J. A. Hamilton, R. L. Blakley, F. D. Looney, and M. E. Winfield, *Biochem. Biophys. Acta*, 1969, **177**, 374.
[8] M. A. Foster, H. A. O. Hill, and R. J. P. Williams, *Biochem. Soc. Symposia*, 1970, **31**, 187.
[9] H. Rudinger, *Europ. J. Biochem.*, 1971, **21**, 264.