Electron Paramagnetic Resonance Studies of Cob(II)alamin and Cob(II)inamides*

J. H. Bayston, F. D. Looney, J. R. Pilbrow,† and M. E. Winfield

ABSTRACT: Although the water molecule in the fifth coordination position of diaquocob(III)inamide is easily replaced by imidazole- or pyridine-like molecules, the other aquo group is replaced less readily. On reduction to the cobaltous state the group in the sixth coordination position detaches. The bond to the fifth ligand is weakened, so that in general there is some replacement by solvent, but ligands such as imidazole or triphenylphosphine are largely retained when their excess concentration is of the order of 10^{-2} M. Electron paramagnetic resonance spectral characteristics are given for cob(II)alamin (vitamin B_{12r}) in a variety of solvents, and for a large range of cob(II)inamides in methanol. Unsaturated heterocyclic nitrogen atoms in general, as well as the nitro group, triphenylphosphine and thiocyanate ion, are especially effective in coordination. Cyanide ion, NH_3, NH_2OH, and a number of amino acids coordinate at high pH but not in neutral solution. Steric effects on the epr spectra are noted when the ligand is triphenylphosphine or dimethylbenzimidazole. Steric hindrance to coordination is found with 8-methylquinoline but not with 2-methylpyridine. Of the biological amino acids examined, only histidine remains attached to the cobalt atom after its reduction at physiological pH (6×10^{-3} M free histidine). At the same pH adenine is detectably retained, but not guanine, cytosine, uracil, or the triphosphates of adenosine, guanosine, cytidine, or uridine. Thus if within a molecule of the ribonucleotide reductase of *Lactobacillus leichmannii* the nucleotide ligand of cob(II)alamin becomes detached, it is likely to be replaced only by a histidine residue or a water molecule. Use of water as solvent leads to broadening of the electron paramagnetic resonance absorption lines. They can be sharpened by addition of a variety of reagents such as 3,3-dimethylglutarate ion, egg albumin, aquocob(III)alamin, or methanol. Even with combinations of enhancers it does not prove possible to match the high degree of resolution attained when cob(II)alamin is formed within a molecule of ribonucleotide reductase. It is confirmed that the splitting of the hyperfine lines into triplets, observed in the spectra of cob(II)alamin and related cob(II)inamides, is due to the axial nitrogen atom. The splitting induced by the four hydropyrrole nitrogen atoms is too small to be detected except as line-width anisotropy.

The principal object of the work to be described was to contribute to the chemical knowledge required for an understanding of the mode of action of those enzymes which function in association with a corrinoid such as deoxyadenosylcobalamin (dimethylbenzimidazole[1] coenzyme). It is generally believed that the functional reactions of the corrinoid coenzymes are ionic, involving diamagnetic species in the Co^{I} and Co^{III} states. Possibly the cobaltous species, which are paramagnetic and whose formation would involve one-electron reduction or oxidation, do not occur during the normal functioning of the enzyme systems. But the cobaltous complexes are amenable to study by electron paramagnetic resonance as well as by optical methods, enabling us to obtain otherwise inaccessible information concerning reaction mixtures. Some of the information can be extrapolated to the diamagnetic states of the complexes. On the other hand, it is unwise to assume that the behavior of the cobaltic corrins is a guide to that of the reduced forms, as this paper will show.

Specific objectives were to learn which axial ligands could survive reduction of the metal atom without detaching; which amino acid residues of a corrinoid-dependent enzyme could replace as ligand the nucleotide side chain of the coenzyme; and to what extent the nature of the coordinated nucleotide determined the electron density at the cobalt atom and therefore its fitness for a specific enzyme reaction. (Little was known about how the nucleotide influenced the cobalt atom except that when the sixth coordination position was filled by an alkyl group the cobalt–nucleotide bond was weakened (Haywood *et al.*, 1965). From this it could be inferred that a nucleotide which formed a weak bond to the metal atom would ease the formation of a cobalt–carbon bond.)

In the course of this investigation some of the conclusions reached were utilized by Hamilton *et al.* (1969) in the interpretation of electron paramagnetic resonance spectra observed during *in vitro* reactions of a ribonucleotide reductase which depended for its action on dimethylbenzimidazole coenzyme. It then became clear that information was also needed regarding which molecular environments led to the greatest resolution of the electron paramagnetic resonance signal of B_{12r}; which cell constituents could, as axial ligand in a cob(II)inamide, result in an electron paramagnetic resonance

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: B_{12r}, cob(II)alamin (there is either no ligand, or a solvent molecule, in the site known as the sixth coordination position, *i.e.*, the axial position which is on the same side of the corrin as the acetamide side chains; in the fifth position is a nitrogen atom of the 5,6-dimethylbenzimidazole group of the nucleotide side chain.)

signal resembling that of B_{12r}; and finally whether B_{12r} has a ligand in the sixth coordination position.

Experimental Section

The techniques used for obtaining electron paramagnetic resonance spectra of cobaltous complexes in an atmosphere of argon, including gas purification, have been dealt with in published papers (Bayston and Winfield, 1967; Bayston *et al.*, 1963, 1969). Coupling constants and g values were also determined as before (Bayston *et al.*, 1969). By averaging measurements from several electron paramagnetic resonance scans of two or more samples, the error was diminished to less than ± 1 G for most of the coupling constants to be presented. For g values the error was usually within the limits ± 0.005.

In many of the experiments to be described the cobaltic forms of the corrinoids, at a concentration of 10^{-2}–10^{-3} M, were reduced with excess potassium formate, which, like CO, yields exclusively cobaltous corrinoids (Bayston *et al.*, 1969). Formaldehyde or dihydrolipoate (prepared by reduction of sodium lipoate with potassium borohydride) was also used on occasion; ascorbic acid was used when the pH was above 9. Reductions of corrinoids were carried out at 17–18° for durations determined by pH, and by the nature of the solvent, the reducing agent, and the complex to be reduced. When water was used as a solvent, or as part of a mixed solvent, it contained buffer to 0.1 M unless otherwise specified.

Reagents and solvents used were Analytical Reagent grade or better with the following exceptions: B.D.H. Laboratory Reagent potassium formate, potassium borohydride, L-ascorbic acid, adenine, L-histidine, L-arginine, L-cysteine, triphenylphosphine, sodium azide, sodium thiosulfate, ammonium chloride, isoquinoline, α-hydroxytoluene, and ethanediol; B.D.H. Biochemical grade guanine and uracil; Hopkin and Williams low in rare earths cerous nitrate; Fluka Purum dimethyl sulfoxide, benzimidazole, and dimethylbenzimidazole; Light and Co. imidazole, L-glutamine, L-tryptophan, and DL-proline; May and Baker thiourea; Eastman Kodak 2-aminopyrimidine; Aldrich 3,3-dimethylglutaric acid and L-lysine; Yeda L-histidylglycine; Mann five-times crystallized egg albumin; Nutritional Biochemicals DL-leucine-DL-tyrosine. Aniline was stored in the dark at low temperature, after redistilling an Analytical Reagent grade in a spinning-band column at reduced pressure. All nucleotides were obtained from P-L Biochemicals.

Glaxo Laboratories supplied dimethylbenzimidazole coenzyme, cyanocobalamin, and aquocobalamin. The latter was recrystallized from acetone–water, stored in a desiccator, and dissolved as required in the solvent appropriate to a given experiment by bubbling argon through the mixture. The preparation and purification of the chloride of diaquocobinamide was essentially the same as described elsewhere (Bayston *et al.*, 1969). It was stored at 0° and protected from attack by polluted air. For comparison with cobinamide obtained by acid hydrolysis, some batches were prepared by hydrolysis in the presence of cerous hydroxide by a modification of the procedure of Friedrich and Bernhauer (1956). The final product from both methods was a red microcrystalline material which on conversion into the dicyanide at pH 8 had an optical absorption spectrum identical (within the experimental error of the spectrophotometric procedure) with a spectrum supplied by Dr. R. Bonnett.

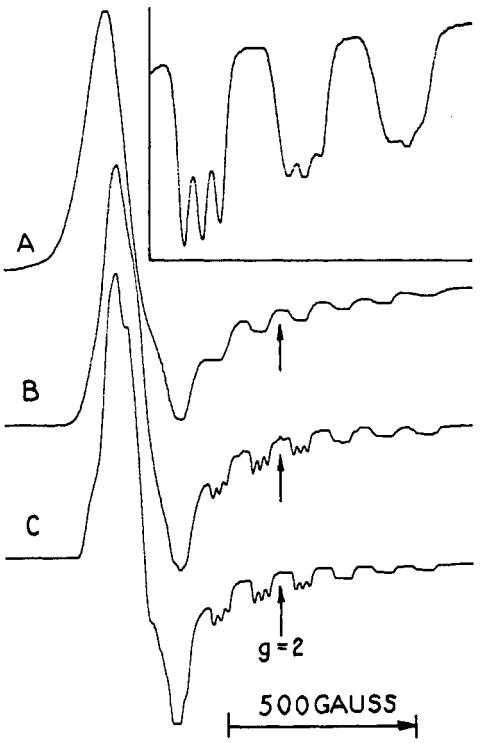

FIGURE 1: Electron paramagnetic resonance spectra of B_{12r}. (A) Crystalline B_{12r} at 298°K, showing flattening of the hyperfine structure at high field strength. (B) B_{12r} at 77°K, formed by photolysis of dimethylbenzimidazole coenzyme in pH 7.3 aqueous phosphate buffer containing dimethylglutarate ion to enhance resolution of the triplets. (The very small singlet at $g = 2$ is due to the liquid nitrogen dewar surrounding the sample.) (C) B_{12r} in ethanol. Inset: high-field end of curve C recorded at high gain and low scanning rate, to show resolution of the triplets of the last three hyperfine lines.

Purified ribonucleotide reductase from *Lactobacillus leichmanii* was provided by Dr. R. L. Blakley of the Australian National University.

Results

Cob(II)alamin. When B_{12r} was formed by anaerobic photolysis of a solution of dimethylbenzimidazole coenzyme in water at 0° (using visible light from a tungsten filament lamp) and the sample examined by electron paramagnetic resonance spectrometry at temperatures well below the freezing point, the signals obtained resembled those first reported by Hogenkamp *et al.* (1963). Buffering the solution at pH 7.3 with phosphate did not affect the result. The same poor resolution was observed when B_{12r} was produced by reduction of aquocobalamin in the pH 7.3 buffer by CO (Bayston and Winfield, 1967), or by HCOOK. A sharper spectrum was obtained from a sample of crystalline B_{12r} in argon (Figure 1A). Some of the hyperfine lines were flattened at the tip, hinting at the presence of unresolved superhyperfine structure. On adding to solutions of B_{12r} in phosphate buffer at pH 7.3 certain solutes such as aquocobalamin, egg albumin, histidylglycine, or methanol (but

TABLE I: Effects of pH, Solvent, and Added Solutes on the Hyperfine and Superhyperfine Structure of Electron Paramagnetic Resonance Spectra of B_{12r} at 77°K.

Solvent	pH	Resolution Enhancer	High-Field Constants: Hyperfine Coupling[a] (G)	High-Field Constants: Superhyperfine Coupling[b] (G)	No. of Triplets Resolved	Highest Peak:Next Highest[c]
Water	13[d]		111		0	~1.2
Water	13[d]	B_{12a}[e]	111	18	3	~1.1
Water	8.3[f]		111		0	~1.4
Water	7.3[f]		110	18	1	~1.1
Water	7.3[f]	Albumin	109	18	5	1.55
Water	7.3[f]	Dimethylglutarate[g]	110	19	5	~1.2
Water	7.3[f]	Dimethylglutarate + MeOH[h]	110	18.5	6	1.5
Water	7.3[f]	Dimethylglutarate + Coenz[i]	111	19	5	1.6
Water	7.3[f]	MeOH[h]	110	18	5–6	1.5
Water	7.3[f]	MeOH[h] + HG[j]	110	18.5	6	1.5
Water	6.0[f]		110		0	~1.6
Water	4.0[f]		110		0	~1.1
			150	0	0	
Water	1.1[k]		156	0	0	~2.4
MeOH			110	19	7	1.5
EtOH			110	19	8	1.25
n-PrOH			110	19	4–5	1.25
n-BuOH			111	18	4	~1.4
α-Hydroxytoluene			110	18.5	4–5	1.25
Ethanediol			110	18.5	5–6	1.4
Dimethylformamide			110	18.5	6–7	1.3
Me_2SO			110	18.5	5	~1.5
Enzyme[l]	7.3	Enzyme	110	18	9–10	1.25
Pyridine			112[m]	19	6	2.2[m]
None[n]			110		0	~1.3

[a] Hyperfine coupling constant due to the cobalt nucleus. The *g* value of the signal was 2.006 ± 0.007. [b] Superhyperfine coupling constant due to the nitrogen nucleus. [c] Ratio of the height of the highest hyperfine line to the height of the next highest. The two lines were about 50 G apart and occurred at a field strength of 2700–2800 G. [d] 0.1 M KOH. [e] Hydroxocobalamin, at a concentration equal to that of the B_{12r}. [f] Sodium phosphate buffer (0.066 M). [g] Sodium 3,3-dimethylglutarate (0.16 M). [h] Methanol to 50% by volume. [i] The B_{12r} was produced in this instance by irradiation of dimethylbenzimidazole coenzyme with visible light, and was therefore accompanied by nucleoside detached by photolysis. [j] Histidylglycine. [k] HCl–KCl buffer. [l] A nucleotide reductase; for specific activity and other experimental details see Hamilton *et al.* (1969). The B_{12r} was formed from dimethylbenzimidazole coenzyme situated within the protein molecules. [m] Some coordination of pyridine occurred. [n] Crystalline B_{12r}.

not formate, chloride, or phosphate) some of the hyperfine lines split into triplets. Figure 1B shows the result of enhancement by 3,3-dimethylglutarate.

When B_{12r} was dissolved in methanol the spectrum resembled that in water containing dimethylglutarate except that the resolution was further improved. With ethanol (Figure 1C) the superhyperfine splitting was not as deep as with methanol, but the total number of lines resolved was a little greater and the detail in the low-field region was distinctly different from that with other solvents. For a series of alcohols the order of decreasing number of lines resolved was ethanol > methanol > ethanediol > 1-propanol = α-hydroxytoluene > 1-butanol (Table I). Observed differences in *g* value and in nitrogen coupling constant were probably not significant. The high-field cobalt coupling constant was in most instances 110 ± 1 G; the high values for acid solutions are dealt with in the next section. Signal characteristics which were sensitive to the environment were the degree of resolution and the relative height of the two strongest hyperfine lines (columns 6 and 7 of Table I). Use of the latter characteristic is *in lieu* of listing *g* values and coupling constants for the two perpendicular signals which comprise the low-field portion of the spectrum, quantities which can be obtained only by computer simulation of the curves. (A program has been developed which is capable of describing many of the features of the cob(II)alamin spectra. A further refinement is needed to describe in detail the low-field signals, since there is anisotropy in the line widths due to unresolved interactions between the unpaired electron and the four hydropyrrole nitrogen atoms. However it is evident from the curve fitting already

attempted that the high-field portion of the spectrum is due to the parallel signal, and that the true values of $g_{||}$ and the related coupling constants are little different from the values used in Table I and estimated as follows: $g_{||}$ is taken to be the value of g at a point midway between the fourth and fifth hyperfine lines; the cobalt coupling constant is the distance between the hyperfine lines, in G, averaged for lines 3–8 inclusive; the nitrogen coupling constant is the distance between the superhyperfine lines of a triplet averaged over those triplets which are resolved at high gain.)

Dilution of methanol with an equal volume of pH 7.3 aqueous phosphate buffer caused only slight loss of resolution of the B_{12r} signal; little further loss was noted on dilution to 1% methanol. Enhancements caused by addition of both dimethylglutarate and methanol to a solution of B_{12r} in pH 7.3 phosphate buffer were partly additive. But combinations of enhancers did not exceed the effectiveness of methanol used as the sole solvent. None of the spectra obtained in the course of the present work matched the resolution achieved when dimethylbenzimidazole coenzyme was converted into B_{12r} at a catalytically active site within molecules of the enzyme ribonucleotide reductase (Hamilton *et al.*, 1969). Nine or more sets of triplets were then resolved. Signal characteristics for the enzymically formed B_{12r} are given in Table I, for comparison with those of related cob(II)inamides and of B_{12r} formed by reduction of aquocobalamin in a variety of media. Within experimental error the coupling constants were unchanged by the special environment.

Detachment of Nucleotide. It has been deduced from optical absorption studies (Hill *et al.*, 1965) that the product of one-electron reduction of cob(III)alamins in acid solution is not always B_{12r}. Detachment of the nucleotide from the cobaltous ion by lowering the pH occurs more readily than from the cobaltic ion. The effect is more clearly detected by electron paramagnetic resonance examination. When the pH of a B_{12r} solution was decreased below about 7 it was found that the signal characterized by a high-field coupling constant of 110 G (Table I) became weaker and mixed with a signal which had a high-field coupling constant of 144.5 G and narrow hyperfine lines which were not split into triplets. The pK for the protonation of the nucleotide (and its detachment from cobalt) was 3.2, in fair agreement with the pK of *ca.* 2.5 found by Hill *et al.* (1965). Little true B_{12r} remained at pH 2.1 (Figure 2A).

As the pH was decreased below about 4, the high-field cobalt coupling constant for the protonated complex began to rise above 145 G; between pH 2 and 1 it increased steeply, to 156 G.

Substitution of solvent for nucleotide as axial ligand seemed also to occur in pyridine solutions (containing 10% of water or methanol to heighten the solubility of the corrinoid). The resulting increase in high-field coupling constant (Table I) was scarcely distinguishable from experimental error; however it was reproducible, and accompanied by significant changes in the low-field region of the spectrum. Superimposed on the principal absorption curve was a weak signal, interpretable by assuming the presence of a strongly coordinating impurity, or of a pyridine ligand in the sixth position in a few per cent of the corrinoid molecules. The shape and relative size of the weak signal were unaltered when the experiment was repeated after redistillation of the pyridine,

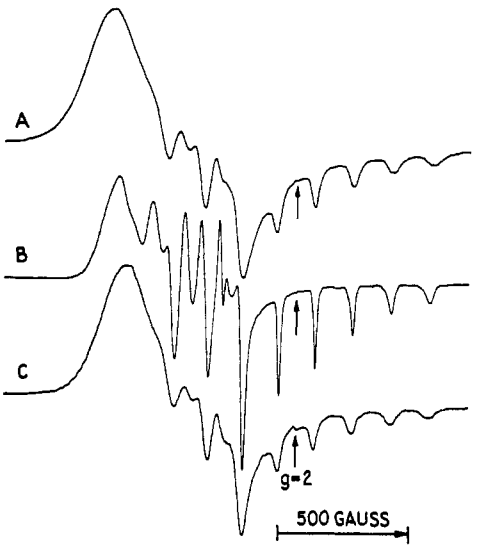

FIGURE 2: Electron paramagnetic resonance spectra at 77°K of acidified B_{12r} and of related cob(II)inamides. (A) B_{12r} in aqueous phosphate at pH 2.1. (B) Methanolcob(II)inamide in methanol. (C) Aquocob(II)inamide in aqueous phosphate buffer at pH 7.3, showing strong resemblance to curve **A**.

or recrystallization of the aquocobalamin used as source of B_{12r}.

Cobinamides. The chloride of diaquocobinamide, dissolved in methanol, was reduced with formate (a more rapid reaction than the reduction of aquocobalamin), then frozen, and the electron paramagnetic resonance spectrum scanned, with the result shown in Figure 2B. The high-field cobalt coupling constant was 30% bigger than for B_{12r} at neutral pH, the tips of the lines were not flattened nor split into triplets, and their width was *ca.* one-third as great. The resolution of the low-field region of the signal was much improved and it could now be seen to consist of eight closely spaced lines, overlapped by the first two of a high-field set of eight more widely spaced.

A different signal to that in Figure 2B resulted with most other solvents, or when the cob(III)inamide which was reduced contained a strongly held fifth ligand. When water acted as solvent as well as providing the fifth ligand, the absorption lines were broadened and therefore less well resolved (Figure 2C), but the high-field coupling constant was almost identical with that in methanol (Table II), as it was in most instances in which the ligand was expected to be weakly held. The changes induced in the signal were then principally changes of shape in the low-field region. Iodide was anomalous as will be seen later.

To prove that the complex responsible for the signal in Figure 2B did not contain formate or chloride as ligand, it was demonstrated that neither their absence from the medium, nor their presence in high concentration, affected the spectrum. Replacement of formate as reductant by formaldehyde (or by KBH_4 or dihydrolipoate provided reaction was halted by freezing before much of the cob(II)-inamide formed was further reduced) did not alter the nature of the electron paramagnetic resonance signal produced. In a later paper, we shall describe the situation which arises when CO is the reducing agent in acid solutions.

TABLE II: Characteristics of High-Field Electron Paramagnetic Resonance Signals of Cob(II)inamides at 77°K, as a Function of the Fifth Ligand.

Fifth Ligand	Solvent	High-Field Constants		
		g	Hyperfine Coupling[a] (G)	Superhyperfine Coupling[b] (G)
α-Hydroxytoluene	α-Hydroxytoluene	2.009	146	0
Ethanediol	Ethanediol	2.01	146	0
Water	Water	2.01	144.5	0
MeOH	MeOH	2.01	144.5	0
EtOH	EtOH	2.01	144	0
I^-	MeOH	1.81	144	*c*
		1.80	143	*c*
Me_2SO	Me_2SO	2.01	143	0
		d	99.5	0
CNS^-	MeOH	2.007	130	0
N_3^-	MeOH	2.005	129	13
Aniline	MeOH	2.002	119.5	14.5
Thiourea	MeOH	2.003	119	0
Adenine	MeOH	2.006	116	19
Isoquinoline	MeOH	2.005	114	19
Benzimidazole	MeOH	2.008	113	18.5
Dimethylbenzimidazole	MeOH	2.01	112.5	18.5
Pyridine	MeOH	2.006	112.5	19
Pyridine	Pyridine	2.01	110	19
		d	109	*d*
NH_2OH	MeOH	2.002	111	20
Imidazole	MeOH	2.005	111	20
Histidine	MeOH	2.004	111	20
NO_2^-[e]	MeOH	2.001	108.5	26
$P(Ph)_3$[f]	MeOH	2.01	93	*c*
		1.92	96	*c*
CN^-	MeOH	2.01	84	0

[a] Hyperfine coupling constant, due to the cobalt nucleus. [b] Superhyperfine coupling constant, due to nucleus of the atom coordinated to cobalt in the fifth position. [c] Unresolved. [d] Unmeasurable due to low concentration of the complex relative to concentration of other paramagnetic species present. [e] Nitro group, formed from nitrite. [f] Triphenylphosphine.

To prove that the fifth ligand was methanol rather than water or OH^- was not possible with the means at our disposal. Progressively diluting the methanolic solution with water did not produce a change of signal which could be correlated with substitution of water for methanol as ligand. With 50% water the signal was almost unchanged in shape, while the resolution was slightly better than in the absence of added water. Even with 99% water the resolution was little inferior, and the signal shape more like that with pure methanol as solvent than with methanol-free water. With ethanol, the signal obtained was much less like that for a solution in water than in methanol, yet clearly distinguishable from the latter. The same may be said for ethanediol and α-hydroxytoluene, when used as solvents.

Insensitivity of the spectrum of Figure 2C to the presence of formate or chloride confirmed that the fifth ligand in this case was either a water molecule or hydroxyl ion. Below a pH of *ca.* 4.5 the signal was accompanied by one of quite different form, consisting mainly or entirely of a line at $g = 2.15$ (Figure 3A). On warming the sample to 18° the new signal gradually disappeared without significant loss of aquocob(II)inamide. From experiments at different pH values it was found that the optimum pH for the line at $g = 2.15$ was between 2 and 3; at pH 1 it was barely detectable.

Splitting of the hyperfine lines into triplets was never observed when the possible axial ligand was nitrogen free. But most ligands containing a nitrogen atom which was both sterically and chemically appropriate for coordination caused splitting. Nitrite ion, after isomerization to a nitro group, was responsible for the largest nitrogen coupling constant yet observed (Figure 3B and Table II). With azide ion, the amplitude of the splitting was small (Figure 3C) due at least in part to a diminished nitrogen coupling constant (Table II). With an unsaturated heterocyclic nitrogen atom bound to cobalt both the extent of splitting and the values of the coupling constants were much the same as for B_{12r}. In general appearance, the spectrum of benzimidazolecob(II)inamide strongly resembled that of B_{12r}, more so than did the spectrum of the dimethylbenzimidazole analog. Imidazole- and histidinecob(II)inamide differed from B_{12r} in that the

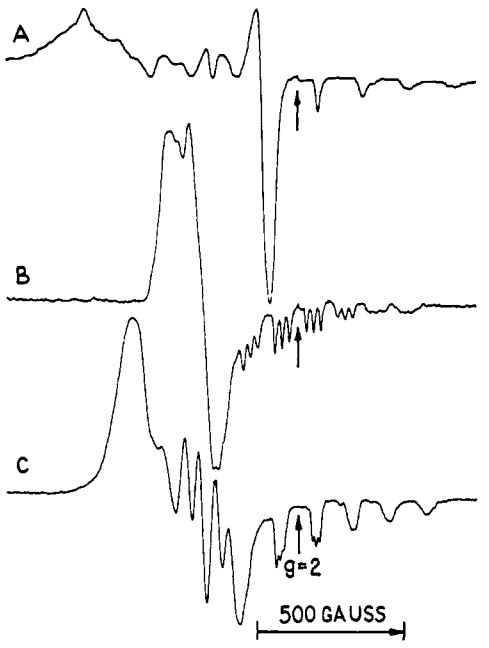

FIGURE 3: Electron paramagnetic resonance spectra at 77°K of cob(II)inamides. (A) Products of reducing diaquocob(III)inamide in aqueous phosphate at pH 2.1, showing new signal arising at $g = 2.15$. (B) Nitrocob(II)inamide in methanol. (C) Azidocob(II)-inamide in methanol.

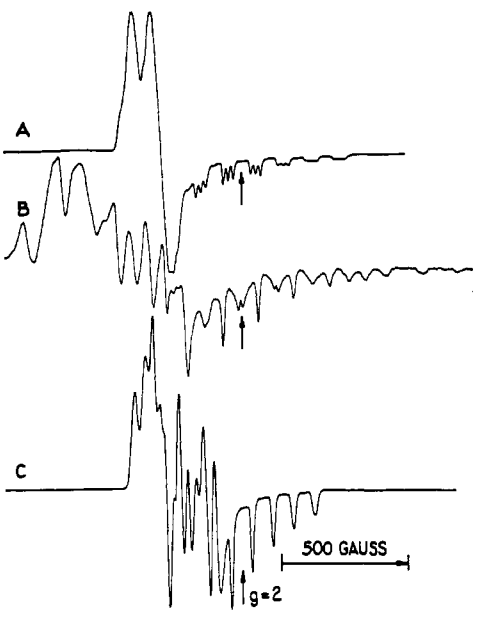

FIGURE 4: Electron paramagnetic resonance spectra at 77°K of cob(II)inamides in methanol. (A) Histidinecob(II)inamide. (B) Two iodocob(II)inamide isomers, mixed with methanolcob(II)-inamide formed by substitution of a solvent molecule for an iodo-ligand during reduction at 291°K. A large excess of KI has been added to increase the proportion of iodo complexes. (C) Cyanocob-(II)inamide in the presence of a large excess of KCN.

second major peak in the low-field region was as high or higher than the first (see Figure 4A).

On reduction in methanol of cob(III)inamide which had a nitrogen atom coordinated in all six positions, the binding of the sixth ligand[2] was weakened to the point of being negligible, as judged by the lack of superhyperfine structure attributable to a sixth ligand and by the relative insensitivity of the spectral shape to the concentration of reactants when imidazole was the complexing agent. In many instances retention of the fifth ligand was also negligible unless the pH was higher than the pK_a of the conjugate acid formed by detachment of the fifth nitrogen atom. The ease of detachment of a large number of ligands was tested in methanol at an apparent pH of 8.5, as determined with a glass electrode. The total cobinamide concentration was 2×10^{-3} M while the concentration of free coordinating agent was 10^{-2} to 4×10^{-2} M except in cases of low solubility, *e.g.*, glutamine (3×10^{-3} M) and guanine (7×10^{-4} M). Because of difficulties in obtaining accurate values of the stability constants the experimental results will not be presented except as follows: Table II lists electron paramagnetic resonance characteristics for those complexes in which some retention was detected at pH 8.5. ATP, 2-aminopyrimidine, ammonia, L-arginine, L-cysteine, CTP, dATP, dCTP, dGTP, cytosine, 2,2′-dipyridyl, egg albumin, formaldehyde, DL-5-hydroxytryptophan, L-glutamine, GTP, DL-leucyltyrosine, L-lysine, methylformate, 8-methylquinoline, L-tryptophan, uracil, and the ions ascorbate, borate, bromide, chloride, dihydrolipoate, dimethylglutarate, ferrocyanide, fluoride, molybdate, and phosphate were completely detached. Signal modifications which could not be attributed definitely to coordination of the additive were induced by guanine, DL-proline, and the ions oxalate and sulfite.

The reduction of iodoaquocobinamide by formate in methanol at pH 8.5 proved to be a complex process, whose detailed study has been postponed. For the present, it suffices to consider the result shown in Figure 4B. About 40% of the reduced corrinoid exhibited the electron paramagnetic resonance signal of methanolcob(II)inamide, while the remainder produced two signals, corresponding to two distinct complexes, each containing iodine (either as a ligand or as both a ligand and a component of the corrin). The unusual appearance of the signals was due to the cobalt coupling constants for the two iodo complexes being almost as large at low field as at high (133 G compared with 144), and to abnormally low g values of 1.80 and 1.81 for the high-field region. Splitting of the hyperfine lines by the iodine nucleus was suggested by their broadness but was not resolved.

Thiocyanate was strongly held, as were the nitro group, triphenylphosphine, and sterically unhindered imidazole- or pyridine-like groups. Histidine was not completely detached at pH 6 and a free histidine concentration of 6×10^{-3} M. Amino groups were retained when the pK_a of their conjugate acid was sufficiently small, *e.g.*, aniline and hydroxylamine.

[2] Spectrophotometrically it can be demonstrated that when a base such as adenine is added to an aqueous solution of hydroxoaquocob-(III)inamide at pH 8 it readily coordinates in the fifth position, but less readily in the sixth. A large excess of adenine is required if most of the cobalt atoms are to have two adenine ligands. When imidazole is the complexing agent the bonding is stronger; even so, adding 2 moles of imidazole/mole of aquohydroxocobinamide results in only three-quarters of the cobalt atoms accepting two new ligands, at 20° and a corrinoid concentration of 7.5×10^{-5} M (J. H. Bayston and M. E. Winfield, 1969, unpublished data).

TABLE III: High-Field Electron Paramagnetic Resonance Characteristics at 77°K in Methanol of Cob(II)inamides Which Are Stable Only at High pH.

Fifth Ligand	App pH	High-Field Constants		
		g	Hyperfine Coupling (G)	Superhyperfine Coupling (G)
NH_3	10.9	2.005	112	17.5
Glutamine	11.8	2.002	109	17.0
Guanine	12.0	2.001	108	19
Proline	11.6	2.004	107	17.5
Tryptophan	10.3	2.005	107	17
Leucyltyrosine	11.1	1.997	106.5	17.5
Cysteine	11.7	2.003	106	17
Arginine	11.8	2.005	106	17.5
Lysine	11.7	2.002	105	17.5
HO-tryptophan	11.6	2.000	103.5	17.5

With triphenylphosphine as ligand there were two signals, in the proportion 3:2 approximately, both ascribed to coordination of triphenylphosphine since they resembled each other more than the spectrum due to cob(II)inamide coordinated to solvent. In neither signal was there any superhyperfine splitting which could be attributed to the phosphorus nucleus. Both had small high- and low-field coupling constants; one was notable in having a g value at high field of only 1.92.

Little cyanide coordinated at pH 8.5. Figure 4C shows the electron paramagnetic resonance spectrum of cyanocob(II)inamide at pH 11. It displays the smallest high-field cobalt coupling constant yet observed, 84 G, and at low field the coupling constants are also small. A parallelism between the values at high and low field was indeed observed for most of the cob(II)inamides studied. Neither cyanoaquocob(III)inamide nor dicyanocob(III)inamide was reduced by formate at room temperature. (With other cobinamides, reduction was possible with the fifth ligand already coordinated, but it was very slow for azido- or nitrocobinamide, which were therefore usually reduced with ascorbate.)

With dimethylformamide as solvent in place of methanol, the retention of iodide and of triphenylphosphine fell to a few per cent. The retention of most other ligands was not greatly affected. When pyridine or highly purified deuteriopyridine was the fifth ligand, change of solvent from methanol to 90% pyridine or deuteriopyridine introduced a few per cent of a second absorption spectrum, possibly that of a dipyridine complex. Detailed description of this and the 2-, 3-, and 4-methylpyridinecob(II)inamides will be given in a later paper. We wish to note here only that coordination of the 2-methyl isomer is not sterically hindered.

Of the compounds listed earlier as unable to coordinate at pH 8.5 in methanol, several were found to bind appreciably when the pH was raised to a value above the pK_a (usually by addition of anhydrous sodium carbonate). The relevant electron paramagnetic resonance characteristics are given in Table III. They indicate that coordination through an amino or imino group attached to an aliphatic carbon atom leads to a high-field cobalt coupling constant about 5 G smaller than that observed when the coordination is through an unsaturated heterocyclic nitrogen atom. Such is the sensitivity of the low-field region of the spectrum to small changes in the nature of the fifth ligand that all of the amino acids studied gave signals of different shape, even though several of them coordinated through their α-amino group.

Discussion

Coordination Number. Changes of solvent have effects on the g values and coupling constants of electron paramagnetic resonance signals of B_{12r} which are much smaller than those induced by changing the fifth ligand. Even when the solvent is pyridine, only a few per cent of what could prove to be a hexacoordinate cobaltous complex are formed. It is therefore a reasonable assumption that bonding of solvent in B_{12r} is weak, *i.e.*, the bond length in the sixth coordination position usually corresponds more nearly to physical than to chemical bonding. Until evidence to the contrary is available, the same assumption is made for all of the cob(II)inamide complexes except when the solvent is pyridine.

Substitution Reactions. There is little substitution of a solvent molecule for the fifth ligand in aquocobalamin dissolved in water unless the pH is well below unity (the pK for the change is about -2.4 (Haywood *et al.*, 1965)). A very different situation prevails after reduction of the metal to the cobaltous state: the pK for displacement of the nucleotide from the metal ion is now 5 or 6 units higher. Nevertheless pyridine is the only solvent investigated which tends to displace the nucleotide of B_{12r} as fifth ligand.

In the case of aquocob(II)inamide, change of solvent usually appears to result in substitution of a molecule of the new solvent for the aquo group. There is no doubt that this has happened when the new solvent brings about a substantial change in coupling constant. But when the observed change is 1% or less, as when methanol is used in place of water, we cannot be certain that substitution has occurred. Since we take the coordination number to be five, the changes observed (but not readily expressed in the tables) in height and position of the low-field lines in the electron paramagnetic resonance spectra are assumed to signify that substitution has taken place. Thus the substance responsible for the signal in Figure 3A is assumed to be methanolcob(II)inamide; the signal could not be mistaken for that obtained when aquocob(II)inamide is dissolved in α-hydroxytoluene or ethanediol, or even in ethanol. Replacement of methanol by pyridine as solvent causes little change in the principal signal when pyridine, which is expected to coordinate more strongly than water and methanol, is the fifth ligand, but a large change if methanol is initially the axial ligand.

When the solvent is neutral methanol, replacement of the methanol ligand assumed to occupy the fifth coordination position occurs readily with unhindered imidazoles and pyridines, but not with adenine, guanine, cytosine, uracil, various biological amino acids other than histidine, nor with the sterically hindered 8-methylquinoline or 2-aminopyrimidine, nor with cyanide ion. At an apparent pH of 8.5, a little replacement by adenine, hydroxylamine, and cyanide can be detected, and at a higher pH still, by ammonia and a number of amino acids.

In Table II the nitrogen coupling constant tends to increase as the cobalt coupling constant decreases, and both vary over a wide range. Keeping in mind that the cobalt coupling constant is usually small at low field when it is small at high field, it is justifiable to conclude that the unpaired electron is to some extent delocalized onto the coordinated nitrogen atom in the fifth position, particularly when the axial ligand is the nitro group. An even greater degree of delocalization, onto a carbon atom in this case, is suggested by the very small cobalt coupling constants of the cyano complex (Figure 4C).

Water and alcohols, which are the most weakly held ligands of Table II since they are displaced by much lower concentrations of the complexing agents listed below them, have the largest cobalt coupling constants. Iodide is exceptional in forming complexes with coupling constants just as large, but with unusually small g values, and in being the only halogen ion to displace the solvent from the fifth coordination position. Hydrogen bonding is possibly responsible: X-ray analysis (Hodgkin, 1965) has shown that ligands such as water form hydrogen bonds to amide and carboxyl groups in some of the cobaltic complexes (and therefore presumably in the cobaltous).

Superhyperfine Structure of the Electron Paramagnetic Resonance Spectra. The only atomic species in B_{12r} whose nucleus could split each of the hyperfine lines into three equal lines is that of ^{14}N ($I = 1$). If the hydropyrrole nitrogen atoms were responsible we should expect to find sets of nine superhyperfine lines, or sets of three in which each line is broadened by three sets of weaker triplets. The accumulated evidence from several hundred B_{12r} and cob(II)inamide electron paramagnetic resonance spectra, measured under a variety of conditions, affirms that only one nitrogen atom is involved. As will be seen in Table II, the one nucleus which can account for the observed triplets is that of an axially bound nitrogen atom. The interactions of the unpaired electron with the four hydropyrrole nitrogen atoms could well be small enough to escape detection except as the hyperfine line-width anisotropy mentioned earlier, especially as the four atoms are not equivalent (or rather they have been shown to be nonequivalent in several cobaltic corrins and are assumed to be nonequivalent in the reduced complexes).

Superhyperfine line broadening, as observed with crystalline B_{12r}, could have several explanations, *e.g.*, exchange interactions made possible by the proximity of the cobalamin molecules to each other, and uncertainties in hydrogen bonding of amide, hydroxyl, and phosphate groups which result in a number of slightly different isomers within the one crystal. Line broadening when the sample is in an aqueous medium lacking resolution-enhancing substances is probably due to separation of crystalline B_{12r} during the freezing process.

Since the magnitudes of the coupling constants are dependent on bond strength, variations in the length or bond angle of the axial coordinate link should cause line broadening. Thus changes in pH in the neighborhood of the axially bound nitrogen, resulting from inhomogeneous concentration changes during freezing, and to variability of packing of solute and solvent molecules and ions between the corrinoid and the axial ligand, are likely to weaken triplet resolution. It has been suggested that a high degree of certainty in the orientation of the sterically variable parts of the B_{12r} molecule, as well as complete isolation of the paramagnetic molecules from each other, was responsible for the outstanding resolution obtained when B_{12r} was formed within an enzyme molecule (Hamilton *et al.*, 1969).

Steric Effects. As judged by electron paramagnetic resonance spectra, benzimidazolecob(II)inamide resembles B_{12r} more closely than does the dimethylbenzimidazole analog. One of the factors responsible is likely to be the steric hindrance between the methyl groups of dimethylbenzimidazole and the corrin, detected in cyanocobalamin by X-ray analysis (Hodgkin *et al.*, 1962). In the absence of the constraining ribose phosphate, which in the nucleotide is attached to dimethylbenzimidazole, dimethylbenzimidazole which is coordinated but not chelated could prefer a different orientation with respect to the corrin. Another factor is the small modification of the pK_a by the combination of dimethylbenzimidazole with ribose phosphate.

With the aid of a Dreiding model it can be seen that free rotation of a triphenylphosphine group about the Co–P bond is not possible, and also, that there is more than one orientation in which the phenyl groups can fit between the corrin side chains. Thus the two different electron paramagnetic resonance spectra resulting from coordination of triphenylphosphine in methanol solution could correspond to two isomers which differ in direction and extent of steric hindrance, with resulting differences in the angle at which the Co–P bond is inclined to the "plane" of the four hydropyrrole nitrogen atoms. The failure to detect doublets in the spectra of the triphenylphosphine complexes is not understood, but it too could be related to orientation of the ligand.

The existence of more than one iodo isomer is difficult to understand except in terms of I–Co–N bond angles which are dictated by weak bonding of iodide to the corrin or to solvent molecules.

Active Sites in Corrinoid Coenzymes. In a preliminary account of the electron paramagnetic resonance spectra of B_{12r} in organic solvents (Looney and Winfield, 1967) it was suggested on the basis of the substantial nitrogen coupling constant of 18.5 G that the nitrogen atom in the fifth coordination position was the long-sought active site in corrinoid coenzymes *in vivo*. We were unaware at that time of the demonstration by Frey and Abeles (1966) that the active site in diol dehydrase is the carbon atom in the sixth coordination position. Nevertheless the participation of a nitrogen atom in the fifth position during the functioning of some of the other coorinoid coenzymes has not yet been ruled out.

In a sense the cobalt atom itself, on its fifth side, can be an active site. In one or both of its reduced states it will be intermittently unbound, or bound to a solvent molecule, instead of to the nucleotide side chain, an effect which will tend to alternately activate and deactivate the coenzyme. In the cobaltous state, the odd electron density at the metal atom will be at a maximum when the fifth position is either unfilled, or filled by an oxygen atom of water or of an amino acid residue. It will be at a minimum when the fifth position is filled by a sterically unhindered imidazole (of the groups likely to be available *in vivo*).

Addendum

Schrauzer and Lee (1968), in a recent communication, quote $g_{||}$ values and nitrogen coupling constants for B_{12r} in a water–ethanol mixture which are in fair agreement with our

present values and those given earlier (Bayston *et al.*, 1969; Looney and Winfield, 1967). For B_{12r} in the presence of imidazole, for adeninecob(II)inamide, and for "factor B_r" the cobalt coupling constants are in considerable disagreement with our values.

In a preliminary note which appeared after submission of our paper, Cockle *et al.* (1969) give coupling constants which agree well with our values (after conversion to the same units), but in addition they find a dependence on the nature of the reducing system. They suggest that the substance referred to as factor B_r by Schrauzer and Lee (1968) has a nitrogenous base as fifth ligand, which would explain why the coupling constant is not appropriate for aquocob(II)-inamide or cyanocob(II)inamide.

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