Biochem. J. (1970) **120**, 263–269
Printed in Great Britain

The Chemistry of Vitamin B_{12}

THE CO-ORDINATION OF BIOLOGICALLY IMPORTANT MOLECULES

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(*Received* 27 *May* 1970)

The following equilibrium constants (given as $\log K$ in units of M^{-1}) were determined for the substitution of co-ordinated H_2O in aquocobalamin by glycine (bound through N) 5.8, cysteine (bound through S) 6.0 or 8.3, depending on the value chosen for the pK of the thiol group, and phenolate 2.9. The spectrum of the phenolate cobalamin shows an additional intense absorption band at 468nm with a molar extinction coefficient of 1.1×10^4, which is assigned to a charge transfer from the phenolate to the cobalt ion. Equilibrium constants have also been determined for the equilibria between adenylcobamide cyanide and CN^-, HO^- and H^+, which show that the adenine is more easily displaced by CN^- and HO^- than is 5,6-dimethylbenziminazole in vitamin B_{12}, but can be protonated by acid while still remaining co-ordinated to the cobalt. It is shown that in the binding of corrinoids to proteins and polypeptides the formation of hydrogen bonds is far more important than co-ordination by the metal.

In previous papers we have determined the formation constants for the binding of a variety of ligands in the axial positions of cobalt(III) corrinoids and have studied the interaction between the ligands as shown by the effect of changing one ligand on the properties of the complex such as the u.v.–visible spectrum of the corrin ring, the proton magnetic resonance of H-10 of the corrin ring, the stretching frequency of cyanide co-ordinated in the *trans*-position and the formation constants for ligand substitution in the *trans*-position (for references see Firth, Hill, Pratt, Thorp & Williams, 1968*b*, 1969; Firth, Hill, Pratt & Thorp, 1968*c*). The aim of the present paper is (1) to extend the study to include more ligands of interest to the biochemistry of the corrinoids, and (2) to discuss the binding of corrinoids by polypeptides and proteins.

Equilibrium constants have already been reported for the substitution of co-ordinated H_2O, usually in aquocobalamin, by several amino acids (e.g. histidine) and ligands containing functional groups of the type present in amino acids (e.g. ammonia, acetate, imidazole) (for references see the Discussion section). We have now determined the equilibrium constants for the substitution of H_2O in aquocobalamin by the phenolate anion, by glycine and by cysteine.

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Most naturally occurring corrinoids possess a nucleotide side chain terminating in a heterocyclic base, which is usually a purine or benziminazole derivative and can co-ordinate to the cobalt. Almost all previous work on the spectra and equilibria has been carried out on the cobalamins and cobinamides. Cobalamins, which include vitamin B_{12} itself (cyanocobalamin), all contain the base 5,6-dimethylbenziminazole. The cobinamides, on the other hand, lack the nucleotide sidechain and this co-ordination position is usually occupied by H_2O; the best known is Factor B (cyanoaquocobinamide). There is, by contrast, no quantitative information on equilibria involving corrinoids that contain purines, even though compounds such as ψ-B_{12}† and adenylcobamide coenzyme, both of which contain adenine, are important products of microbial biosynthesis. We have therefore studied the co-ordinating ability of adenosine in ψ-B_{12} and determined the equilibrium constants for reaction with H^+, HO^- and CN^-.

MATERIALS AND METHODS

Samples of cyano- and aquo-cobalamin were kindly given by Dr L. Mervyn of Glaxo Laboratories Ltd., Greenford, Middx., U.K., and a sample of ψ-B_{12} was kindly given by Professor K. Bernhauer, Stuttgart, W. Germany. A.R. reagents were used whenever possible ($HClO_4$, NaOH, KCN, K_2HPO_4, sodium acetate, phenol);

† Abbreviation: ψ-B_{12}, adenylcobamide cyanide.

glycine, cysteine hydrochloride and quinol were reagent grade.

Equilibrium constants were determined spectrophotometrically in aqueous solution at room temperature (except for ψ-B_{12} and CN^-, where the solutions were thermostatically controlled at 20°C). The nature of the ligands involved in each equilibrium is given below. These constants are stoicheiometric equilibrium constants, i.e. the terms in parentheses refer to concentrations under the experimental conditions and not to thermodynamic activities.

Spectra were recorded on either a Beckman DK 2 or a Unicam SP.700 spectrophotometer, with 1 cm quartz cells. Molar extinction coefficients are based on the value of 3.04×10^4 for the γ-band (367 nm) of dicyanocobalamin, into which other cobalamins can easily be converted (Hill, Pratt & Williams, 1964).

In studying the equilibrium between ψ-B_{12} and acid, the pH of the solution in the spectrophotometer cell (approx. 2 ml) was measured with a Radiometer pH-meter and a Radiometer GK 264 combined electrode.

The electrophoresis of ψ-B_{12} with vitamin B_{12} as standard was carried out in a modified Baird and Tatlock C6/5400 horizontal electrophoresis tank at 200 V.

RESULTS

Aquocobalamin and glycine. Aquocobalamin reacts readily with glycine in approximately neutral solution, and isosbestic points are observed. The γ-band of the product is situated at 360 nm. Since cobalamins containing H_2O and $CH_3 \cdot CO_2^-$ have γ-bands at 350 and 352 nm respectively, whereas those containing nitrogenous bases have them at longer wavelength, e.g. NH_3 356 nm, imidazole 358 nm and pyridine 360 nm (Firth *et al.* 1969), we conclude that glycine is co-ordinated through the nitrogen atom, i.e. as $^-O_2C \cdot CH_2 \cdot NH_2 \rightarrow Co$. The rate of equilibration is rather low under the conditions required to observe an equilibrium, and solutions of aquocobalamin in acetate buffer, pH 5.5, were therefore allowed to come to equilibrium in the presence of 0 M, 0.02 M-, 0.20 M- and 2.0 M-glycine. The degree of complex-formation in the second and third solutions (43 and 91%) gave apparent equilibrium constants under the experimental conditions of 20 and 27. Allowance for the formation of acetatocobalamin (Firth *et al.* 1969) and use of the value of $pK_a = 9.7$ for the removal of the proton from glycine (Sillén & Martell, 1964) gave the value for the equilibrium constant:

$$K = \frac{[\text{Co–NH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2^-]}{[\text{Co–OH}_2][\text{NH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2^-]}$$

of $\log K = 5.8 \pm 0.2$.

Aquocobalamin and cysteine. The formation of a complex from aquocobalamin and cysteine has already been reported (Hill, Pratt & Williams, 1962; Pratt, 1964; Alder, Medwick & Poznanski, 1966). The cobalt(III) ion is reduced to cobalt(II) by cysteine in alkaline solution (Hill *et al.* 1962; Peel, 1963; Pratt, 1964), and the equilibrium has therefore been studied in faintly acid solution. The spectrum of the product is similar to that of thiosulphatocobalamin in having an 'atypical' spectrum, characterized by the occurrence of the γ-band at longer wavelength with considerably diminished intensity and the appearance of other bands of comparable intensity in the 300–350 nm region (Pratt & Thorp, 1966); the comparable wavelengths (with molar extinction coefficients $\times 10^{-4}$ in parentheses) are: cysteinatocobalamin, 338 (1.52), 350 (shoulder) (1.35) and 371 nm (1.41); thiosulphatocobalamin, 330 (1.55), 353 (1.49) and 367 nm (1.55). Adler *et al.* (1966) also found that similar 'atypical' spectra were shown by cobalamins containing ethanethiol, cysteine and GSH. One can therefore conclude that cysteine is co-ordinated through the sulphur atom. Wagner & Bernhauer (1964) showed by electrophoresis that the GSH complex was uncharged at pH 2.5, had one negative charge over the range pH 4–7 and two negative charges at pH 11. At pH 4–7 the co-ordinated GSH has therefore lost two protons, presumably from the carboxylic acid and thiol groups. We assume that the thiol group of cysteine also becomes ionized on co-ordination to the cobalt. Solutions of aquocobalamin in acetate buffer, pH 5.5, were allowed to come to equilibrium in the presence of 0 M-, 0.001 M-, 0.002 M- and 0.1 M (excess)-cysteine. The degree of complex-formation in the second and third solutions (61 and 80%) gave apparent equilibrium constants under the experimental conditions of 1.6×10^3 and 2.0×10^3 respectively. Cysteine shows two pK values in alkaline solution of approx. 8 and 10; there is, however, no agreement on which corresponds to the ionization of the thiol and which to that of the ammonium group (see the discussion by Wallenfels & Streffer, 1966). After allowing for the formation of acetatocobalamin, use of the value pK_a 8.2 for the ionization of the thiol group in cysteine leads to the value $\log K$ 6.0 and of the value pK_a 10.5 to $\log K$ 8.3, where the equilibrium constant is:

$$K = \frac{[\text{Co–SR}]}{[\text{Co–OH}_2][\text{RS}^-]}$$

Aquocobalamin and phenol. Aquocobalamin reacts rapidly with phenol in mildly alkaline solution to give a new complex with a rather low equilibrium constant and an unusual spectrum. The pK_a of phenol is 10.0. The equilibrium constant was determined in phosphate buffer, pH 10.5; at higher pH phenol undergoes oxidation to give coloured products. Since the formation constant is low and no end point could be obtained corresponding to 100% formation, the constant was calculated by

the method of Lunn & Morton (1952) and Newton & Arcand (1953). No complex-formation was observed at pH 7 and below, from which we conclude that the ligand is the phenolate anion. The equilibrium was shown to involve one phenol per cobalt and the formation constant found to be K 800 or $\log K$ 2.9 where:

$$K = \frac{[\text{Co–OC}_6\text{H}_5]}{[\text{Co–OH}_2][\text{C}_6\text{H}_5\text{O}^-]}$$

The spectrum of the fully formed phenolate complex was determined over the range 350–600nm by extrapolation from mixtures with known ratios of the hydroxide and phenolate complexes. The α- and γ-bands of the phenolate [λ_α 533nm (shoulder), ϵ_M approx. 0.93×10^4; λ_γ 355.5nm, ϵ_M approx. 1.9×10^4] are similar in energy and intensity to those of the hydroxide (λ_α 533nm, ϵ_M 0.92×10^4; λ_γ 356.5nm, ϵ_M 1.93×10^4). But the phenolate also shows additional absorption in the region between the α- and γ-bands with an apparent peak at 468nm (ϵ_M 1.1×10^4). If one assumes that this additional absorption is due to transitions of the charge-transfer type (see the Discussion section) and that the absorption due to the π–π transitions alone would be the same as that of the hydroxide, then one can isolate the spectrum due to charge transfer alone by subtracting out the spectrum of hydroxocobalamin; this gives a very broad and featureless absorption extending from approx. 370 to approx. 530nm with a flat maximum at approx. 455nm with ϵ_M approx. 0.7×10^4.

Yurkevich, Rudakova & Pospelova (1966) have also reported the preparation of a complex from aquocobalamin and phenol that could be isolated as a solid. No equilibrium constant was reported and the phenol/cobalt ratio was not determined either for the solid or in solution. But the spectrum in aqueous solution showed an intense hand at 425nm (ϵ 0.6×10^4) in addition to the α-, β- and γ-bands at 540 (0.6×10^4), 510 (0.6×10^4) and 360nm (2.8×10^4). This complex could be the same as we have studied, although the low value of the equilibrium constant that we obtained makes it difficult to see how they obtained the spectrum of the apparently fully formed complex by dissolving the crystalline complex in water; in addition, the spectra are slightly different. They also reported that the spectrum of aquocobalamin was altered by the addition of quinol or resorcinol without stating whether a reaction or equilibrium was involved. In preliminary experiments we found that aquocobalamin reacted rapidly and irreversibly with quinol in alkaline solution to give products that absorb at about 400nm, i.e. quinol is involved in a reaction, not an equilibrium.

Equilibria involving ψ-B_{12}. The spectra of aqueous solutions of ψ-B_{12} were studied over the range of pH 0 (1M-perchloric acid) to 14.7 (5M-sodium hydroxide). Reversible equilibria were observed in strongly alkaline and weakly acid solution. Electrophoresis at pH 6 (0.05M-phosphate buffer) confirmed that the complex possessed zero overall charge, as expected for adenylcobamide cyanide. At this pH the spectrum shows α-, β- and γ-bands at 546, 516 and 359nm respectively. The alkaline equilibrium was studied in solutions of 0–5M-sodium hydroxide. Isosbestic points were observed, and from the position and relative heights of the absorption bands (α-, β- and γ-bands at 553, 520 and 362nm) the axial ligands in the product were identified as CN^- and HO^- (cf. cyanohydroxocobinamide 550, 525 and 362nm), i.e. adenine has been displaced by hydroxide. The equilibrium constant was determined from the point of inflexion of the graph of extinction at 359nm against HO^- concentration; pK is 13.8 and $\log K$ is 0.2 ± 0.2, where:

$$K = \frac{[\text{NC–Co–OH}]}{[\text{NC–Co–Ad}][\text{OH}]}$$

and Ad is the adenine of the nucleotide side chain. No analogous change was observed for cyanocobalamin (vitamin B_{12}) in solutions containing up to 2M-sodium hydroxide, i.e. the corresponding $\log K$ is less than -0.5.

The acid equilibrium was studied in solutions of 0–1M-perchloric acid. The change in spectrum is relatively small, and the position of the α-, β- and γ-bands of the product (541, 513 and 358nm) shows that the axial ligands are not CN^- and H_2O (cf. cyanoaquocobinamide 525, 494 and 353nm), i.e. the adenine has not been displaced from co-ordination to the cobalt. Excellent isosbestic points were observed, and the plot of extinction at 359nm against the pH of the solution, measured with a hydrogen electrode, gave pK 2.3 ± 0.1.

The reaction of ψ-B_{12} with potassium cyanide shows a simple equilibrium; good isosbestic points are observed and no further reaction is noted (for over 2h). The product has α-, β- and γ-bands at 579, 540 and 368nm (cf. dicyanocobinamide 580, 540 and 367nm). The equilibrium therefore involves one cyanide and the displacement of adenine from co-ordination by cyanide. The equilibrium constant was determined under the same conditions as those used previously for the analogous equilibrium of cyanocobalamin with cyanide, namely 20°C, phosphate buffer, pH 11.4, and low concentrations of potassium cyanide (Firth *et al.* 1969). Qualitative experiments indicated an equilibrium constant of approx. 10^5. At the very low cyanide concentrations required by the high equilibrium constant, equilibrium is not established instantaneously. Solutions of 2.95M-ψ-B_{12} were therefore allowed to come to

Table 1. *Comparison of equilibria involving vitamin* B_{12} *and* ψ-B_{12}

	Reagent	Axial ligands in product	Conditions	log K (M^{-1})	Reference
Vitamin B_{12}	H^+	NC-Co-OH_2	H_2SO_4-H_2O mixtures	0.1 ± 0.1	Hayward, Hill, Pratt, Vanston & Williams (1965)
	HO^-	NC-Co-OH	0–2 M-NaOH	<−0.5	This paper
	CN^-	NC-Co-CN	Phosphate buffer, pH 11.4, with various KCN concns.	3·8 ± 0·2	Firth *et al.* (1968*b*)
ψ-B_{12}	H^+	NC-Co-AdH*	0–1 M-$HClO_4$	2.3 ± 0.1	This paper
	HO^-	NC-Co-OH	0–5 M-NaOH	0.2 ± 0.2	This paper
	CN^-	NC-Co-CN	Phosphate buffer, pH 11.4, with various KCN concns.	5.1 ± 0.2	This paper

* See the text for explanation.

equilibrium (30 min) in the presence of 0.02 mM- and 0.03 mM-potassium cyanide. The following values of K, where:

$$K = \frac{[\text{NC-Co-CN}]}{[\text{NC-Co-Ad}][\text{CN}]}$$

were obtained: K 1.1×10^5 and 1.6×10^5 or log K 5.0 and 5.2, i.e. log K 5.1 ± 0.2.

The above equilibrium constants involving ψ-B_{12} are given in Table 1 together with analogous data for cyanocobalamin.

DISCUSSION

Equilibrium constants of the cobalamins. All the cobalamins that have been prepared (both in the present work and previously) with amino acids or with ligands containing donor groups of the type present in amino acids have been listed in Table 2 with (1) the equilibrium constants (log K) where:

$$K = \frac{[\text{Co-X}]}{[\text{Co-OH}_2][\text{X}]}$$

and (2) the apparent equilibrium constants at pH 7 (log K') where:

$$K' = \frac{[\text{Co-X}]}{[\text{Co-OH}_2]([\text{X}]+[\text{XH}])}$$

The values of K (or K') reported in the literature have been converted into K' (or K) by the use of the values of pK listed in column 2. Values of K' provide a measure of the strength of binding of amino acids and other ligands to the cobalt under physiological conditions. Certain qualitative observations on thiols and organic sulphides have also been included in Table 2. Adler *et al.* (1966) found no interaction between aquocobalamin and methionine, cystine or GSSG; but no concentrations were reported and the maximum value of the equilibrium constant cannot therefore be calculated. Bauriedel (1956) states that of 20 (unspecified) common amino acids studied at pH4.5 only histidine reacted with aquocobalamin. Pyridine, imidazole and other imidazole-containing ligands such as histamine, carnosine and histidylhistidine in addition to histidine also reacted (Bauriedel, Picken & Underkofler, 1956). Data are also included for CN^- and SO_3^{2-}, which have the highest values of K' of all cobalamins so far studied; it is of note that these cobalamins are both produced as artifacts during the isolation of corrinoids. The identification of the ligand atom is discussed below under 'Spectra of the cobalamins'.

The data in Table 2 show that oxygen, nitrogen and sulphur atoms may all act as donors, though the equilibrium constants vary widely depending on the exact nature of the functional group; thus the values of log K' for S are in SO_3^{2-} 6.8, in thiolates 3–5 and in dialkylsulphides <−1. The following order of complexing ability is observed at pH7 with the donor groups present in amino acids: N (amine or imidazole) ≈ S (thiolate) ≫ 0 (carboxylate, phenolate). The very similar values of K' for amine, imidazole and thiolate mean that an amino acid or polypeptide with more than one of these functional groups can co-ordinate in more than one way, although one isomer will predominate; under certain conditions kinetic factors, such as the differing rates of co-ordination of amines and imidazoles, may play an important role.

Spectra of the cobalamins. A noticeable change in the spectrum accompanies the substitution of co-ordinated H_2O by all the ligands listed in Table 2 with the exception of acetate. The γ-band shifts, but there is little change in the general shape of the absorption spectrum when a nitrogen atom is co-ordinated, whether in a base (ammonia, imidazole,

Table 2. *Equilibrium constants of cobalamins containing amino acids or ligands with related groups*

For definition of K and K' see the text. †, Complex observed, but no quantitative data available.

Ligand	Ligand atom	Free base		Complex		
		pK	Reference	log K (M^{-1})	log K' (M^{-1})	Reference
Acetate ($CH_3 \cdot CO_2^-$)	O	4.6	Sillén & Martell (1964)	0.7	0.7	Firth *et al.* (1969)
Phenolate ($C_6H_5 \cdot O^-$)	O	10.0	Sillén & Martell (1964)	2.9	−0.1	This paper
NH_3	N	9.4	Sillén & Martell (1964)	≈7	≈4.6	Hayward, Hill, Pratt & Williams (1970)
Imidazole ($C_3H_4N_2$)	N	7.2	Albert (1959)	4.6	4.2	Hanania & Irvine (1964)
Imidazolate ($C_3H_3N_2^-$)	N	14.5	Albert (1959)	8.8*	1.3	Hanania & Irvine (1964)
Glycine	N	9.7	Sillén & Martell (1964)	5.8	3.1	This paper
Histidine	N	9.2	Sillén & Martell (1964)	5.8	3.6	Bauriedel (1956)
Ethanethiolate ($C_2H_5 \cdot S^-$)	S	—	—	+	+	Adler *et al.* (1966)
Thioglycollate ($OC \cdot CH_2 \cdot S^-$)	S	—	—	+	+	Hill *et al.* (1962); Hayward *et al.* (1965)
Dimethyl sulphide	S	—	—	<−1†	<−1†	Firth *et al.* (1969)
Cysteinate (RS^-)	S	8.2 or 10.5	Wallenfels & Streffer (1966)	6.0 or 8.3	4.8	This paper
Glutathionate (RS^-)	S	8.6 or 9.5	Wallenfels & Streffer (1966)	4.9 or 5.8	3.3‡	Adler *et al.* (1966)
CN^-	C	9.3	Sillén & Martell (1964)	≪12	≪10	Hayward *et al.* (1965)
SO_3^{2-}	S	7.0	Sillén & Martell (1964)	7.3	7.0	Firth *et al.* (1969)

* Calculated from data of Hanania & Irvine (1964) by using the equation $\log K_2 = \log K_1 + pK_{B2} - pK_{C2}$ (see Hayward *et al.* 1970).

† No change in spectrum observed in 1 M-dimethyl sulphide.

‡ The reported equilibrium constant (K'') was determined in acetate buffer, pH 4·7, whence $\log K' = \log K'' + (7\text{–}4.7)$

pyridine) or an anion (N_3^-, NCO^-, NO_2^-). HO^- causes a similar shift in wavelength, but the intensity of the γ-band is much lower, whereas phenolate produces a very unusual spectrum (see below). Ligands such as thiols, sulphite and thiosulphate (but not NCS^-), which co-ordinate through a sulphur atom, all produce 'atypical' spectra, characterized by the presence of a γ-band at longer wavelength with low intensity and the appearance of new bands of comparable intensity in the region 300–350nm. Carbanions (as in 5,6-dimethylbenziminazolylcobamide coenzyme) and other very polarizable ligands such as I^- and $NCSe^-$ also produce 'atypical' spectra. Further details of spectra are given by Pratt & Thorp (1966) and Firth *et al.* (1969). It is therefore easy to detect the formation of a new complex as the result of the substitution of co-ordinated H_2O and to identify the ligand atom (except in the case of carboxylates), e.g. whether cysteine and GSH are co-ordinated through oxygen, nitrogen or sulphur. It is much more difficult, however, to detect the substitution of 5,6-dimethylbenziminazole by another nitrogenous base or to decide whether histidine is co-ordinated through the imidazole or amine nitrogen atom. Since, however, histidine has a higher equilibrium constant at pH 7 ($\log K'$ 3.6) than glycine ($\log K'$ 3.1) and histidine alone out of 20 amino acids tested reacted with aquocobalamin under the experimental conditions (Bauriedel, 1956), it seems reasonable to conclude that histidine is co-ordinated through the imidazole nitrogen atom.

The unusual spectrum of the phenolate complex deserves further comment. It clearly contains an additional intense band centred at about 455nm that is superimposed on the normal pattern of α-, β- and γ-bands. Similar intense bands or unusual colours have been reported for complexes of various phenolates with silver(I) (see, e.g., Blanchard, Finkbeiner & Russell, 1962), copper(II) (Blanchard *et al.* 1962; Harrod, 1969), titanium(IV) (Watenpaugh & Caughlin, 1966) and iron(III) (Ackermann & Hesse, 1969; Limb & Robinson, 1969). The phenolatopenta-aquoiron(III) complex shows an intense band (ϵ 1200) at 560nm (Ackermann & Hesse, 1969). In all these complexes the metal ion is known to be readily reduced, whereas phenols are, of course, oxidizable, and the transitions giving rise to the intense absorption can be ascribed to a charge-transfer transition from the phenolate to the metal ion. Harrod (1969) did, however, conclude that charge transfer occurred from the copper(II) to the phenolate, but his arguments were based mainly on trends in thermal stability and cannot be accepted.

Co-ordination of adenine in ψ-B_{12} and adenylcobamide coenzyme. The equilibrium constants presented in Table 1 are the first quantitative data relating to the co-ordination of the nucleotide adenine in ψ-B_{12}. There are two main points of interest. First, comparison with the analogous equilibrium constants for vitamin B_{12} (see Table 1) with CN^- and HO^- shows that the cobalt–adenine bond is about an order of magnitude weaker than the cobalt–5,6-dimethylbenziminazole bond. Secondly, ψ-B_{12} differs from vitamin B_{12} in its behaviour towards acid. Both bases can be protonated, but adenine remains co-ordinated whereas 5,6-dimethylbenziminazole is displaced from the cobalt. Since substituted anilines usually have pK values in the range 1–5, the co-ordinated adenine is probably protonated on the amino group. Heterocyclic bases can therefore co-ordinate to the cobalt ion of corrinoids in three forms: (1) as the neutral base, (2) in the anionic form, as shown for imidazolate, benziminazolate and adeninate anions (Hayward *et al.* 1970), and now (3) as a protonated base.

These results can be used to explain the structure and spectra of adenylcobamide coenzyme. Adenylcobamide coenzyme has the same spectrum at all pH values as 5,6-dimethylbenziminazolylcobamide coenzyme in acid solution (Bernhauer, Gaiser, Müller & Wagner, 1960), i.e. the adenine is not co-ordinated to the cobalt. This is clearly due to a combination of (i) the strong trans-weakening effect of the organo ligand together with (ii) the weakness of the cobalt–adenine bond compared with the cobalt–5,6-dimethylbenziminazole bond. For a fuller discussion of the *trans*-effect in relation to the equilibrium constants for ligand substitution in the corrinoids see Firth *et al.* (1968*b*, 1969) and Hayward *et al.* (1970). The indirect evidence adduced for five-co-ordination in the alkyl corrinoids (Firth *et al.* 1968*a*) can now be considered as supported by the X-ray analysis of *NN'*-ethylenebis(acetylacetoneiminato)methylcobalt(III), which shows the complex to be five-co-ordinate (Brückner, Calligaris, Nardin & Randaccio, 1969). In 5,6-dimethylbenziminazolylcobamide coenzyme the cobalt–5,6-dimethylbenziminazole bond is weakened to such an extent that the complex exists in aqueous solution as a mixture of about 90% of the expected six-co-ordinate complex and about 10% of the five-co-ordinate complex, in which 5-6,dimethylbenziminazole is no longer co-ordinated. By analogy with ethylcobinamide (Firth *et al.* 1968*a*) we can conclude that adenylcobamide coenzyme exists mainly as the five-co-ordinate complex. It has also been shown that the rate of interconversion of the five- and six-co-ordinate corrinoids is extremely rapid (Firth *et al.* 1968*a*). Since the formation constants for the binding of all ligands in the position *trans* to methyl, ethyl and 5-deoxyadenosyl are low ($\log K$ 2 or less), i.e. the cobalt ion in this environ-

ment shows no strong preference for any particular type of ligand (Pailes & Hogenkamp, 1968; Firth *et al.* 1969) and the rates of substitution are high, the cobalt ion is obviously open to attack by a wide range of potential ligands or substrates.

Binding of corrinoids to polypeptides and proteins. A complex of aquocobalamin with a polypeptide has been isolated and the composition of the 80 amino acid residues determined (Hedbom, 1960). The cobalamin is firmly held and the spectrum shows a γ-band at 350nm (i.e. probably aquocobalamin). On treatment with cyanide the γ-band moves to 361nm (i.e. cyanocobalamin) and the cobalamin is released from the complex. It is clear that the spectrum excludes the possibility of the co-ordination of any nitrogen or sulphur atom from an amino acid side chain. The co-ordination of a carboxylate group cannot be so firmly excluded, but in this case the formation constant at pH 7, $\log K'$, is less than 1. The firm binding of aquocobalamin to the polypeptide cannot therefore be explained by co-ordination of an amino acid to the cobalt. Similar arguments can be used to show that the binding of vitamin B_{12} and 5,6-dimethylbenziminazolylcobamide coenzyme to various proteins can also not be due primarily to changes in the co-ordination sphere. Spectroscopic and other studies have shown that neither the cyanide nor the alkyl ligand is displaced on binding to the protein, and that if 5,6-dimethylbenziminazole is displaced it can only be displaced by a ligand that has an almost identical effect on the spectrum (see, e.g., Wijmenga, Thompson, Stern & O'Connell, 1954; Gregory & Holdsworth, 1955; Babior & Li, 1969; Babior, Kon & Lecar, 1969). Examination of the known equilibrium constants in solution shows that for the binding of either vitamin B_{12} or 5,6-dimethylbenziminazolylcobamide coenzyme by the displacement of 5,6-dimethylbenziminazole by any amino acid functional group the formation constant will be less than 1. We conclude that the major factor in the binding of corrinoids to proteins or polypeptides is the formation of hydrogen bonds.

This work was supported by the Medical Research Council (U.K.). We thank Dr L. Mervyn and Glaxo Laboratories Ltd. for samples of cyano- and aquocobalamin, and Professor K. Bernhauer for a sample of adenylcobamide cyanide (ψ-B_{12}). This is paper no. 13 of the series 'The chemistry of vitamin B_{12}' [paper no. 12: Hayward *et al.* (1970)].

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