1196. *The Chemistry of Vitamin* B_{12}. *Part IV.*[1] *The Thermodynamic* trans-*Effect*

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Equilibrium constants are reported for several ligand replacement reactions of cobalamins and cobinamides (see Figure) and are interpreted in terms of the thermodynamic *trans*-effect of the ligand which is not replaced. It is shown that as this ligand is changed in the order H_2O, NC^-, $HC{\equiv}C^-$, $H_2C{=}CH^-$, CH_3^-, so the *trans* cobalt–cyanide bond becomes less stable relative to the cobalt–benziminazole bond which in turn becomes less stable relative to the cobalt–water bond. The work involved the identification of certain cobalamins and cobinamides.

This Paper presents a study of equilibria involving the binding of axial ligands to the cobalt of vitamin B_{12} (see Figure). The results are discussed in terms of the thermodynamic *trans*-effect of one ligand X on the equilibria for the replacement of other possible axial ligands Y by Z. The corrinoids are particularly well suited for the study of this effect in general for the following reasons.

(1) Octahedral co-ordination eliminates the uncertainty concerning the binding of further ligands which is inherent in studying square-coplanar complexes.

(2) The equatorial ring prevents *cis–trans*-isomerisation.

(3) The equilibria are, in most cases, fairly rapidly established.

(4) It has proved possible to study a wider range of ligands than in simpler cobalt(III) complexes.

Particular attention has been paid to complexes containing carbon ligands. They provide a useful, graded series of ligands, are fairly stable to acid, alkali, and cyanide, and give many equilibrium constants within an easily measurable range. Additional interest in these organometallic complexes is provided by the fact that the naturally-occurring coenzymes are also alkyl complexes.

Before commencing the study of the equilibrium it is necessary to establish the structures of the molecules and the valency of the cobalt. Knowledge of the structures depends ultimately on the *X*-ray analysis of, for example, B_{12}[2] and DBC.[3] * Both of these are

* DBC is α-(5,6-dimethylbenziminazolyl)cobinamide coenzyme.

[1] Part III, H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *J.*, 1965, 2859.

[2] D. C. Hodgkin, *Fortschr. Chem. org. Naturstoffe*, 1958, **15**, 167.

[3] P. G. Lenhert and D. C. Hodgkin, in " Vitamin B_{12} and Intrinsic Factor," ed. Heinrich, Ferdinand Enke, Stuttgart, 1962, p. 105.

diamagnetic cobalt(III) complexes.[4] Other compounds have been related to them either by the stoicheiometry of a reaction or of an equilibrium (*e.g.*, dicyanocobalamin [5]) or by the method of preparation (*e.g.*, methylcobalamin [6,7]) and similar methods have been used in the present work. In addition we have determined the oxidation number of the cobalt and the charge on the complex (and hence the nature of the ligand) of ethynyl- and vinylcobalamin by e.s.r. and electrophoresis, respectively. Octahedral co-ordination, where one of the ligands is a solvent molecule (H_2O), rather than square-pyramidal configuration is difficult to establish conclusively. But in view of the known strong preference of cobalt(III) for octahedral co-ordination and the parallel in spectra between "aquo"-corrinoids and others known to possess six ligands,[8] it will be assumed that all the complexes to be discussed in this Paper are octahedral. A subsidiary uncertainty regarding these complexes is the orientation of the two axial ligands. Because of the relative orientation of the side-chains and the slight deviation from planarity of the corrin ring the equatorial ligand has an "upper" and a "lower" side and two isomers are theoretically possible when the axial ligands are not identical. The benziminazole occupies the same co-ordination site in both vitamin B_{12} and DBC, namely on that side of the ring towards which the nucleotide side-chain projects; the same orientation is assumed for the other cobalamins. No *X*-ray analysis has yet been reported of a cobinamide, but in Factor V_{1a} the cyanide is on the side opposite to that in vitamin B_{12}.[9] No assumptions can therefore be made about the configuration of cobinamides containing two different axial ligands. However, as shown in the Experimental section, the spectrum of methylcobinamide is identical within experimental error with that of the corresponding cobalamin in strong acid, where the base becomes protonated and replaced by H_2O as ligand. This means either that the configuration round the cobalt is the same in the cobalamin and cobinamide or that a change in configuration has no observable effect on the corrin ring, in which case it is reasonable to assume that the equilibrium constant for ligand exchange will be virtually unaffected by a change in configuration. The possibility of co-ordination isomerism will therefore be neglected in discussing the results.

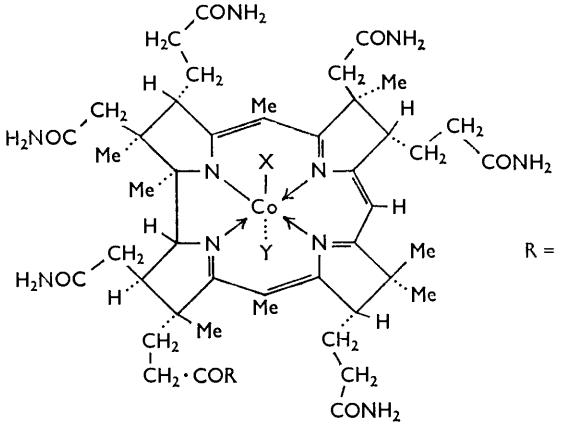
Results

Characterisation of the Compounds.—(*a*) *Vinyl- and ethynylcobalamins.* Although the method of preparation of these cobalamins [7] suggests their formulation as cobalt(III) complexes containing the groups $C_2H_3^-$ and C_2H^- respectively, this has not been proved. We have therefore sought evidence for the presence of unpaired electrons in the compounds and have determined the charge carried by the compounds. Solutions (0·004M) of the vinyl- and ethynyl-cobalamins in water when cooled to 77° K showed no detectable e.s.r. signal, *i.e.*, any signal present was less than 1/400 of that of a corresponding solution of the cobalt(II) derivative, vitamin B_{12r}. Electrophoresis in acetate buffer, pH 5·0, using cyanocobalamin and aquocobalamin as reference compounds (uncharged and one positive charge, respectively) showed that both compounds were uncharged; the spectra in acetate buffer were the same as in water and showed no change over a period longer than the electrophoresis experiment. It is concluded that these compounds, like DBC, can be regarded as diamagnetic cobalt(III) complexes in which the ligand is present as a carbanion ($HC{\equiv}C^-$ and $H_2C{=}CH^-$, respectively) and not as the uncharged hydrocarbon (π-complexes of C_2H_2 and C_2H_4).

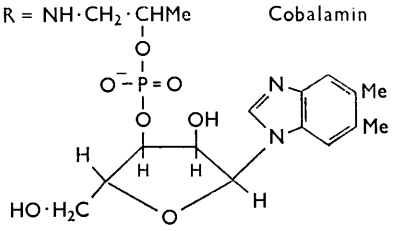
(*b*) *Cobinamides.* There are six possible cobinamides with CN^-, H_2O, and OH^- as the only axial ligands. The identity of dicyanocobinamide has been established; it has the same spectrum (λ_γ = 367 mμ) as dicyanocobalamin [5,10] and is uncharged.[10]

[4] R. Bonnett, *Chem. Rev.*, 1963, **63**, 573.
[5] P. George, D. H. Irvine, and S. C. Glauser, *Ann. New York Acad. Sci.*, 1960, **88**, 393.
[6] O. Müller and G. Müller, *Biochem. Z.*, 1962, **336**, 299.
[7] A. W. Johnson, L. Mervyn, N. Shaw, and E. L. Smith, *J.*, 1963, 4146.
[8] H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Proc. Roy. Soc.*, 1965, in the press.
[9] K. Venkatesan, D. H. Dale, and D. C. Hodgkin, Paper presented at the Brussels International Symposium on Organic Chemistry, 1962.
[10] J. B. Armitage, J. R. Cannon, A. W. Johnson, L. F. J. Parker, E. Lester Smith, W. H. Stafford, and A. R. Todd, *J.*, 1953, 3849.

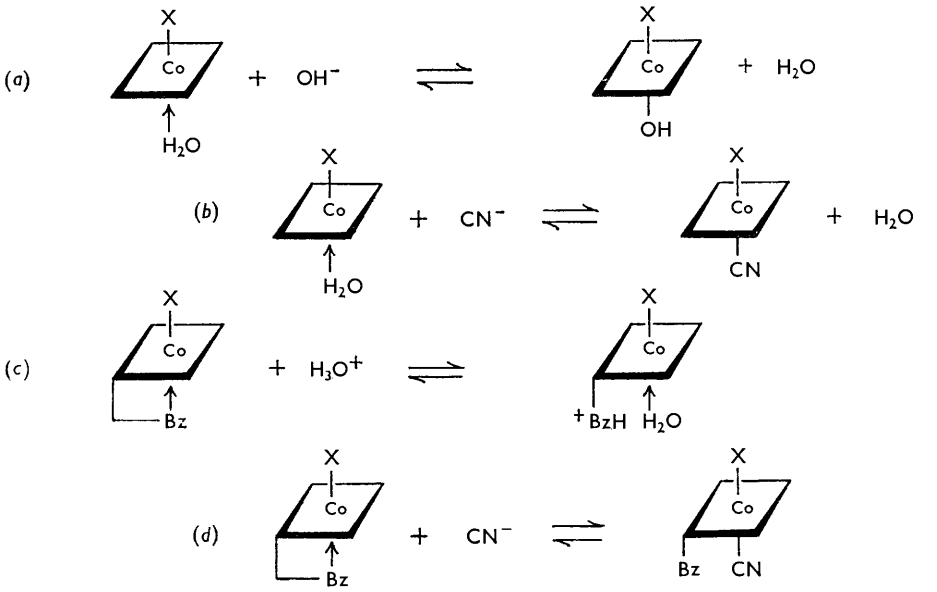
There is some discrepancy in the literature regarding Factor B, which can be formed from the dicyanocobinamide by acidification. Armitage *et al.*[10] concluded from the i.r. spectrum that this compound (λ_γ = 354 mμ) contained no cyanide. Bernhauer, Renz, and Wagner,[11] however, concluded from the infrared spectrum that cyanide was indeed present, and pointed out that the spectrum was identical with that of Factor V_{1a} (λ_γ = 353 mμ) which is known to contain one cyanide.[9,12] Our own work (described in the notes to Table 1) shows that the cyanides of dicyanocobinamide are removed in two steps with very different formation constants (see Table 1). The monocyanide shows a spectrum with λ_γ = 353 mμ in agreement with that given by Bernhauer *et al.*[11,12] The electrophoretic behaviour of Factor B in acid shows that it has one positive charge, establishing the complex as cyanoaquobinamide.[11] It undergoes a reversible change, involving one proton, with a pK = 11 to give the cyanohydroxocobinamide.[13]



R = NH·CH$_2$·CH(OH)·CH$_3$ Cobinamide



R = NH·CH$_2$·CHMe Cobalamin



George *et al.*[5] claim that the cyanide is lost from Factor B on acidification without, however, affecting the spectrum (λ_γ = 355 mμ in both cases). Bernhauer *et al.*,[11] on the other hand,

[11] K. Bernhauer, P. Renz, and F. Wagner, *Biochem. Z.*, 1962, **335**, 443.
[12] K. Bernhauer, F. Wagner, and D. Wahl, *Biochem. Z.*, 1961, **334**, 279.
[13] B. H. Offenhartz and P. George, *Biochemistry*, 1963, **2**, 142.

claim that the cyanide is not displaced by acid even in light, and give $\lambda_\gamma = 348$ mμ for the cyanide-free complex, which they state without evidence to be diaquocobinamide. We found that a 0·0001M-solution of cyanoaquocobinamide in 0·001N-sulphuric acid showed no change when kept in the dark for several days. On irradiation, however, the γ-band moves from 353 to 348 mμ. If the solution is contained in a sealed cell to prevent the loss of HCN the reaction is slowly reversed on standing in the dark. If the HCN is removed by a stream of air, no regeneration occurs in the dark until a small quantity of KCN is added. The product of photolysis has p*K*s at 6 and ~10·5 (Table 1). The spectrum of the compound present below pH 6 is identical with that of aquocobalamin in strong acid, where the base is protonated and replaced by water, and it is therefore identified as diaquocobinamide. Loss of one and two protons gives aquohydroxo- and dihydroxo-cobinamide, respectively.

Equilibria in Aqueous Solution.—The main equilibria investigated were:

Cobinamides:	replacement of H_2O by OH^- (*a* in Figure)	
	,,	H_2O by CN^- (*b* in Figure)
Cobalamins:	,,	benzimidazole by H_2O (with protonation of base) (*c* in Figure)
	,,	benzimidazole by CN^- (*d* in Figure)

The equilibria were all studied spectrophotometrically and therefore reflect only those changes directly involving the chromophore (*i.e.*, changes in the conjugated ring itself and in the co-ordination of the cobalt ion) and not changes occurring at the periphery of the molecule (*e.g.*, the protonation of adenine or phosphate in DBC [14] or the possible hydrolysis of the amide side-chains).[10,15] The natures of the complexes were established by the stoicheiometry of the equilibrium together with certain features of the spectra, *e.g.*, comparison of the spectra of the cobalamins and cobinamides above 300 mμ to show whether the co-ordination around the cobalt was the same, and the shift of the benzimidazole band from 288 to 285 mμ when protonated.[16,17] In all cases except where otherwise stated the stoicheiometry of the reaction was 1·0 ± 0·02.

The values for the *stoicheiometric* equilibrium constants, *K*, for reactions (*b*) and (*d*) are listed in Table 1. The constants were calculated either directly from the known total free ligand concentration when this was in large excess, or from the known concentrations of reactant and product complexes (known from the spectrophotometric determinations) and the deduced concentration of free ligand, when the total complex and free ligand concentrations were comparable. The free ligand concentration is, of course, the total ligand concentration less the bound ligand. The equilibrium constants for reactions (*a*) and (*c*) are expressed in Table 1 as the acid dissociation constants, pK_a, for the reactions

$$Co(H_2O) \rightleftharpoons Co(OH^-) + H^+$$

and

$$\{Co(H_2O) + BzH^+\} \rightleftharpoons Co(Bz) + H_3O^+$$

respectively, as this is conventional in the literature. The logarithms of the stability constants for the replacement of water by hydroxide (*a*) are the differences between pK_w and pK_a where K_w is the hydrolysis constant. The logarithms of the stability constants for the replacement of benzimidazole by water (*c*), at the same time protonating the benzimidazole, are identical with the pK_a values given in the Table.

Reversible changes with $pK > 0$ are also shown by solutions of aquocobalamin in the presence of sulphur-containing ligands such as sulphite, thiosulphate, sulphide, cysteine, and thioglycollate. The p*K* in 0·02M-sodium sulphite has already been given as 2·0.[18] In each case the acid solution is yellow and the benzimidazole band occurs at 284 mμ, whilst the neutral solution is red, the band occurring at 288 mμ.

Both cobalamins and cobinamides show the presence of one or more additional equilibria

[14] J. N. Ladd, H. P. C. Hogenkamp, and H. A. Barker, *J. Biol. Chem.*, 1961, **236**, 2114.
[15] R. Bonnett, J. R. Cannon, A. W. Johnson, and A. Todd, *J.*, 1957, 1148.
[16] G. R. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, P. Mamalis, V. Petrow, and B. Sturgeon, *J. Pharm. Pharmacol.*, 1949, **1**, 957.
[17] G. H. Beaven, E. R. Holiday, E. A. Johnson, B. Ellis, and V. Petrow, *J. Pharm. Pharmacol.*, 1950, **2**, 944.
[18] J. A. Hill, J. M. Pratt, and R. J. P. Williams, *J. Theor. Biol.*, 1962, **3**, 423.

in strong acid (conc. H_2SO_4 or $HClO_4$). The number and nature of these complexes has not yet been established, but the previous conclusion [18] that the benziminazole remains bound to the cobalt in the cobalamins has now been shown to be incorrect; in concentrated sulphuric acid the cobalamins and cobinamides have identical spectra above 300 mμ, and the nucleotide shows the same band at 288 mμ whether free or bound in a cobalamin.

TABLE 1

Equilibrium constant measurements

	System	Method	Reagents	pK_a	Note	Ref.
$H_2O \longrightarrow OH^-$	Aquocobalamin	1A	0·05M-Phosphate buffers	7·8 (6·9, 7·5, 7·65)	(*a*)	19–21
	Diaquocobinamide	2A	0·001M-H_2SO_4 + NaOH	6·0	(*b*)	
		2B		6·1		
	Cyanocobinamide	2	,,	11·0	(*c*)	
			Ionic strength $\longrightarrow$ 0	(11·0)		13
	Methylcobinamide	1	Strong NaOH	$\geqslant$14	(*d*)	
			?	(>12) log K		6
$H_2O \longrightarrow CN^-$	Diaquocobinamide	3C	0·001M-H_2SO_4	$\geqslant$14	(*e*)	
	Aquocobalamin	3C	0·2N-Sodium acetate + 0·08N-HCl pH = 4·75	$\geqslant$12	(*e*)	
	Cyanocobinamide		?	(8)		5
	Methylcobinamide	2	1N-NaOH + 1N-KCN	3·0 ± 0·2 pK_a	(*f*)	
Benz $\longrightarrow$ H_2O	Aquocobalamin	1B	Conc. H_2SO_4 + H_2O	−2·4 ± 0·2	(*g*)	
	Cyanocobalamin	1B	,, ,,	+0·1	(*h*)	
	Ethynylcobalamin	2A	Dilute H_2SO_4	+0·7	(*i*)	
	Vinylcobalamin	2A	Dilute H_2SO_4	+2·4	(*i*)	
	Methylcobalamin	1A	0·2N-Acetate/0·08N-HCl buffers	+2·5	(*i*)	
			?	(+2·7)		22
	DBC	1A	0·2N-Acetate/0·08N-HCl buffers	+3·35	(*i*)	
			Various	(3·3—3·5, 3·52) log K		14, 22
Benz $\longrightarrow$ CN^-	Cyanocobalamin	?	?	(4)		5
	Ethynylcobalamin	2A	1N-NaOH + KCN	2·7	(*j*)	
	Vinylcobalamin	1A	,,	0·7	(*j*)	
	Methylcobalamin	1B	,,	0·1	(*j*)	

An estimated error of ±0·1 applies to all log (equilibrium constants) except where otherwise stated.

Notes on Table 1. (*a*) There is no spectrophotometric evidence for the formation of a phosphate complex under these conditions. The difference between our value and that of Hanania and Irvine (7·65),[21] the most reliable in the literature, is within experimental error.

(*b*) Diaquocobinamide was prepared by photolysing a 0·0001—0·001M-solution of Factor B in $HClO_4$ or H_2SO_4, pH 2—4, until no further change in spectrum was observed, the HCN being removed by a stream of air passing through the solution. The resulting solution showed the same spectrum above 300 mμ as vitamin B_{12a} in strong acid [see under (*h*)]:

	λ_γ	λ_β	λ_α	ε_{molar}
Diaquocobinamide	348	490	519	$2·8 \times 10^4$
B_{12a} in strong acid	348	491	518	$2·8 \times 10^4$

The constants were determined in perchloric or sulphuric (~0·001M) rather than in hyrochloric acid because of the formation of chloride complexes. In a separate experiment in strong acid

[19] R. P. Birks, E. G. Newstead, and N. R. Trenner, *Science*, 1951, **113**, 625.
[20] E. L. Smith K. H. Fantes, S. Ball, J. G. Waller, M. B. Emery, W. K. Anslow, and A. D. Walker, *Biochem. J.*, 1952, **52**, 389.
[21] G. I. H. Hanania and D. H. Irvine, Paper presented at the 8th International Conference on Co-ordination Chemistry, Vienna, 1964.
[22] D. Dolphin, A. W. Johnson, and R. Rodrigo, *Ann. New York Acad. Sci.*, 1964, **112**, 590.

solutions (2N-H_2SO_4) the stability constant of the chloride complex of diaquocobinamide has been shown to be $3{\cdot}3 \pm 0{\cdot}2$. Difficulties were also experienced owing to the sulphur dioxide in the atmosphere. Solutions of diaquocobinamide at very low or high pH show little or no change in spectrum over long periods, but over the range pH 5—11 the solutions gradually become yellow. The position of the isosbestic points during this reaction and the spectrum and light-sensitivity of the product identified it as sulphitocobinamide, and the source of contamination was shown to be the atmosphere. In 0·05M-sodium borate the log (stability constant) of the sulphite complex of aquohydroxocobinamide, which is formed rapidly, exceeds 8. Despite these experimental problems the agreement between the two methods of determining the constant is good.

(*c*) This equilibrium was studied to check the value given by Offenhartz and George.[13] Armitage *et al.*[10] had reported a pK of 6·8 for Factor B, but it is not clear from their Paper whether they were studying the diaquo- or the monocyano-cobinamide, though Bonnett [4] assumes it was the latter. Our value agrees with that of Offenhartz and George.[13] Factor B, like diaquocobinamide, reacts with sulphite to give sulphitocobinamide, but the reaction is slower and far less serious. This may have been the reaction observed by Offenhartz and George [13] and attributed by them to undefined " aggregation phenomena."

(*d*) No change in spectrum was observed between 1N-sulphuric acid and 10N-potassium hydroxide. Thus, the ionisation of the bound H_2O to OH^- does not occur in an observable pH range. The appearance of the band at 285 mμ indicates that the benziminazole is protonated in the acid form.[16,17]

(*e*) Vitamin B_{12} and Factor B were photolysed until complete conversion into B_{12a} and diaquocobinamide had occurred, and then left in the dark at room temperature to allow reformation of the cyanide complex. The experimental data were as follows:

	B_{12}	Factor B
Concentration	$2{\cdot}5 \times 10^{-5}$M	$2{\cdot}9 \times 10^{-5}$M
Solution	Acetate buffer	0·01N-H_2SO_4
pH	4·75	2·0
$t_{\frac{1}{2}}$ of dark reaction	4½ hr.	~2 days
% reformation (time)	>98% (after 6 days)	>93% (after 17 days)

The minimum constants given in Table 1 were calculated on the basis of the pK of HCN being 9·3.[23] George *et al.*[5] also give figures for these equilibrium constants, but the calculations were based on wrong assumptions as to the nature of the equilibria observed [see under (*h*)].

(*f*) Slight decomposition of the methylcyanocobinamide was noted at high cyanide concentrations, which may have been due to the extreme light-sensitivity of the complex. A weak complex between methylcobinamide and cyanide has already been noted by Müller and Müller.[6] The result quoted is the mean of three determinations.

(*g*) The acid end-point cannot be observed because of overlap with a further equilibrium (or equilibria) involving the complexes present in concentrated sulphuric acid. The spectrum of the fully-formed complex can, however, be obtained by diluting a solution of vitamin B_{12a} in concentrated acid with water, since the equilibrium involing the reversible displacement of the base is set up relatively slowly in acid. The benziminazole band occurs at 284 mμ, indicating that the base is protonated,[16,17] and the spectrum above 300 mμ is identical with that of the diaquocobinamide [see under (*b*)]. The equilibrium constant was derived using the Hammett acidity function H_0.[24]

(*h*) A further equilibrium also obscures the acid end-point in the case of vitamin B_{12}. Thus again method 1B was used and the equilibrium constant derived using the Hammett acidity function H_0. Beaven *et al.*[17] observed a shift in the γ-band of B_{12} to shorter wavelength at pH <1 and concluded that the base was reversibly displaced and protonated with pK ~0. George *et al.*[5] apparently observed the same equilibrium but because their experimental data could not be fitted to a single equilibrium they concluded that both the cyanide and the benziminazole were displaced. They used hydrochloric acid solutions, however, and since chloride can act as a ligand (see (*b*)) their results probably reflect the displacement of the base by both Cl^- and H_2O.

(*i*) The acid forms of ethynyl-, vinyl-, and methyl-cobalamin and DBC are all yellow and

[23] *Chem. Soc. Special Publ.* No. 7, Part II, 1958.
[24] M. A. Paul and F. A. Long, *Chem. Rev.*, 1957, **57**, 1.

in each case the benziminazole band occurs at 284 mμ. Acid catalyses the decomposition of ethynyl- to acetyl-cobalamin, identified by comparison of the spectra in acid and alkali with those of a sample of acetylcobalamin ($t_{\frac{1}{2}}$ ~10 min. in 1N-sulphuric acid at room temperature). Acid catalysed the decomposition of vinylcobalamin to vitamin B_{12a} ($t_{\frac{1}{2}}$ ~45 min. in 10N-sulphuric acid at room temperature). In addition a further reaction or equilibrium of vinylcobalamin was observed in strong acid, marked by an increase in absorption around 310 mμ. The acid end-points could not, therefore, be obtained in these two cases. Methylcobalamin, however, is completely stable to acid, and DBC is stable over the range required for determining the pK. The acid form of methylcobalamin has the same spectrum above 300 mμ as methylcobinamide:

		Main bands (mμ)			$\varepsilon_{303} : \varepsilon_{458}$
Methylcobinamide in water	—	302·5	372	458	2·40
Methylcobalamin in 1N-H_2SO_4	285	303	372	458	2·35

Ladd *et al.*[14] report that the equilibrium of DBC, as determined by titration and paper ionophoresis, involves two protons, but conclude that only one proton is involved in the change of spectrum (in agreement with our result), the other being associated with the adenine nucleotide. The buffers used in these determinations do not affect the spectra of the complexes.

(*j*) The equilibrium constants for replacing benziminazole by cyanide in aquocobalamin and DBC cannot be determined. Cyanide decomposes DBC to dicyanocobalamin; excellent isosbestic points were observed during the reaction in 0·1N-potassium cyanide and no evidence was obtained for any intermediate. Vitamin B_{12a} reacts with one mole of cyanide to give vitamin B_{12} and with an excess to give dicyanocobalamin. Ethynyl-, vinyl-, and methylcobalamin, however, show a reversible reaction with cyanide. The spectra of methylcobinamide and methylcobalamin in excess of cyanide appear to be the same above 300 mμ (main bands at 388 and 568 mμ and ~390 and ~565 mμ, respectively). Dolphin *et al.*[22] claim that cyanide decomposes alkylcobalamins to dicyanocobalamin, but do not state which compounds they have studied. The constants were all determined at high sodium hydroxide concentration so as to control ionic strength and pH.

Discussion

The data in Table 2 show, first of all, that very large changes in the equilibrium constants for ligand substitution can be brought about simply by changing the *trans*-ligand (a variation of over 10^{11} for the replacement of H_2O by CN^-); secondly, that the stability constant order for the ligands is the same for each type of equilibrium (*a*), (*b*), and (*d*) but is reversed for (*c*) where the entering ligand is the weaker donor; and, thirdly, that as the fixed ligand is changed in the order H_2O, NC^-, $HC{\equiv}C^-$, $H_2C{=}CH^-$, CH_3^- (and then C′$_5$-deoxyadenosyl in the one equilibrium for which a constant is reported) so the stability of the *trans* cobalt–cyanide bond decreases relative to that of the cobalt–benziminazole bond (*d*) which in turn decreases relative to that of the cobalt–water bond, *i.e.*, $-pK_a$ in (*c*).

Previous measurements[25] on the thermodynamic *trans*-effect using Pt^{II}, Co^{III}, Fe^{II}, and Rh^{III} complexes have shown it to be small but in all these cases closely similar ligands were involved in the equilibria. As a consequence it has proved very difficult to establish the cause of the *trans*-effect. Both the thermodynamic and the kinetic *trans*-effects have been discussed in terms of inductive effects, π-bonding, polarisation, etc. Of the two, the thermodynamic equilibrium constants should be the easier to interpret because they involve only ground states of known stereochemistry. We hope to study further equilibrium constants of the corrinoids with a view to separating out the several possible effects. However, the results obtained so far—the similar effect of carbon and sulphur anions in general and the order cyanide < ethynyl < vinyl < methyl in particular—support the correlation of the *trans*-effect with the ability of the ligand atom to reduce the effective nuclear charge on the cobalt atom (see, for example, Basolo and Pearson[25]). The influence

[25] F. Basolo and R. G. Pearson, *Progr. Inorg. Chem.*, 1962, **4**, 381, Pt^{II} Co^{III}; B. A. Jillot and R. J. P. Williams, *J.* 1958, 462, and R. C. Davies, D.Phil. Thesis (Oxford) 1963, Fe^{II}; E. J. Bounsall and A. J. Poë, Abstracts of The Eighth International Co-ordination Chemistry Conference, Vienna (1964), p. 313, Rh^{III}.

of the carbon ligands on the wavelength of the γ-band in the cobalamins follows the same order and has been correlated with the electronegativity of the carbon atom.[4]

It is interesting to note that the cobalt atom when attached to an alkyl group shows no marked preference for carbon, nitrogen, or oxygen as the sixth ligand atom. Furthermore, all the corrinoids studied are unusual in being kinetically labile (towards the replacement

TABLE 2

Summary of equilibrium constants *

	Cobalamins		Cobinamides	
	(*c*)	(*d*)	(*a*)	(*b*)
	Benz ⟶ H_2O	Benz ⟶ CN^-	H_2O ⟶ OH^-	H_2O ⟶ CN^-
trans-Ligand	log K (= pK_a) *	log *K*	pK_a *	log *K*
H_2O	−2·4	(⟶ B_{12})	6·0	⩾14
^-CN	+0·1	4	11·0	8
$^-C{:}CH$	+0·7	2·7	—	—
$^-CH{:}CH_2$	+2·4	0·7	—	—
$^-CH_3$	+2·5	0·1	>14	3·0
			(No p*K* detected)	
$^-C_5'$-Deoxyadenosyl (DBC)	3·4	(⟶ dicyanide)	—	—

* See the Figure for the chemical formulation of the equilibria. The stability constant for reaction (*a*) is $pK_w - pK_a$ and for reaction (*c*) is equal to pK_a, see p. 6488.

of at least one axial ligand), in contrast with the inertness of most cobaltic complexes.[26] Both thermodynamically and kinetically, therefore, the cobalt atom of the coenzymes could be reactive towards a wide range of compounds *in vivo*.

EXPERIMENTAL

Reagents.—Samples of the corrinoids were given by Dr. E. Lester Smith, F.R.S., and Professor D. C. Hodgkin, F.R.S. With the exception of Factor B the samples were used without further purification. The samples of Factor B contained an excess of cyanide which was easily removed by passing a stream of air through the solution in the dark at pH 3 to leave the pure monocyanide (see under Results). AnalaR reagents were used wherever possible. [It should be noted that two of the compounds are unstable to strong acid. Acid catalyses the conversion of ethynyl- into acetyl-cobalamin, recognised by its spectrum. Compare the analogous hydration of acetylenes to aldehydes, catalysed by mercury(II) salts. Vinylcobalamin decomposes to aquocobalamin, perhaps *via* the addition of a proton to give the π-olefin complex.]

Absorption Spectra.—The spectra were taken with a Beckmann DK ratio-recording spectrophotometer and also with a Unicam S.P. 600, using 1 cm. silica cells. Molar extinction coefficients were determined by conversion into the dicyanide using the molar extinction coefficient [4] of λ_γ of the dicyanide = $3{\cdot}04 \times 10^4$.

Photolysis.—The solutions to be photolysed were contained in a sealed cell and placed 10—25 cm. from a 230 v, 125 w Osram mercury lamp.

Measurement of pH.—Most pH measurements were made on small (~2 ml.) quantities of solution in a spectrophotometer cell. We used either a Radiometer or Cambridge pH meter in conjunction with either a Radiometer GK 264 combined electrode which fitted into the B10 socket of a cell, or very slim glass and calomel electrodes which fitted into a straight-sided cell.

Electron Spin Resonance.—The samples, cooled in liquid nitrogen, were examined in a Varian X-band spectrometer, V 4500, in a double-cavity cell, using a Mn^{2+} in MgO sample as reference; the spectrum was run from 0·5 to 4 kilogauss with a scan rate of 350 gauss per inch; the modulation width was 0·5 gauss at 100 kc./sec. No signal was detected from solutions of vinyl- or ethynyl-cobalamins; under the same conditions solutions containing the same concentration of vitamin B_{12r} exhibited prominent signals.[27]

Electrophoresis.—Experiments were carried out in a modified B.T.L. C6/5400 horizontal electrophoresis tank at 200 v. The following Table gives the conditions and rates of migration

[26] D. R. Stranks, " The Reaction Rates of Transition Metal Complexes " in " Modern Co-ordination Chemistry," ed. J. Lewis and R. G. Wilkins, Interscience, New York, 1960, p. 78.

[27] H. P. C. Hogenkamp, H. A. Barker, and H. S. Mason, *Arch. Biochem. Biophys.*, 1963, **100**, 353.

of the different compounds. The charge on the vinyl and ethynyl compounds are obviously the same as that on B_{12} at all the recorded pH values.

Supporting electrolyte	Volts	Time (hr.)	Movement (cm.) B_{12}	B_{12a}	Vinyl	Ethynyl
0·01M-NaOH	200	2	1·8	1·8	1·8	1·8
Acetate/HCl buffer pH 5·0	200	2½	0·8	−0·2	0·8	0·7
0·02N-CH_3CO_2H	400	2½	−2·7	−9·0	−2·9	

Determination of Equilibrium Constants.—All equilibrium constants were determined spectrophotometrically. Three different methods of adding the reagent (1—3) and three different methods of analysing the results were used (A—C).

(1) A range of solutions of varying reagent concentration was used.

(2) The corrinoid solution in a 1-cm. cell was titrated with a solution of the reagent (a series of increasingly concentrated solutions was often used) from a micrometer syringe fitted with a hypodermic needle.

(3) In order to obtain an estimate of the equilibria between cyanide and aquocobalamin and diaquocobinamide, which have high formation constants ($\geqslant 10^{12}$), a known, very low concentration of cyanide was generated *in situ* by the complete photolysis of a solution of the cyano-complex in acid inside a sealed spectrophotometer cell to prevent loss of HCN. The concentration of cyanide ion was calculated from the pK of HCN, the pH of the solution and the concentration of the corrinoid.

All the equilibria studied involved the substitution, by a reagent (Y) such as CN^-, of either H_2O in the cobinamides or of the nucleotide benziminazole in the cobalamins; the concentration of water or benziminazole can therefore be neglected in the equilibrium $A + nY \rightleftharpoons B$, where A and B are both corrinoids. The experimental parameters were: the optical density at the given wavelength corresponding to 100% A (ε_A), 100% B (ε_B), or intermediate values (ε_Y) in equilibrium with a known concentration of the reagent, Y.

$$\therefore \quad K = \frac{[B]}{[A][Y]^n} = \frac{\varepsilon_Y - \varepsilon_A}{(\varepsilon_Y - \varepsilon_B)[Y]^n}$$

(A) Where ε_B was experimentally observable the experimental data were plotted as log [Y] against log [B]/[A]. Unit slope proved the participation of one mole of reagent in the equilibrium; $K = 1/[Y]$ when log [B]/[A] = 0.

(B) When ε_B was not experimentally observable because of a second overlapping equilibrium, irreversible decomposition at high concentrations of the reagent, or a very low formation constan the data were analysed by the following method.[28,29] From the above equation

$$(\varepsilon_B - \varepsilon_Y) = \frac{\varepsilon_A - \varepsilon_Y}{K[Y]^n} \text{ or } -\varepsilon_Y = \frac{\varepsilon_A - \varepsilon_Y}{K[Y]^n} - \varepsilon_B$$

A plot of ε_Y against $(\varepsilon_A - \varepsilon_Y)/[Y]$ yields a straight line if $n = 1$, the slope of which is equal to $1/K$ and the intercept to ε_B. The experimental results could be re-evaluated by the first method using a value of ε_B obtained either by this second method (referred to in Table 1 as method A_B) or, where deviation occurs only over the last 5% or so of the equilibrium, by first determining an approximate equilibrium constant by the first method (A_A).

(C) Minimum values only for the equilibrium constant between CN^- and aquocobalamin and diaquocobinamide were obtained from the degree of reformation of the cyano-complex observed after standing in the dark.

In all cases, values of [Y] were corrected for reagent bound by the complex, and values of ε for dilution if necessary.

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[28] A. K. Lunn and R. A. Morton, *Analyst*, 1952, **77**, 718.
[29] T. W. Newton and G. M. Arcand, *J. Amer. Chem. Soc.*, 1953, **75**, 2449.