The Chemistry of Vitamin B_{12}. Part 18.[1] Nature of the Equilibria exhibited by Organocobalamins

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The equilibria between the ' base-on ' and unprotonated ' base-off ' forms have been studied, and values of ΔH and ΔS determined, for a series of organocobalamins. The equilibrium is increasingly displaced in favour of the ' base-off ' form as the ligand is varied in the order: cyclopropyl ≤ methyl < 5'-deoxy-5'-adenosyl (in the dimethylbenzimidazolylcobamide coenzyme, dbc) < ethyl ~ propyl < isobutyl ~ cyclobutyl < neopentyl ~ cyclopentyl < isopropyl ~ cyclohexyl. The equilibrium constants and the spectra of the ' base-off ' forms are anomalous for complexes such as ethylcobalamin, and it is suggested that in their unprotonated ' base-off ' forms the heterocyclic base interacts with the corrin ring and its substituents through hydrophobic bonding. The experimental evidence for the various forms exhibited by organocobalamins is summarised and assessed.

THE large body of experimental evidence available on the *cis* and *trans* effects of the axial ligands in cobalt corrinoids provides a remarkably self-consistent pattern in which the main variable is the σ-donor power of the axial ligands.[2,3] For most ligands so far studied steric factors have been minimal. We now wish to study systematically the effect of steric factors in a series of simple alkyl ligands on spectra, equilibria, and reactivity, with the aim of providing support for the mechanism proposed[4] for the isomerase reactions which involves labilisation of the Co–C bond in the coenzyme through distortion of the Co–C_α–C_β bond angle by the protein.

The basic types of complex and equilibria involving changes in the axial ligands, which are exhibited by organocobalamins and organocobinamides in aqueous solution in the absence of any added ligands, are shown schematically in Figure 1 A—E and F—G respectively.[2,5] These complexes may also differ in the conformation of the corrin ring, in the position of the Co atom with respect to the equatorial plane, and in various intramolecular interactions (see below), as well as in the number and nature of the axial ligands; and the organo-ligand R may affect the observed equilibria either through a purely electronic effect *via* the Co atom or through a variety of steric repulsions, *e.g.* between the ligand atom itself and the equatorial N atoms or between the more distant atoms of a larger ligand and the corrin ring or its substituents. A good understanding of the nature of these various changes, and of the interplay between them, is therefore an essential prerequisite to any attempt to study and interpret the steric effects of organo-ligands and the possible labilisation of the Co–C bond in the coenzyme form of vitamin B_{12} by distortion.

Some confusion has, unfortunately, arisen over the nature of these complexes and equilibria because of the difficulty of distinguishing between changes in conformation and co-ordination number and between changes in conformation involving the side chains and those involving the conjugated corrin ring (see, for example, refs. 6—8). The aim of this paper is therefore to establish, as conclusively as possible, the general pattern of spectroscopically detectable equilibria shown by the organocobalamins, while in the preceding paper[1] we examine the effect of individual organo-ligands on specific equilibrium constants and on the spectra of the complexes involved.

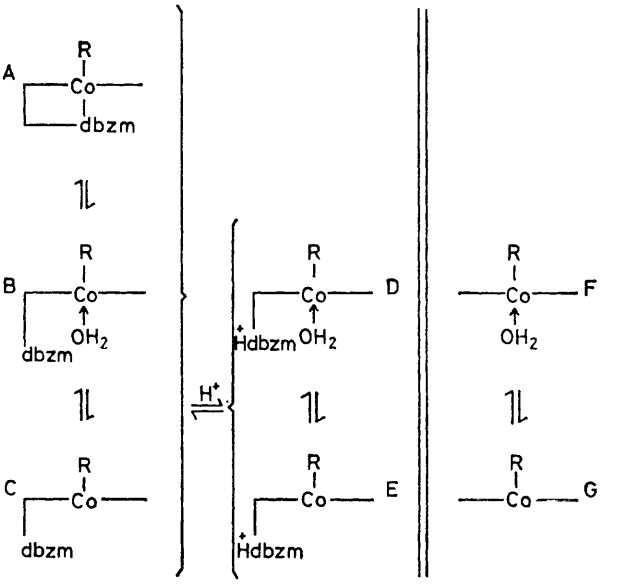

FIGURE 1 Schematic summary (see refs. 2 and 5) of the equilibria involving a change in the axial ligands exhibited by organocobalamins (A—E) and organocobinamides (F and G). R is the organo-ligand. C, E, and G are five-co-ordinate complexes, the others are six-co-ordinate complexes. Complexes in the same vertical column are involved in pH-independent equilibria, in which increasing temperature favours the five- over the six-co-ordinate forms. Further evidence (see text) indicates (i) that the Co atom is displaced out of the equatorial plane towards the axial ligand atom in the five-co-ordinate complexes, (ii) that the corrin ring has the ' cobalamin ' conformation in A and the ' normal ' conformation in all the others, *unless* R is sufficiently bulky to suffer strong steric interaction with the ring or its substituents, (iii) that the B and D forms can be neglected where the ligand atom is a tetrahedral C atom bonded only to other C and H atoms, and (iv) that the C form may occur as a variant (C^{dbzm}) in which dbzm interacts with the corrin ring + substituents through hydrophobic bonding

The evidence regarding the conformation of the corrin ring can be summarised as follows. Lenhert[9] has pointed out that all the conformations of the corrin ring

which have so far been established for the naturally occurring (and closely related) corrinoids by *X*-ray analysis fall into two groups, which we shall term ' cobalamin ' and ' normal '. These groups differ mainly in the C(5)–C(6) region, where the ' cobalamin ' forms show a much more pronounced bend due to contact with the H atom on C'(4) of dbzm (5,6-dimethylbenzimidazole). Virtually the same ' cobalamin ' conformation is found in both the ' wet ' [10] and ' dry ' [11] forms of vitamin B_{12} (cyanocobalamin), vitamin B_{12} 5'-phosphate,[12] neovitamin B_{12} (cyano-13-epicobalamin),[13] and the coenzyme dbc (5'-deoxy-5'-adenosylcobalamin),[9] *i.e.* with all the corrinoids containing co-ordinated dbzm, irrespective of changes in the conformation of the side chains (*cf.* wet and dry B_{12}), in the exocyclic ring (*cf.* B_{12} and neo-B_{12}), or in the axial ligand and Co–N bond lengths (*cf.* B_{12} and dbc). The ' normal ' conformation is found in the cyanoaquo-complex of cobyric acid (Factor V_{1a}) [14] and the cyanochloro-complex of the hexacarboxylic acid,[15] as well as in both halves of the iodide-bridged dimeric cobalt(III) complex of cobyrinic heptamethyl ester where the Co atom is five-co-ordinate and displaced by 0.11 and 0.13 Å from the mean plane of the four atoms towards the iodine.[16–18]

The electronic spectra in the 300—600 nm region, which are due to π–π^* transitions within the conjugated corrin ring, are very sensitive to changes in the number and nature of the axial ligands (*cf.* B_{12} and dbc), though apparently not so much to their relative orientation; [2] they are relatively insensitive to changes in the exocyclic ring (*cf.* the dicyanide complexes of B_{12} and neo-B_{12}) [19] and the side chains.[2] A comparison of the absorption spectra of thin films of solid B_{12} and dbc, as well as of the reflection spectra of their solids and of the absorption spectra of their solutions, has demonstrated that large changes in spectra (*i.e.* in electronic structure) can occur even when there is no significant change in the conformation of the conjugated ring.[5,20]

Hydrogen-1 and ^{13}C n.m.r. spectroscopy have, unfortunately, provided no unambiguous evidence on conformational changes. There is a correlation between the chemical shift of C(10)–H and changes in the electronic spectra,[5] but there is no correlation at all between the chemical shifts [21,22] of the methyl protons on C(5) and C(15) and the electronic spectra (see for example, refs. 2 and 3). Brodie and Poe [22] have, in fact, concluded from their ^1H n.m.r. studies that ' the conformations of the corrinoid rings in methylcobinamide and methylcobalamin appear to be very similar ' and that ' the differences between the n.m.r. spectra of the cobinamides and cobalamins (can be) ascribed to changes in the conformation of groups extending from the corrin ring, with the corrin ring essentially unchanged,' *i.e.* any small changes in the n.m.r. spectra which might serve as fingerprints for the two (or more) conformations of the conjugated ring are obviously swamped by effects due to changes in the side chains; *cf.* also refs. 23 and 24.

We now wish to establish whether changes in conformation alone (as between ' cobalamin ' and ' normal ') cause significant changes in the electronic structure of the chromophore (the ring) or not, *e.g.* by comparing the spectra of methylcobalamin (with the ' cobalamin ' conformation) and of the imidazole adduct of methylcobinamide (with some other, presumably the ' normal ', conformation). Pailes and Hogenkamp [25] have reported that methylcobinamide forms an adduct with imidazole with a low formation constant (K = 11 dm^3 mol^{-1}) and have given brief details of the spectrum, which appears to be similar to that of methylcobalamin. We have therefore made a direct comparison of their spectra in order to determine the change in energy of the first electronic transition ($\alpha\beta$ region) due to a change in conformation.

It is clearly impossible to change the conformation without simultaneously changing several other factors. Because of its interaction with the corrin ring, dbzm is unsymmetrically co-ordinated to the Co with Co–N–C bond angles of 116 and 132° in dry B_{12} [11] and 123 and 132° in dbc; [9] the bonding would presumably be much more symmetrical in the case of imidazole. For the same reason the Co–N bond length may be slightly longer in the case of dbzm. The differences in the pK_a of the free base and in the bond length and bond angles involving the co-ordinated base will all superimpose a slight, but unknown, ' electronic ' effect on top of the ' steric ' effect due to the release of pressure between dbzm and the corrin ring and the associated change in conformation, not to mention any more subtle effects due to removing the nucleotide side chains or replacing the hydrophobic dbzm by the hydrophilic imidazole. Any observed differences in spectrum between these two complexes will therefore be the resultant of several changes and can only provide a qualitative indicator of the effect of a change in conformation alone.

The aims of this paper are (i) to provide evidence for, and discuss the nature of, the further equilibria involving the ' base-off ' forms (C) of organocobalamins in neutral solution, (ii) to check the effect of a change in conformation alone on the spectrum (and hence the electronic structure) of the corrin ring, and (iii) to summarise and discuss the available evidence regarding the equilibria exhibited by organocobalamins.

EXPERIMENTAL

Materials and methods have been described in Part 17.[1] Ethylcobinamide was prepared in the same way as methylcobinamide.

RESULTS

1. *Temperature-dependent Equilibria of Organocobalamins in Neutral Solution.*—The spectra of the methyl, ethyl, isobutyl, and cyclobutyl cobalamins show reversible temperature-dependent changes with good isosbestic points between 15 and *ca.* 80 °C in both deionised water (pH 6.8) and dilute Na[OH] (pH 9.8), *i.e.* the equilibria are pH-independent. The changes in spectrum observed on raising the temperature correspond to a shift in equilibrium between a typical red ' base-on ' (A) form at low temperature and a typical yellow ' base-off ' (C) form at high

temperature. The changes found for the ethyl complex are shown in Figure 2. The cyclopropyl complex shows changes similar to those of the methyl complex, but has not been studied in detail. The spectrum of the neopentyl (np) complex under N_2, which is unusual due to the presence of *ca.* 40% of the 'base-off' form (see below), also shows temperature-dependent and apparently reversible changes at pH 6.8 involving a shift from the red to the yellow form on raising the temperature, but even under nitrogen the rate of decomposition is sufficient to destroy any isosbestic points. The existence of reversible temperature-dependent equilibria in neutral and alkaline solutions of the isopropyl, cyclopentyl, and cyclohexyl complexes could not be proved directly by studying the effect of temperature owing to their rapid rate of decomposition even under N_2. Their spectra are, however, similar to that of neopentylcobalamin and clearly indicate the presence of both A and C forms.

The changes in spectrum with temperature were studied in more detail for the cobalamins of Me, Et, Bu^i, and C_4 at pH 9.8 and evaluated in terms of a simple equilibrium between the red (A) form at low temperature and the yellow (C) form at higher temperature (C_3, C_4, C_5, and C_6 denote

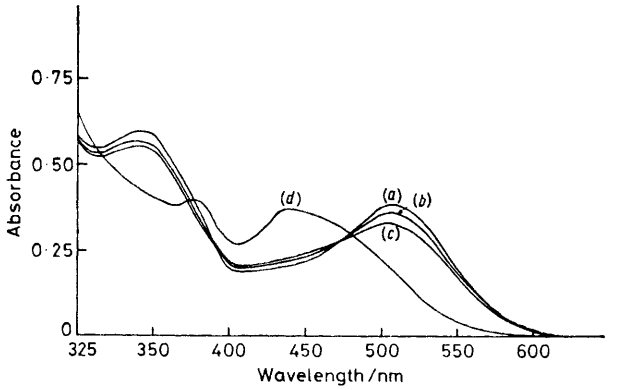

FIGURE 2 Spectra of 4.3×10^{-5} mol dm^{-3} ethylcobalamin at pH 9.8 at (*a*) 20, (*b*) 48, and (*c*) 68 °C; and (*d*) at pH 1 at 25 °C

cyclo-propyl, -butyl, -pentyl, and -hexyl respectively). The spectra corresponding to pure A and pure C could not be obtained directly for any of these complexes over the accessible range (0—90 °C) and end-points for the changes in optical density at the wavelengths used were obtained by graphical extrapolation. Plots of log K_{AC}, where K_{AC} = [C]/[A], against $1/T$ (see Figure 3) gave values of ΔH, ΔS, and x (fraction present as C at 25 °C) which are listed in Table 1. The linearity of the plots in Figure 3 suggests that the end-points obtained by extrapolation cannot be too inaccurate, though we wish to point out that, because of the existence of a third species (see below), linearity may not be a good check on the accuracy of the end-points.

The apparent percent of C present in solutions of the isopropyl, cyclopentyl, and cyclohexyl cobalamins in neutral solution at room temperature can be estimated by assuming that the spectrum of 100% C is the same as that of the E form in acid and that the spectrum of the A form is similar to that of the A form of ethylcobalamin; these results are also given in Table 1.

We have previously reported experimentally determined values for x at 20 °C in dbc and ethylcobalamin; [5] these are noted in Table 1. Values of x have also been calculated

TABLE 1

Data for the temperature-dependent equilibria of organocobalamins in neutral solution

	Conversion of low- to high-temperature forms				
	ΔH		ΔS		x [a]
Ligand	kcal mol^{-1}	kJ mol^{-1}	cal K^{-1} mol^{-1}	J K^{-1} mol^{-1}	
R_{dbc}					0.10 [b]
Me	3.6 ± 1	16 ± 5	7.5 ± 2	33 ± 10	0.05
Et	3.8 ± 0.5	16 ± 2	10 ± 2	43 ± 10	0.17 [c]
Pr^i					0.80
Et	3.8 ± 0.5	16 ± 2	10 ± 2	43 ± 10	0.17
Pr					0.17
Bu^i	3.5 ± 1	15 ± 5	9 ± 2	38 ± 10	0.23
np					0.40
C_3					≤0.05
C_4	5.3 ± 0.5	22 ± 2	16 ± 2	66 ± 10	0.25
C_5					0.40
C_6					0.80 ± 0.10

[a] x = Fraction present as the high-temperature 'base-off' form (C) at 25 °C. [b] Value determined at 20 °C (see ref. 5). [c] Compare x = 0.13—0.15 determined at 20 °C (see ref. 5).

for certain organocobalamins from values of $pK_{obs.}$ and an assumed pK for dbzm itself; [28] since the basis of the calculation can now be shown to be incorrect (see below), these have not been included.

The data of Table 1 show that the apparent concentration of C at equilibrium at 25 °C increases quite regularly with increasing substitution on C_α (Me < Et < Pr^i) or C_β

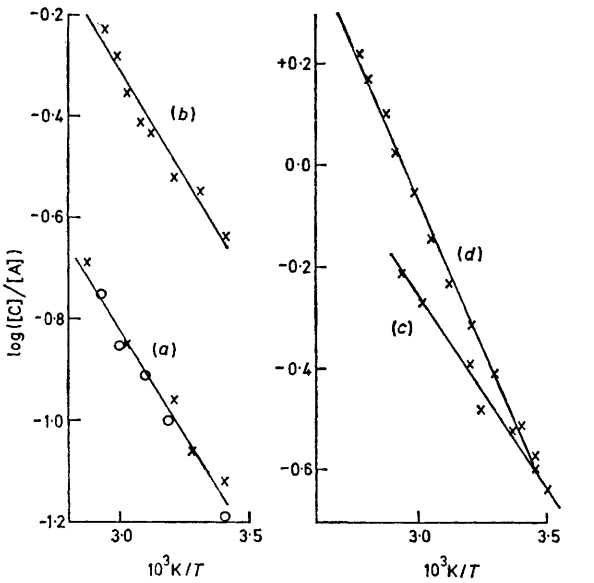

FIGURE 3 Experimental plots for the determination of ΔH for the pH-independent equilibria of (*a*) methyl (two separate experiments denoted by × and ○ respectively), (*b*) ethyl, (*c*) isobutyl, and (*d*) cyclobutyl cobalamins at pH 9.8

(Et ~ Pr < Bu^i < np) or increasing size of the alicyclic ring ($C_3 < C_4 < C_5 < C_6$). This shows a parallel with the variation in $pK_{obs.}$,[1] but closer examination reveals an anomaly.

2. *Comparison of Variations in* $pK_{obs.}$ *and* K_{AC}.—For all the organocobalamins studied in the present series of

papers the spectra in acid show no variation with temperature, which indicates that these acidified cobalamins exist only as the E form, with no detectable amount of the D form.[1] By analogy, it is reasonable to assume that there is no significant concentration of the B form in equilibrium with the C form in neutral solution. One should then be able to explain all the observed equilibria in terms of three species (A, C, and E) linked by two independent equilibria for which we have used the equilibrium constants $K_{obs.}$ and K_{AC}, where $K_{obs.} = [H^+]([A] + [C])/[E]$ and $K_{AC} = [C]/[A]$.

The validity of this scheme involving only three complexes can be checked as follows. A third equilibrium constant $K_{EC} = [C][H^+]/[E]$ is the same as the dissociation constant of the protonated dbzm in the pendant side chain, *i.e.* $K_{dbzm} = K_{EC}$ and $pK_{dbzm} = -\log_{10}K_{EC}$. At the pH corresponding to $pK_{obs.}$, 50% of the cobalamin should be present as E, $50x$% as C, and $50(1 - x)$% as A (where x is the fraction of the cobalamin present as C in neutral solution), so that $K_{EC} = x[H^+]$ and $\log K_{EC} = \log x + \log [H^+]$ or $pK_{dbzm} = pK_{obs.} - \log x$. Values of pK_{dbzm} calculated by this method for the different organocobalamins should be consistent with each other and with the value of 5.0, which has recently been determined for the pK of dbzm in dicyanocobalamin.[27] The relevant data are given in Table 2, with the ligands listed in order of increasing $pK_{obs.}$.

TABLE 2

Calculation of the pK of the pendant dbzm (pK_{dbzm}) *

Ligand	$pK_{obs.}$	x	pK_{dbzm}
Me	2.5—2.7	0.05	3.8—4.0
C_3	2.9 ± 0.1	≤0.05	≥4.1
R_{dbc}	3.3—3.5	0.10	4.3—4.5
Et	3.87	0.19	4.6
Pr	3.84	0.17	4.6
C_4	4.16—4.18	0.25	4.7—4.8
Bu^i	4.15—4.20	0.23	4.8
C_5	≥4.5	0.40	≥4.9
C_6	4.7 ± 0.2	0.80	4.6—5.0
Pr^i	≥4.5	0.80	≥4.6
np	4.7 ± 0.2	0.40	4.9—5.3

* $pK_{dbzm} = pK_{obs.} - \log x$, where x = fraction present as C at 25 °C and $K_{obs.} = [H^+]([A] + [C])/[E]$. Values of x and $pK_{obs.}$ are taken from ref. 1 and Table 1 respectively.

In spite of the relatively large errors involved, it appears that the last four complexes (of C_5, C_6, Pr^i, and np) give calculated values which are compatible with $pK_{dbzm} = 4.9$—5.0. A scheme involving only three types of complex (A, C, and E) is therefore adequate for explaining the observed equilibria for these four complexes. On the other hand, the values of pK_{dbzm} calculated for methylcobalamin and dbc (and probably cyclopropylcobalamin) are obviously anomalous, and even those for the ethyl, propyl, cyclobutyl, and isobutyl complexes appear to be slightly anomalous. The data indicate that the supposed C form of methylcobalamin is about ten times more stable than expected, relative to the A form. These results are inconsistent with the unmodified scheme involving only A, C, and E complexes.

3. *Comparison of the Spectra of the 'Base-off' C and E Forms.*—The analogous C and E forms of the cobalamins and the G forms of the cobinamides should have identical spectra above 300 nm, provided that there is no significant difference in the degree of interaction (whether coulombic, hydrogen-bonding, or hydrophobic) between the relevant side chains and the main part of the corrin structure, which could affect the electronic structure of the chromophore. Comparison of the pure C and E forms is, unfortunately, not possible since complexes such as methylcobalamin are still only partially converted from A to C at *ca.* 90 °C while complexes such as isopropylcobalamin contain *ca.* 80% C at room temperature but decompose even without raising the temperature. A comparison of the changes observed during acidification (*i.e.* A ⟶ E) and on raising the temperature (*i.e.* A ⟶ C) is, however, sufficient to show that the C and E forms do not always have identical spectra.

In the case of ethylcobalamin, acidification at room temperature converts a shoulder at *ca.* 380 nm into a more pronounced band at 376 nm and an isosbestic point is observed at *ca.* 380 nm. Raising the temperature in neutral solution, however, leads to the gradual disappearance of the shoulder at 380 nm while the isosbestic point occurs at 390 nm, as shown in Figure 2. A similar decrease in the prominence of the bands around 370—380 nm was also noted on heating neutral or basic solutions of dbc and the isobutyl and cyclobutyl cobalamins, but could not be established conclusively for methylcobalamin because of the much smaller changes observed. In all these cases the acidified (E) form has a pronounced band in this position which did not disappear on raising the temperature. By contrast, neutral solutions of the isopropyl, neopentyl, cyclopentyl, and cyclohexyl cobalamins, which contain 40—80% C (see Table 1), do show prominent bands in the 370—380 nm region and, within the larger error involved in studying these unstable complexes, there appear to be no significant differences between the C and E forms. Finally, a comparison of the spectra of ethylcobalamin in acid solution (E) with that of ethylcobinamide (G) in both acid and neutral solution revealed no significant differences.

Our results show that for certain complexes (*e.g.* that of neopentyl) there appear to be no obvious differences in spectrum between the C and E forms. However, for many others the spectrum of the C form (hereafter referred to as C^{dbzm}) is slightly different from that of the E and, at least in the case of ethyl, of the G form. The close similarity between the spectra of the C and C^{dbzm} forms, except in the 370 nm region, suggests that the latter have the same basic, five-co-ordinate structure as the E and normal C forms, but that there is weak additional interaction with some part of the unprotonated side chain.

If we assume that the C and C^{dbzm} forms are characterised by the presence and absence respectively of an obvious band at *ca.* 380 nm, then we can conclude that C is the predominant form of the unprotonated 'base-off' forms of the cyclopentyl, cyclohexyl, isopropyl, and neopentyl cobalamins at room temperature, while C^{dbzm} is the predominant form for the other cobalamins and dbc at elevated temperature and probably also at room temperature. The differences in spectra are clearly too small to allow a more quantitative study of the equilibrium between the two forms.

4. *Comparison of the Spectra of Methylcobalamin and of the Imidazole Adduct of Methylcobinamide.*—The spectrum of methylcobinamide was studied in aqueous solutions of 0—10 mol dm^{-3} imidazole over the range 350—600 nm. Solutions of methylcobinamide were made up with several different concentrations of imidazole and the concentration of free base (pK 7.2) [28] varied by the addition of concentrated H_2SO_4 to lower the pH. Equilibrium was set up 'instantaneously' with no further slow change in the shape of the spectrum, and an isosbestic point observed

at 500 nm. Direct comparison of the spectra of methylcobinamide in 10 mol dm^{-3} imidazole and of methylcobalamin in water showed that the shapes of the αβ bands were similar but not identical. The cobinamide has a more pronounced shoulder at *ca.* 480 nm, but both show maxima at 517—518 nm. The former spectrum probably represents *ca.* 99% of the imidazole complex, while the latter represents the sum of the spectra of *ca.* 95% of the red ' base-on ' form and *ca.* 5% of the yellow ' base-off ' form (see Table 1), which could cause a slight displacement of the true maximum of the red form to higher energy. We therefore conclude that the change of conformation (and change of ligand from dbzm to imidazole) affects the position of the maximum at *ca.* 518 nm by $\leqslant 2$ nm. The γ bands appear to be situated at 373—374 nm in both cobinamide and cobalamin.

DISCUSSION

We discuss here the evidence for the main changes in structure and intramolecular interaction, which may be observed in the usual stereoisomers of organocobalamins (*i.e.* with the alkyl group in the ' upper ' co-ordination site opposite to that usually occupied by dbzm; see ref. 2). These include (i) the number and nature of the co-ordinated axial ligands, (ii) the position of the cobalt atom with respect to the mean square plane of the four equatorial N atoms, (iii) the conformation of the corrin ring, and (iv) other non-bonded interactions such as steric compression between the H atom on B(4) of the co-ordinated dbzm and C(5) of the corrin ring, and the (probable) hydrophobic interaction between non-co-ordinated dbzm and hydrophobic portions of the corrin ring substituents.

Changes in the Axial Ligands and the Stereochemistry of the Cobalt Ion.—The various types of complex and equilibria involving changes in the axial ligands of the organocobalamins are depicted in Figure 1 A—E. The evidence for this scheme is based on the nature of the analogous complexes and equilibria observed for organocobinamides (Figure 1 F—G) and has been fully discussed elsewhere.[2,5] The Co atom has been shown to be five-co-ordinate in [*NN'*-ethylenebis(acetylacetoneiminato)]-methylcobalt, [CoMe(acen)],[29] but conclusive proof of five-co-ordination in an organocorrinoid by *X*-ray analysis would be welcome. Although forms B, D, and F play an important part in the equilibria of the vinyl complexes,[2,5] present evidence [1] suggests that B and D forms may be neglected for organocobalamins where the ligand atom is a tetrahedral C atom bonded only to other C and H atoms.

Hogenkamp *et al.*[7] imply that their ^{13}C n.m.r. results do not support the pH-independent equilibria shown in Figure 1, although they do not explicitly exclude them. Their arguments are, however, indirect and the key statement ' that the Me resonances of [^{13}C]methylaquocobinamide and [^{13}C]methylcobalamin . . . show very similar . . . upfield shifts with increasing temperature ' contradicts the experimental results of their Figure 1, which show that the Me resonance (in Hz) of the former *increases* from *ca.* 32 320 to over 32 350 on raising the temperature, while that of the latter *decreases* from 32 580 to below 32 550 (note the confusing orders along the *y* axis). They offer no explanation for the marked change in the electronic spectrum shown by, for example, methylcobinamide below room temperature. They did, however, find that the ^{13}C chemical shifts were temperature-dependent even in the case of methylcobinamide above room temperature, where the electronic spectra [5] show no significant change, and concluded that these shifts ' can best be accounted for by invoking a temperature-dependent conformational change involving substituents on the corrin ring ', in agreement with Brodie and Poe's conclusion [22] from their n.m.r. studies (see Introduction). There appears to be no experimental evidence which conflicts with the scheme of Figure 1 as expanded below.

The Co atom will obviously lie approximately in the equatorial plane in the six-co-ordinate complexes. *X*-Ray analysis shows that in [CoMe(acen)] [29] and in both halves of the dimeric iodide-bridged cobalt(II) complex of cobyrinic acid heptamethyl ester [16-18] the Co has a square-pyramidal configuration and in each case is displaced by *ca.* 0.12 Å from the mean square plane of the equatorial ligand atoms towards the fifth ligand. It is reasonable to assume a similar displacement of the Co atom in the five-co-ordinate C, E, and G forms of Figure 1.

Changes in the Conformation of the Corrin Ring.—Comparison of the spectra of methylcobalamin and the imidazole adduct of methylcobinamide shows that, although there are slight differences in the shape of the band envelope of the first (αβ) electronic transition, the wavelength of the maximum at *ca.* 518 nm differs by $\leqslant 2$ nm. It appears from the published data that the adducts of methylcobinamide with imidazole, 1-methylimidazole, pyridine, NH_3, and ethanolamine all have very similar spectra.[25,30] We conclude that a change in conformation alone, while keeping both axial ligands as constant as possible, affects the energy of the transition by only $\leqslant 2$ nm.

The key pieces of evidence regarding conformational changes are, therefore, (i) that *X*-ray analysis shows the occurrence of only two main types of conformation (*viz.* normal and cobalamin) amongst the naturally occurring cobalt corrinoids and their derivatives, whether Co^{III} or Co^{II} and six-co-ordinate or five-co-ordinate (see Introduction), (ii) that relatively large changes in electronic structure may occur without any significant change in conformation (*cf.* B_{12} and dbc [5,20]), and (iii) that changes in conformation may occur without any significant change in electronic structure (present results). The last is not unexpected, as shown by the following qualitative argument.

Hodgkin and co-workers [14] and Lenhert [9] have both concluded from their *X*-ray work that the conformation adopted by the corrin ring is governed mainly by the need to reduce repulsions between the substituents on the corrin ring (in both normal and cobalamin forms) and also between the corrin ring and the H atom on C'(4) of

dbzm (cobalamin form only). Let us, however, ignore the presence of these substituents and consider only the conjugated π system and then treat the conversion of the 'normal' to the 'cobalamin' conformation as equivalent to rotations of +10 and −10° in two successive three-atom segments [N(21)–C(5) and C(5)–N(22)] of the chain. The change in potential energy of each such rotation would probably be intermediate between those for 10° rotations in a four-atom segment with conjugation in a single plane (*viz.* butadiene) and a three-atom segment with conjugation in two planes (*viz.* allene). The calculations of Radom and Pople [see Figure 1(*c*) of ref. 31] give ≤0.2 kcal mol^{-1} * for the former, while the calculations of Dewar and Hasselbach (see Figure 2 of ref. 32) give *ca.* 1 kcal mol^{-1} for the latter, *i.e.* the total change in energy for the double rotation would probably be ≤1 kcal mol^{-1}. Even if the whole of this energy were reflected in a change in the electronic spectrum, it would correspond to a shift of ≤10 nm in an absorption band at 500 nm, in agreement with our experimentally observed shift of ≤2 nm.

The simplest explanation of the presently available evidence on conformation is (i) that relatively little energy would be required for a significant change (*i.e.* detectable by *X*-ray analysis) in the conformation of segments of the corrin ring between the fixed N atoms, *if* the conjugated ring could be treated in isolation from its substituents, but (ii) that the conformation is determined primarily by the need to reduce non-bonded repulsions, as suggested by Hodgkin and co-workers [14] and Lenhert,[9] while (iii) the electronic energy levels are determined primarily by the σ-donor power of the axial ligand(s) and are not significantly affected by changes in conformation of the magnitude observed, so that changes in conformation and in electronic structure can in effect occur independently of each other. This means that for the scheme of complexes shown in Figure 1 we can assign the 'cobalamin' conformation to type A and the 'normal' conformation to all the others *except* when R is sufficiently bulky to cause a change in conformation through severe interaction with the ring or its substituents; this would probably have no detectable effect on the electronic spectrum, but might affect the n.m.r. spectra [*cf.* the C(5), C(15) methyl groups in cyclohexylcobinamide].[22]

Other Interactions of dbzm.—Several lines of evidence indicate that organocobalamins such as methylcobalamin and dbc form at least one type of complex in neutral solution in addition to those shown in Figure 1. We shall assume that in all these cases we are dealing with one and the same type of additional complex (to be labelled C^{dbzm}).

The data of sections 2 and 3 (see Results) show that the spectra and equilibria observed for the cobalamins of Pr^i, np, C_5, and C_6 are, within experimental error, consistent with the occurrence of forms A, C, and E only and with pK_{dbzm} = 4.9—5.0. This is close to the value of 5.0 found for dicyanocobalamin [33] and slightly higher than the value of 4.7 found for 1-β-D-ribo-5,6-dimethylbenzimidazole [27] (*N.B.* dbzm in the cobalamin side chain has the α link), which we have used previously.[2,5] The equilibrium constants for methylcobalamin, on the other hand, lead to an apparent pK_{dbzm} of 3.8—4.0 (see Table 2), which is clearly anomalous. The spectrum of the supposed C form of ethylcobalamin is similar, but not identical, to those of the E and G forms and is distinguished by the partial or complete disappearance of the band in the 370—380 nm region (see Figure 2). Our results show the existence of a new variant (C^{dbzm}) which is spectroscopically distinct from the C forms and can be more stable relative to the A forms and in which there must be some interaction between the unprotonated dbzm side chain and the chromophore. The data of Table 2 show that there is a good parallel between the effect of the ligand on $pK_{obs.}$ and on the stability of the C^{dbzm} relative to the C forms.

Brown *et al.* [34] have studied the kinetics of the base-on/off equilibrium of methylcobalamin by temperature-jump spectrophotometry at 5 °C. They observed two pH-independent relaxations over the whole range of pH studied (0.46—7.42) which, as they stated, must correspond to the presence of three types of complex in pH-independent equilibria, in addition to the protonated base-off form A (I in their paper). Assuming that these three complexes were types A, B, and C (III, II, and IV in their paper) led to inconsistencies, including 'the rather startling conclusion that the base-on species of methylcobalamin is only some 30% co-ordinated to benzimidazole at 5 °C' and that 'the pK_a for the base-off benzimidazolium species of methylcobalamin must be 4.0 or less' in striking quantitative agreement with the discrepancy noted in Table 2. We interpret their data and observed inconsistencies as providing further evidence for the presence of some additional species (*viz.* C^{dbzm}).

It has been known for some time that the ^{1}H n.m.r. of the C(20) methyl group, which projects down from the corrin ring, shows an unusual shift in the A forms of organocobalamins due to the proximity of the co-ordinated dbzm and becomes normal when the dbzm is protonated in the E forms, but that heating neutral solutions of dbc (to afford partial conversion of the A to C^{dbzm} form) appears to have no affect on the anomalous shift.[35,36] This provides evidence that the dbzm in form C^{dbzm} (at least in the case of dbc) remains in the neighbourhood of C(20), in agreement with the spectroscopic evidence that there is some interaction between the dbzm side chain and the chromophore in the C^{dbzm} forms.

What is the nature of the interaction which keeps dbzm near C(20) in the C^{dbzm} forms? Unprotonated dbzm is, of course, fairly hydrophobic and, when not co-ordinated to the cobalt, might be expected to interact with some hydrophobic part of the corrinoid molecule; *cf.* the hydrophobic interaction of adenine with the C(46) methyl revealed by the *X*-ray analysis of dbc.[9] An examination of models suggests that likely positions for the unco-ordinated dbzm would include the hydro-

* Throughout this paper: 1 cal = 4.184 J.

phobic clefts either (*a*) between C(20) + C(30) and C(41) (*i.e.* a position very close to that occupied by dbzm when co-ordinated) or (*b*) between C(20) and C(48); either geometry would explain the observed effect of dbzm on the C(20) methyl resonance. We therefore suggest that the C^{dbzm} forms have a structure in which the Co is essentially five-co-ordinate (hence the electronic spectra) and the dbzm is held, mainly by hydrophobic interaction, in one or both of the hydrophobic clefts mentioned (hence the n.m.r. spectra). The possibility of additional charge-transfer interaction with the conjugated ring or of weak coulombic interaction with a residual positive charge on the cobalt cannot be excluded, and may explain the variation in stability with the ligand (see Table 2). Cockle *et al.*[8] have previously concluded from the n.m.r. data that the dbzm could be held near C(20) only if it remained co-ordinated to the cobalt by an unusual and very long bond, but they did not consider other possible alternatives. We agree that N(7) of dbzm is probably the donor atom situated closest to the Co in the 'lower' co-ordination site but, since the electronic spectra do not provide evidence for any meaningful degree of interaction with the Co, we prefer to assign the major component of the interaction to hydrophobic bonding.

The data of Table 1 and Figure 3 show that the conversion of the six-co-ordinate A to the five-co-ordinate C^{dbzm} forms involves an increase in enthalpy (4—5 kcal mol^{-1}) and in entropy (7—16 cal K^{-1} mol^{-1}) which are comparable to the values (4.5 ± 2 kcal mol^{-1} and 16 ± 7 cal K^{-1} mol^{-1}) which we found[5] for the conversion of the six-co-ordinate F to the five-co-ordinate G forms of methyl- and vinyl-cobinamide in methanol. However, it is probably not wise to draw conclusions from the thermodynamic data, which involve relatively small changes in energy. Table 1 also shows that, although the isobutyl and cyclobutyl cobalamins have the same value of x (% 'base-off' form) at 25 °C, they have very different values of ΔH.

The presently available evidence therefore strongly suggests, though it cannot prove, that for the organo-cobalamins which we are studying at present we have to consider equilibria involving four types of complex, *viz.* A, C, C^{dbzm}, and E; that A is six-co-ordinate with the 'cobalamin' conformation and with Co in the equatorial plane, while the others are five-co-ordinate with the 'normal' conformation and Co displaced out of the plane towards the fifth ligand atom; and that dbzm is co-ordinated in A, not (to any significant degree) co-ordinated in C^{dbzm} but held against the lower part of the corrin ring by hydrophobic interaction, free and unprotonated in C, and free but protonated in E. (*N.B.* Because C^{dbzm} is merely a 'variant' of C and is often not experimentally distinguishable, its concentration is included in that of C for the purposes of determining equilibrium constants.)

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