The Circular Dichroism and Absorption Spectra of Some Vitamin B_{12} Derivatives*

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ABSTRACT: The absorption spectra and circular dichroism of three groups of vitamin B_{12} derivatives (cobalamins and cobinamides) have been studied: (1) organocyanocobalamins and organocyanocobinamides, (2) organocobalamins, and (3) cobalamins. These compounds differ in axial ligands bound to the cobalt(III) while they all have the same corrin ring system. The absorption spectra are classified in terms of a four-band system, decreasing in wavelength in the order: $\alpha\beta$, D, E, and γ, and correlated with the circular dichroism spectra. Within each group of compounds both the absorption and the circular dichroism spectra show a marked dependence on the axial ligand, their wavelength increasing with the increasing nephelauxetic (electron donor) effect of the ligand. Simultaneously there is a gross variation of the $\Delta\epsilon$ value of the bands in the circular dichroism leading in extreme cases to an inversion of sign. The change in sign is not due to inversion in the relative positions of the axial ligands. The circular dichroism spectra of particular compounds also show a complete inversion between -100 and $-180°$. These inversions indicate that the magnitude or even the sign of the circular dichroism spectrum (or of the rotational strength) is a very sensitive property of this coenzyme molecule, depending upon such variables as small changes in chemical substituents, temperature, or even solvent. The assignment of the electronic transitions and the relationship between the absorption and the circular dichroism spectra are discussed. The dependence of both on the axial ligand is considered in terms of possible electronic and conformational changes within the corrin chromophore. The temperature dependence of the circular dichroism is considered good evidence for the importance of conformational effects. This series of observations on model compounds should prove valuable in the examination of the interaction between B_{12} coenzymes, their apoenzymes, and substrates. The inversion in sign of a coenzyme circular dichroism spectrum may further be of general importance since it is shown to arise both from electronic and conformational effects without regard for the chirality of the protein to which it becomes bound.

In previous papers (Hill *et al.*, 1964, 1965; Pratt and Thorp, 1966) the absorption spectra of a large number of derivatives of vitamin B_{12} have been described. Despite the fact that the spectra of different cobalamins, *e.g.*, aquocobalamin and ethylcobalamin, are extremely dissimilar, it was shown that the low-energy absorption bands between 350 and 600 mμ were closely related in all the compounds examined. The isolation by Toohey (1965) of a B_{12} derivative which does not contain cobalt yet has a spectrum which is very similar to those of many which do, further shows that the absorption bands in the region under discussion are due to transitions which involve electrons of the conjugated corrin chromophore and not of the cobalt atom. Despite the considerable spectroscopic changes from one compound to another, there is no reason to suppose that the chromophore is altered chemically in any of the B_{12} derivatives. This has been decisively demonstrated by X-ray diffraction investigations of the yellow nickel-containing compound, "nirrin" (Dunitz and Meyer, 1965), the orange cobalamin coenzyme, DBC[1] (Lenhert and Hodgkin, 1961), and the red cyanocobalamin (Hodgkin *et al.*, 1962), which show the presence of the same number of conjugated double bonds in each compound. Thus the changes in the spectra of the vitamin B_{12} derivatives must be due to electronic or conformational effects, or both, induced by the additional ligands bound to the metal along an axis perpendicular to the corrin plane. These ligands will be called axial ligands. The examination of the circular dichroism of the complexes provides a possible method of uncovering the relative importance of the electronic and conformational effects.

A brief earlier study of the circular dichroism of a few derivatives of vitamin B_{12} has been carried out by Legrand and Viennet (1962) who showed that it is more sensitive to changes in the axial ligands than the absorption spectrum. Because of the limited range of compounds studied, no detailed interpretation of these results was attempted. In the present study, the circular dichroism of a greatly extended range of compounds has

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[1] Abbreviation used: DBC, dimethylbenzimidazolecobalamin coenzyme; NADH, reduced nicotinamide–adenine dinucleotide; FAD, flavin–adenine dinucleotide; FMN, flavin mononucleotide.

been examined and the measurements have been extended over a wide temperature range. As a result it is possible (1) to correlate circular dichroism bands with corresponding bands in the absorption spectra and to make tentative assignments of both; (2) to make inferences about conformation changes in the corrin ring; (3) to illustrate the potential use of circular dichroism as a method of investigating the properties of cobalamins in biological systems; and (4) to make certain general observations on the potential significance and basis of the sign of circular dichroism of biological compounds.

Materials

Aquocobalamin, cyanocobalamin, and the cobalamin coenzyme were supplied by Glaxo Laboratories. A sample of nucleoside was kindly provided by Dr. Offenhartz. The alkylcobalamins, cobinamides, and aquocyanocobinamide were prepared with the assistance of Mr. R. G. Thorp in these laboratories by methods based on those of previous workers (Johnson *et al.*, 1963; Friedrich and Bernhauer, 1964) and were isolated as solids. Full details of the preparations will be given elsewhere (R. A. Firth, H. A. O. Hill, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, to be published). All vitamin B_{12} derivatives are soluble in dimethyl sulfoxide, water, methanol, and ethanol, except for the cobalamin coenzyme which is only sparingly soluble in the alcohols. The samples of B_{12r} and B_{12s} were prepared from B_{12a} by controlled potential reduction (P. K. Das, H. A. O. Hill, and R. J. P. Williams, to be published).

The inorganic salts used to prepare complexes of aquocobalamin and aquocyanocobinamide were AnalaR reagents except in the case of KOCN and KSeCN which decompose on recrystallization and were therefore used without purification. Spectra show that the most likely impurity, KCN, was not present. The complexes were fully formed by suitable addition of the inorganic salt to a neutral solution of aquocobalamin in water. Known stability constants made this possible (Pratt and Thorp, 1966; R. A. Firth, H. A. O. Hill, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, to be published). Hydroxocobalamin and hydroxocyanocobinamide are formed by raising the pH to $\geqslant 10$ and $\geqslant 13$, respectively, with alkali.

The ethanol used for low-temperature studies was of two types. Reagent grade ethanol forms clear and reasonably stable glasses, and this was used for low-temperature absorption spectra. Absolute ethanol forms much more unstable glasses, and this was diluted to an ethanol–methanol (4:1) mixture with reagent grade methanol for use in the circular dichroism measurements. Methanol was also employed in order to obtain high enough concentrations of the alkylcobalamins in solution for circular dichroism measurements. Dimethyl sulfoxide was purified by reduced pressure distillation.

Absorption Spectra. Room temperature spectra in water and dimethyl sulfoxide were measured with a Unicam SP 700 or Beckman DK 2 spectrophotometer, using 1-cm silica cells. In the case of ethylcyanocobinamide the decomposition of the compound by excess cyanide restricted measurements to dimethyl sulfoxide solutions. To obtain the molar absorptivities, all compounds were converted to the dicyanide form. Conversion of a sample of cyanocobalamin, the molecular weight of which had been determined by X-ray studies, to the dicyanide has previously been used to determine the molar absorptivity of dicyanocobalamin (Hill *et al.*, 1964). The values for dicyanocobinamide are assumed to be equal to those of dicyanocobalamin for absorption bands at wavelengths greater than 300 mμ where the uncoordinated nucleotide side chain makes no contribution to the spectrum. Conversion to the dicyanide is affected by simple addition of cyanide for complexes of aquocobalamin with various ligands, or by photolysis in presence of cyanide and oxygen for the alkyl and alkylcyano compounds.

Low-temperature spectra were obtained using a Unicam SP 700 spectrophotometer and a Research and Industrial Instruments Co. variable temperature cell (VLT-2) fitted with two silica windows separated by a 1-mm Teflon spacer. All measurements were carried out at the lowest temperature obtainable when liquid nitrogen was used as a coolant (about $-180°$).

Circular dichroism spectra were recorded with a Roussel Jouan dichrograph. The instrument was calibrated in $\Delta\epsilon$ by use of a standard substance, isoandosterone. Initial experiments were carried out using the cobalamin (1 mg) or cobinamide (0.7 mg) in 2 ml of solvent for the tungsten lamp region (400–600 mμ) and diluting these samples by a factor of 5 for the hydrogen lamp region (300–400 mμ). Measurements at wavelengths longer than 600 mμ were kindly made for us by Dr. F. Woldbye.

The photocell of the dichrograph must receive constant energy from the sample, and hence the slit width of the monochromator alters accordingly. It soon became apparent that, in certain regions of the spectrum, the slit width reached a high value and caused a substantial decrease in the $\Delta\epsilon$ value of sharp peaks. Therefore, all spectra were recorded by taking solutions of the concentrations shown above, and successively diluting them until $\Delta\epsilon$ varied linearly with concentration. Only in these circumstances was a calculation of $\Delta\epsilon$ made for a sharp peak. In the previous work of Legrand and Viennet (1962), concentrations up to twice those which we employed, were used. This probably accounts for the considerable variation of intensity values exhibited in the two sets of results. The concentrations of solutions used for circular dichroism were estimated by conversion to the dicyano form.

For the variable low-temperature work, the VLT-2 cell was used with a 1-mm path length. The temperature is varied by balancing the variable heat input, *via* a 50-w heating coil surrounding the sample, with liquid nitrogen in the dewar. The temperature was measured using an iron-constant thermocouple connected to a 10-mv recorder.

Results

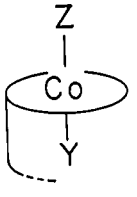
The absorption and circular dichroism spectra of a large number of vitamin B_{12} derivatives have been

TABLE I: Absorption Spectra and Circular Dichroism of a Number of Specimen Derivatives of Vitamin B_{12}.[a]

				Absorption Spectra						
Dicyanocobinamide										
λ	266	277	(301)	(308)	314			(353)		
ε	0.96	1.15	1.02	1.12	1.16			1.46		
	γ		*E*	*D*				β	α	
λ	368	(385)	(402)	420	(475)	(512)		542	583	
ε	3.04	1.25	0.38	0.29	0.45	0.66		0.90	0.99	
				Circular Dichroism						
λ	304	316	320	327	345		363			
Δε	−12.1	−4.6	−5.7	−4.6*	−9.8		[−2.3]			
λ	365	368	395	422	464		482 > 520			
Δε	0	[+6.9]	+19.6	[+12.6]	0		−0.7	0		

[a] This table has been deposited in full as Document No. 9494 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington, D. C. 20540. A copy may be secured by citing the document number and by remitting $2.50 for photoprints, or $1.75 for 35-mm microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress. For the absorption spectra band positions (λ) are given in millimicrons, and shoulders are given in brackets. For the circular dichroism studies band positions are given in millimicrons. Square brackets signify a shoulder, an asterisk signifies a negative or positive *minimum*. Solvents used were water, ethanol, dimethyl sulfoxide (DMSO), and 12 N H_2SO_4 and are indicated. Spectra were measured at both room temperature and liquid nitrogen temperature as shown. The compounds studied were (1) dicyanocobinamide, (2) dicyanocobalamin, (3) ethynylcyanocobalamin, (4) vinylcyanocobinamide, (5) methylcyanocobinamide, (6) methylcyanocobalamin, (7) ethylcyanocobalamin, (8) cyanocobalamin, (9) ethylnylcobalamin, (10) vinylcobalamin, (11) methylcobalamin, (12) ethylcobalamin, (13) cobalamin coenzyme, (14) aquocobalamin, (15) hydroxocobalamin, (16) bromocobalamin, (17) iodocobalamin, (18) cyanatocobalamin, (19) thiocyanatocobalamin, (20) selenocyanatocobalamin, (21) thiosulfatocobalamin, (22) nitritocobalamin, (23) ammoniacobalamin, (24) pyridinecobalamin, (25) azidocobalamin, (26) azidocyanocobinamide, (27) ammoniacyanocobinamide, (28) pyridinecyanocobinamide, (29) aquocyanocobinamide, (30) hydroxocyanocobinamide, (31) cyanocobalamin coenzyme, (32) B_{12r}, (33) B_{12s}, (34) cobyric acid and nucleoside, (35) cobyric acid (factor V_{1a}), (36) cyanocobalamin (base off), (37) methylcobalamin (base off), (38) vinylcobinamide, (39) methylcobinamide, and (40) ethylcobinamide.

studied. To facilitate the description of the vast collection of data (Table I) the compounds have been divided into three main groups. The ligand in the lower axial coordination site (5,6-dimethylbenzimidazole in the cobalamins) is defined as ligand Y, and the ligand in the upper coordination site as ligand Z. Bonnett (1963) has discussed nomenclature and structures for the compounds.



GROUP 1. ORGANOCYANOCOBALAMINS AND ORGANOCYANOCOBINAMIDES. Ligand Y is always cyanide, and ligand Z is also coordinated *via* a carbon atom. The series of Z ligands studied was cyanide (CN^-), ethynyl (Ey), vinyl (Vi), methyl (Me), and ethyl (Et). The presence of the uncoordinated nucleotide in the cyanocobalamins affects the spectrum only below 300 mμ as shown by a comparison of the absorption spectra and circular dichroism of a corresponding pair of cyanocobalamins and cyanocobinamides, *e.g.*, the dicyano compounds, so either the cobalamin or cobinamide may be used to obtain the equivalent cyanocompound. The absorption and circular dichroism spectra of ethynylcyano- and dicyanocobalamins, and methylcyano-, vinylcyano- and dicyanocobinamides were recorded in aqueous solution. The ethylcyanocobinamide was measured only in dimethyl sulfoxide (see Methods).

GROUP 2. ORGANOCOBALAMINS. Ligand Y is the usual nucleotide containing 5,6-dimethylbenziminazole, and ligand Z is coordinated *via* a carbon atom. Below pH ≃0 the benziminazole is replaced by water, but the resulting series of compounds will not be treated as members of group 2 as their spectra and circular dichroism are anomalous, *vide infra*. The same series of carbon Z ligands as above was used. Cobalamin coenzyme, in which ligand Z is C_5'-deoxyadenosyl, was also included.

GROUP 3. COBALAMINS. Ligand Y is the nucleotide containing 5,6-dimethylbenziminazole, and ligand Z is varied by reacting aquocobalamin with suitable anions or bases. The Z ligands used were H_2O, OCN^-, Pyr, NH_3, Br^-, OH^-, NO_2^-, I^-, SCN^-, $SeCN^-$, and $S_2O_3^{2-}$.

The details of the absorption spectra and circular

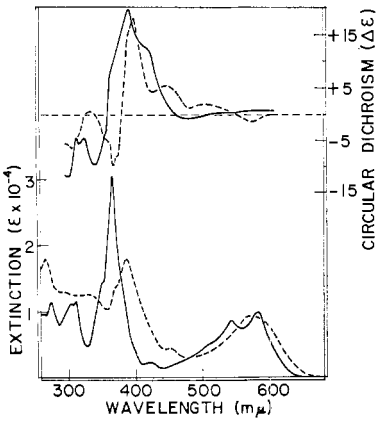


FIGURE 1: Absorption spectra and circular dichroism of dicyanocobinamide (———) and vinylcyanocobinamide (– – – –). The solvent was water; room temperature.

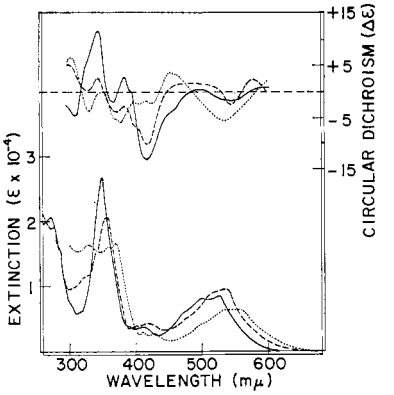


FIGURE 3: Absorption spectra and circular dichroism of aquocobalamin (———), hydroxocobalamin (– – – –), and selenocyanatocobalamin (· · · · · ·). The solvent was water; room temperature.

dichroism of these three groups of compounds are presented in Table I. Examples from each group are given in Figures 1, 2, and 3, respectively, in order to show the range of spectra observed.

Several compounds of very different kinds from those in groups 1–3 have also been studied. The resulting data are presented in Table I including the absorption spectra and circular dichroism of the reduced forms of vitamin B_{12a}, *i.e.*, B_{12r} and B_{12s}, and of the acid forms of the group 2 series of compounds, which show anomalous properties. In the latter compounds water occupies the position Y.

Analysis of Spectra. The extensive data in Table I can be summarized and interpreted conveniently by contrasting the major freatures of the spectra in the different compounds. This is achieved in generalized form in Figure 4. Figure 4A is the simplest type of spectrum, here called *normal, e.g.*, dicyanocobalamin. Figure 4D is the extreme *anomalous* type of spectrum, *e.g.*, methylcobalamin in acid solution, while Figure 4B,C are typical intermediate spectra. The main feature of the series of four spectra is that a set of bands (γ_1, D, E, and $\beta_1\alpha_1$) diminishes while a second set of bands (γ_2 and β_2) increases through Figure 4A–D. This change can be best detailed in series of compounds by considering the spectra in three regions: 500–600 ($\beta_1\alpha_1$), 400–500 (D, E, and β_2), and 300–400 mμ (γ_1 and γ_2).

Region 1 (500–600 mμ). In the normal spectrum the longest wavelength band system consists of two prominent bands, the α_1 and β_1 bands, plus one or more shoulders at shorter wavelengths. The low-temperature spectra show these bands to be a vibrational progression separated, in the case of dicyanocobinamide, by 1300 cm^{-1} (Figure 5).

There is a marked difference in the relative intensities of the α_1 and β_1 bands, *i.e.*, 0–0 and 0–1 vibrational components of the band. For example, the α_1 band is more intense than the β_1 band in dicyanocobinamide but gradually becomes less intense relative to the β_1 band when the Z ligand is varied from CN^- to Ey, Vi, Me, C_5'-deoxyadenosyl, and Et. This intensity variation is one criterion of the change from a *normal* toward an

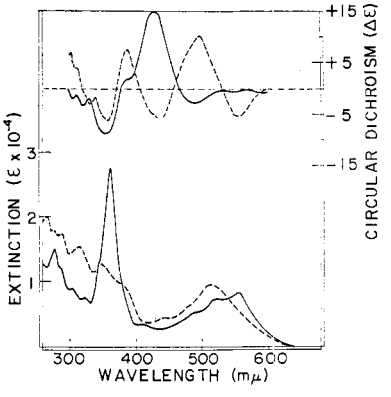


FIGURE 2: Absorption spectra and circular dichroism of cyanocobalamin (———) and ethylcobalamin (– – – –). The solvent was water; room temperature.

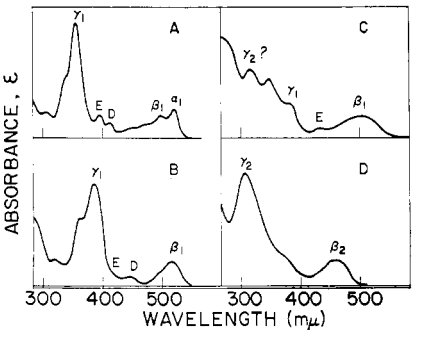


FIGURE 4: Illustration of the changes in spectrum from a normal to that of an extremely anomalous B_{12} derivative.

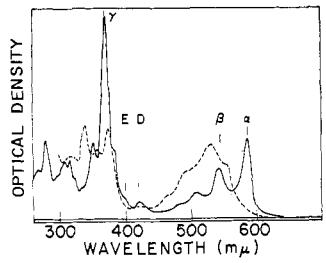


FIGURE 5: Low-temperature absorption spectra of dicyanocobinamide (———) and methylcobalamin (– – – –). The solvent was ethanol; temperature, ~—180°. The letters refer to the bands of dicyanocobinamide.

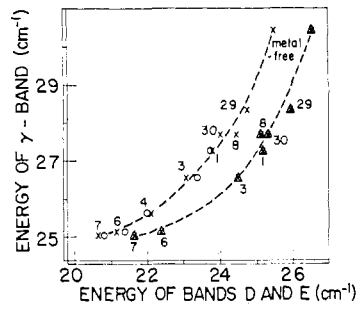


FIGURE 6: Absorption spectra and circular dichroism of cyanocobalamins and cyanocobinamides. Graph of the energies of the absorption bands D (X) and E (▲) together with the energy of the circular dichroism band D (O) against the energy of the absorption γ band. The points are numbered, the key being presented in Table I.

anomalous type of spectrum (Figure 4). Replacement of the Y ligand CN^- by 5,6-dimethylbenziminazole giving compounds of group 2, has little effect on the intensities, but tends to produce less sharp spectra. The band profiles are relatively unaffected at liquid nitrogen temperature.

In compounds of groups 1–3 the wavelength of the α_1 band varies from 500 to 630 mμ and depends on the axial ligands. Variation of the ligand Z along the series from CN^- to Et, or replacement of the Y ligand, 5,6-dimethylbenziminazole, by CN^- moves the band to longer wavelength.

Comparison of the wavelengths of the α_1 band in all these groups of compounds with the nephelauxetic series shows that its dependence on the ligand approximately parallels the nephelauxetic effect. It would appear that increase of electron density on the cobalt due to the axial ligands moves the absorption band to longer wavelengths.

This generalization does not hold in the series of compounds where the benzimidazole base is removed from the cobalt and replaced by H_2O instead of CN^-, *i.e.*, in the acid forms of group 2 compounds. Starting from CN^-/H_2O (compare CN^-/Bz or CN^-/CN^-) and replacing the CN^- by C_2H^-, $C_2H_3^-$, CH_3^-, $C_2H_5^-$, and C_5'-deoxyadenosyl, the spectrum changes through a series of red, orange, and yellow compounds while the longest wavelength band falls from 550 to below 475 mμ. The spectra are now closely related to those of vitamin B_{12r} [cobalt(II)] and nirrin [nickel(II)]. Thus the *abnormal* features of the spectra are gradually generated as CN^- is replaced by benzimidazole and then water on one side of the cobalt or as CN^- is replaced by a more powerful electron-donor ligand on the other side of the cobalt.

The longest wavelength bands in the metal-free compounds are at 525 and 497 mμ, whereas in B_{12r} and nirrin the lowest energy maxima are at 470 and 440 mμ, respectively (see Figure 4D). These bands should be compared with that at 470 mμ in Figure 4D which is labeled β_2 for reasons given in the discussion.

Region 2 (400–500 mμ). Between the high-intensity α_1, β_1, and γ_1 bands in the normal spectra (Figure 4A), there are two bands of low intensity which are labeled D (long wavelength) and E (short wavelength). The high-intensity bands often obscure one of these two bands and their proximity always makes it difficult to discuss intensities or band profiles.

In the case of group 1 compounds and a number of other compounds with cyanide as the Y ligand, it is possible to observe clearly both low-intensity bands. The energies of these bands have been plotted against the energy of the γ_1 band (defined as the highest wavelength high-intensity band below 400 mμ) (Figure 6). To this plot the two bands seen clearly in the metal-free corrin spectrum at 395 and 376 mμ, and the one band observed in vinylcyanocobinamide have been added.

The plot in Figure 6 has been further extended by including the variation of the energy of a characteristic circular dichroism band throughout the group 1 series of compounds. This circular dichroism band correlates well with band D in the spectrum where both can be clearly seen *vide infra*. The dependence of the wavelengths of the D and E bands on Z ligand again follow the nephelauxetic effect, although the energy movement with change of ligand is greater than for either the α_1, β_1, or γ_1 bands.

In the case of the cobalamins of groups 2 and 3, it is common to find only one low-intensity absorption band in this region of the spectrum. However, by plotting the energies of the bands in cases where two bands are observable, and following a characteristic circular dichroism band through each of the groups of compounds (see later), it is possible to show (Figure 7) that the curves representing bands D and E are again approximately parallel to one another, and their energies again move according to the nephelauxetic effect of the ligand. It will be seen that replacement of the Y-ligand 5,6-dimethylbenziminazole by cyanide moves both D and E to higher energy (shorter wavelength). This is quite the opposite effect to that caused by the same change of the Y ligand in the α_1, β_1, and γ_1 regions. The only exception to this rule is cyanocobalamin, band D, appearing to move to longer wavelength when 5,6-dimethyl-

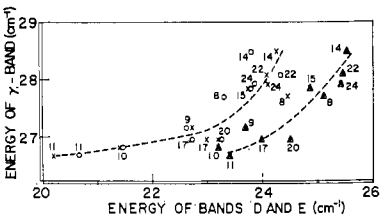


FIGURE 7: Absorption spectra and circular dichroism of cobalamins. Graph of the energies of the absorption bands D (X) and E (▲), together with the energy of the circular dichroism band D (O), against the energy of the absorption γ bands. The points are numbered, the key being presented in Table I.

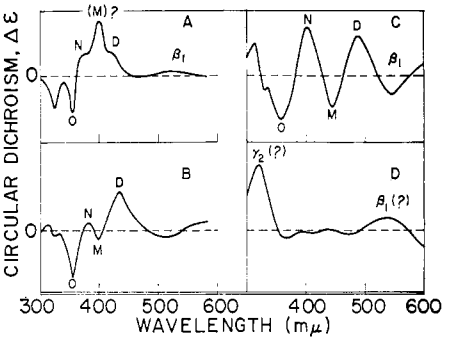


FIGURE 8: Illustration of the changes in circular dichroism from that of a normal compound (A) to that of an extreme anomalous compound (D).

benziminazole is replaced by cyanide. In Figure 7 band D of cyanocobalamin is at a different energy to that suggested by the circular dichroism, and it is presumed that band D is either missing from the absorption spectrum, or shifted by some specific property of this ligand.

A small band in this region of the spectrum can be observed in the cobalt(II) complexes (at ~400 mμ) but none is apparent in the spectrum of the nickel(II) compound (nirrin) or the acid forms of the group 2 compounds. Bands D and E cannot be located in any of the *anomalous* spectra (See Figure 4D).

Region 3 (300–400 mμ). The γ_1 band is defined as the highest wavelength, high-intensity band below 400 mμ, and is easily recognized in all spectra except the most anomalous (see Figure 4A–D). As the Z ligand is varied along, for example, group 2 in the series CN^-, Ey, Vi, Me, C_5'-deoxyadenosyl, and Et, there is a very marked change in the intensity profile in the 300–400-mμ region. The γ_1 band falls steadily eventually reaching one-third of its value in dicyanocobalamin, and other bands at lower wavelengths become more prominent. This intensity variation, like the variation in the $\alpha_1\beta_1$ system, is a criterion of the change from a *normal* toward an *anomalous* type of spectrum. All compounds having anomalous spectra in the $\alpha_1\beta_1$ region are also *anomalous* in the γ_1-band region, but the converse is not invariably true. Certain cobalamins, namely, iodo-, thiosulfato-, and selenocyanatocobalamins have somewhat anomalous spectra in the γ_1 region, yet the $\alpha_1\beta_1$ region is almost normal.

In both normal and intermediate spectra (Figure 4A–C), a shoulder can often be resolved some 20 mμ on the short-wavelength side of the γ_1 band and a weaker band is seen around 320 mμ. In the case of more anomalous spectra these weaker bands increase in strength. For example, in the spectrum of methylcobalamin (see Figure 5), there are strong bands at 339 and 316 mμ. The metal-free compound has a similar γ_1-band profile to dicyanocobalamin, with the γ_1 band at 329 mμ.

The wavelength of the γ_1 band again depends on the axial ligands and closely follows the nephelauxetic effect. In the case of group 1, and related compounds, the wavelength increases for the Z ligand, in the order H_2O $< NH_3 \sim OH^- \sim Pyr \sim N_3^- < CN^- <$ Ey $<$ Vi $< C_5'$-deoxyadenosyl $<$ Me $\sim$ Et. Similarly, in groups 2 (alkali forms) and 3 related compounds, the following order is observed: $H_2O < Br^- < NH_3 \sim NO_2^- < OCN^- \sim SCN^- \sim Pyr < N_3^- < OH^- < CN^- < S_2O_3^- <$ Ey $< SeCN^- \sim I^- <$ Vi $<$ Me $\sim C_5'$-deoxyadenosyl $<$ Et. Replacement of the Y-ligand 5,6-dimethylbenziminazole by CN^- always moves the γ_1 band to higher wavelength.

Extreme anomalies in this region of the spectrum are again notable in the compounds which have one water molecule bound to the cobalt. Here the γ_1 band at 355 mμ in the H_2O/CN^- falls ($H_2O/C_2H_2^-$) while moving to longer wavelength but is finally totally replaced by a band at 310–320 mμ in H_2O/Et^- with only a minor inflection in the region of 380–400 mμ. The spectrum of the final compound of the series closely resembles those of B_{12r} and nirrin (see Figure 4D). The band at about 320 mμ in these spectra is labeled γ_2 for reasons given in the discussion.

Analysis of Circular Dichroism. In describing the circular dichroism spectra, compounds will be divided according to the type of absorption spectrum which they exhibit, *i.e.*, they will be called *normal* or *anomalous*. Figures 8A–D provide a series of circular dichroism spectra to match the full range from a *normal* to an *anomalous* spectrum (see Figure 4A–D). It must be remembered of course that circular dichroism bands can be of either sign. Figure 8A is that of the *normal* series of compounds while Figure 8D is that of the anomalous compounds. In the next sections we describe the regions of the circular dichroism spectrum in more detail for individual series of compounds, treating the three regions of the spectrum in turn as in the sections on absorption spectra. Finally the effect of change of solvent and temperature on the circular dichroism is described. Data are accumulated in the tables.

GROUP 1. ORGANOCYANOCOBALAMINS AND ORGANOCYANOCOBINAMIDES. Examples of the circular dichroism of these compounds are shown in Figure 1, which should be compared with Figure 8A–C.

Region 1 (500–600 mμ). $\Delta\epsilon$ is never large, but increases through the series CN^- to Et, or, in other words, with

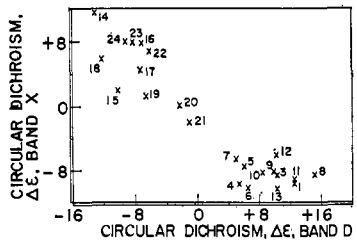


FIGURE 9: Circular dichroism. Graph of $\Delta\epsilon$ for band X against $\Delta\epsilon$ for band D. The points are numbered, the key being presented in Table I.

increasing anomaly in the absorption spectrum. This increase is observed in all compounds which also exhibit anomalous spectra in the γ_1-band region. This is idealized in Figure 8A–C. In this group of compounds, energy correspondence between the maxima of the circular dichroism and absorption spectra is good.

Region 2 (400–500 mμ). The band at ~400 mμ with the largest $\Delta\epsilon$ has approximately the same intensity and wavelength throughout the series, while the long-wavelength shoulder moves sharply out to longer wavelength in the series CN^- to Et. As it moves out it becomes an independent peak and is then followed by a negative peak (see Figure 8A–C). This shoulder correlates well with band D in the absorption spectra, as shown in Figure 6 or 7, and is itself labeled D in the circular dichroism. However, it is not immediately possible to correlate the large $\Delta\epsilon$ band with any band in the absorption spectrum and it is, therefore, not labeled in Figure 4A.

Region 3 (300–400 mμ). From the high-intensity positive band at 400 mμ, the circular dichroism always shows a positive shoulder and then moves sharply over to a series of negative peaks ranging between 370 and 300 mμ. The positive shoulder, N, correlates reasonably with the γ_1 band, and the first strong negative peak labeled O with its short-wavelength shoulder.

GROUP 2. ORGANOCOBALAMINS. Examples of the circular dichroism spectra are shown in Figure 2, which should be compared with Figure 8A–C.

Region 1 (500–600 mμ). Again the correlation with the absorption spectra is good, $\Delta\epsilon$ increasing as before across the series CN^- to Et.

Region 2 (400–500 mμ). Between 400 and 500 mμ there are usually two bands, one positive and one negative. The latter, we assume, is compensated in the early members of the series, *e.g.*, cyanocobalamin; but appears fully in the case of ethylcobalamin (see Figures 4C and 2). The positive band is shown in Figure 7 and correlates well with band D in the absorption spectra, and is, therefore, labeled D. The negative band correlate quite well with band E but is not labeled as such as this is not easily justified in the group 1 compounds. In Figure 4 it is labeled M.

Region 3 (300–400 mμ). Between 300 and 400 mμ there are at least two bands, one positive and one negative. Again the correlation of these bands with the γ bands is uncertain. They are labeled N and O, respectively, in keeping with the system used for compounds of group 1.

GROUP 3. COBALAMINS. Figure 3 shows examples of the circular dichroism. The spectra of these compounds are often inversions of those of the two previous groups.

Region 1 (500–600 mμ). There is no difficulty in finding a correspondence between absorption and circular dichroism spectra, and as before the compounds which have more anomalous spectra show greater intensity of the circular dichroism bands in this region.

Region 2 (400–500 mμ). The most striking feature in the 400–500-mμ region is the band which starts negative, but while moving to higher wavelength becomes positive along the series, H_2O, OCN^-, OH^-, Pyr, NH_3, I^-, Br^-, SCN^-, NO_2^-, $SeCN^-$, and $S_2O_3^{2-}$. From Figure 7, we see that this band can be correlated with band D in the absorption spectrum, and therefore it is labeled D. In the early members of the series its sign is the opposite to that found in groups 1 and 2 compounds. Immediately following band D there is often a circular dichroism band of opposite sign, labeled M, which correlates with band E.

Region 3 (300–400 mμ). In most of the complexes there is one negative and one positive band but there is additional structure in some spectra. The correlation of the positive band with the γ band is possible but uncertain for it could be related to the short-wavelength shoulder of this band. The fact that the γ band correlates best with band N in the circular dichroism spectra of compounds of groups 1 and 2 suggests that this correlation should be made here also when the positive peak of the circular dichroism band, labeled O, becomes the shoulder of the γ band. The sign inversions of the bands in this region of the spectrum are then related to that found for band D (Figure 9). Roughly group 3 compounds have a spectrum of type 8B but completely inverted.

The metal-free corrin has the following circular dichroism (A. J. Thomson, unpublished work). In the $\alpha_1\beta_1$ region, there is a weak negative band with exactly the same energy and intensity profile as the absorption spectrum. There are no bands in this spectrum at 395 and 376 mμ corresponding to absorption bands D and E. Then follows a positive band at 329 mμ and a negative shoulder at 285 mμ and a negative band at 270 mμ. The Cotton effects in the optical rotatory dispersion are of the corresponding signs. Correlation of *all* bands in the spectrum with bands in the circular dichroism is therefore immediately possible. The simplicity of the circular dichroism spectrum of the metal-free corrin as compared with the cobalt-containing derivatives especially in the region between 350 and 450 mμ implies that the magnetic components of the transitions of metal corrins are largely dominated by the metal ion. It is very probably that the *d–d* absorption bands of cobalt(III) lie in this region of the spectrum.

THE COMPOUNDS WITH H_2O–Z AS LIGANDS. Finally the circular dichroism of the compounds with the most anomalous spectra will be described. They all follow the pattern shown in Figure 8D.

Region 1 (500–600 mμ). There is a strong positive

TABLE II: Effect of Solvent on the Principal Peaks in the Absorption Spectra and Circular Dichroism of Cyano- and Methylcobalamins.[a]

	Solvent	Absorption (λ)	Spectra (*E*)	Circular (mμ)	Dichroism (Δε)
Cyanocobalamin	DMSO	360	1.00	360	−6.0
		519	0.29	430	+11.5
		546	0.33		
	H_2O	360	1.00	357	−8.6
		518	0.27	430	+15.1
		549	0.30		
	MeOH–EtOH (1:4)	360	1.00	355	−8.9
		518	0.28	430	+13.3
		547	0.31		
Methylcobalamin	MeOH	339	1.16	355	−7.0
		373	1.00	380	+6.0
		520	0.75	427	−2.0
				480	+10.0
	H_2O	340	1.21	355	−9.3
		373	1.00	384	+7.4
		520	0.75	428	−3.0
				484	+12.6
	MeOH–EtOH (1:4)	339	1.13	360	−4.5
		374	1.00	385	+4.9
		520	0.73	425	−2.5
				480	+15.5

[a] Wavelength (γ) in millimicrons; *E* is the optical density of a band relative to unit optical density of the γ band.

circular dichroism band at above 525 mμ which increases in strength and moves to longer wavelengths along the series CN^-, H_2O, $C_2H_3^-$, CH_3^-, $C_2H_5^-$, and C-adenosyl. Apart from the H_2O–CN^- and H_2O–H_2O compounds there is very weak and diminishing absorption in this region of the spectrum. B_{12r} shows a similar very high ratio of circular dichroism (Δε) to absorption above 525 mμ. Thus this series of compounds can again be considered as a continuation from the extreme members of the series CN^-–Z and Bz–Z.

Region 2 (400–500 mμ). The region between 400 and 500 mμ where there is considerably greater absorption than in the previous series of compounds has relatively little rotational strength in any of the derivatives. This is also true of B_{12r}. The anomalous compounds have band β_2 in this region while the normal compounds have bands D and E.

Region 3 (300–400 mμ). An intense circular dichroism band is found at 321–329 mμ just ahead of the main absorption band around 310 mμ. The intensity of this band increases as intensity falls in the wavelength region from 350 to 400 mμ (see Figure 8C,D). A similar short-wave band is given by B_{12r}. Now that the spectra and circular dichroism in water at room temperature have been described the influence of changing solvent and temperature will be outlined.

Effects of Solvent and Temperature. The effect of solvent on the principal circular dichroism and absorption bands of cyano- and methylcobalamins is insignificant (Table II). In general, therefore, the use of various solvents in this work has no effect on the comparability of the results. However, a marked solvent effect on the absorption and circular dichroism spectrum of the coenzyme cobalamin and ethylcobalamin (Hill *et al.*, 1964) is observed. This is only true of these two compounds and will be discussed in full detail in a future publication (R. A. Firth, H. A. O. Hill, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, to be published).

Preliminary investigations have been made on the effect of temperature on the circular dichroism and absorption spectra of cyano-, dicyano-, methyl-, thiocyanato-, and pyridinocobalamins. In all cases the absorption spectra change but slightly. Only in the first three cases has it proved possible to measure the circular dichroism over the whole wavelength range due to the instability of the glass in experiments with the last two compounds. The results at a number of temperatures between room temperature and ~−180°, and are shown for cyanocobalamin in Figure 10. (The Δε values are not corrected for contraction.) In all experiments, inversion of the spectrum had occurred at liquid nitrogen temperature, except for a few shoulders whose sign could easily depend on the sharpness of the spectrum. The picture emerging from the variable temperature studies is a gradual sharpening of the bands down to ~−100°, followed by a gradual inversion with temperature of some bands down to ~−180°. Some of the bands in the spectrum invert much more rapidly with temperature,

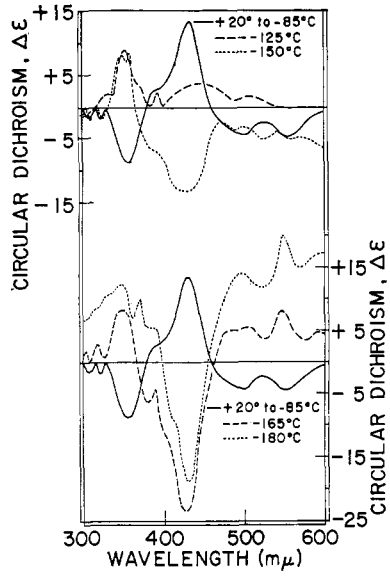
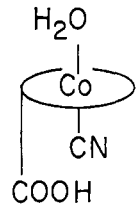


FIGURE 10: Circular dichroism of cyanocobalamin at various temperatures. The solvent was methanol–ethanol (1:4).

and the spectra at −180° then approximates to a mirror image of that at room temperature.

Note on the Effect of Ligand Inversion on the Circular Dichroism. Cobyric acid, factor V_{1a}, an aquocyanocobinamide lacking the aminopropanol group in the side-chain has been shown by X-ray studies to have the following structure. Comparison of the circular dichroism



of this compound and of its complex formed with the nucleoside, with base off and normal cyanocobalamin, respectively, in which the ligands are oppositely orientated shows that inversion of the axial ligands does not greatly affect the circular dichroism.

Friedrich (1966), however, has reported a separation of the aquocyano forms of cobyric acid and cobinamide into two isomers which he believes differ in that the ligands are oppositely orientated. These isomers are interconvertible at room temperature. He has shown then to have circular dichroism spectra differing slightly in $\Delta\epsilon$ value at 430 and 490 mμ, but there is no question of difference in sign.

Discussion

This detailed study of the effects of ligand bonding to the cobalt(III) atom of vitamin B_{12}, of the effects of solvents, and of the effects of temperature on both the spectra and the circular dichroism of the B_{12} derivatives was intended to serve several objectives. The spectra and circular dichroism of the many B_{12} derivatives studied here were essentially unknown. The response of these properties to the systematic variation of the ligands in the compounds should greatly add to the understanding of the electronic structure of the molecule itself. Beyond this, however, spectral properties and optical activity of coenzymes generally, and of B_{12} in particular, have become progressively important in the understanding of coenzyme interaction with apoenzymes both through spectral shifts and the resultant extrinsic Cotton effects (Ulmer and Vallee, 1965). While the rotatory dispersion of NAD(H), pyridoxal phosphate, FAD, FMN, and biotin bound to their respective apoenzymes, has been studied to varying degrees, there are no comparable data for the stereospecific interaction of B_{12} and its derivatives with its apoenzymes. The data presented here form an essential background for any such future investigation. Now the previous studies of the interaction of apoenzymes with their coenzymes have shown extrinsic Cotton effects which are quite specific for the individual systems. Since stereospecificity is one of the inherent characteristics of enzymic catalysis, the origin of the resultant apoenzyme–coenzyme spectra and circular dichroism has been the source of new insight into the mechanism of action of enzymes (Ulmer and Vallee, 1965).

Both in regard to such extrinsic Cotton effects and when investigating intrinsic and side-chain Cotton effects the significance of their signs, *i.e.*, positive or negative, has been the object of much conjecture (Schellman and Schellman, 1964). Thus, it has been thought that the sign of Cotton effects might well be related to the presumable left- or right-handedness of the protein helix, or, perhaps reflect changes in conformation of the enzyme–coenzyme complex (Stryer and Blout, 1961). The very large number of derivatives in the B_{12} series of complexes which have been prepared and described in previous reports from this laboratory are particularly suitable for the critical examination of such postulates. The results of measurements presented here show that changes in signs of Cotton effects can be brought about through variations in temperature and ligand substitution, suggesting yet additional alternatives in the interpretations of the meaning of the signs of extrinsic Cotton effects exhibited by coenzyme–enzyme complexes. These data demonstrate, in fact, that both the substitutions in the B_{12} molecule and the ambient environment itself may alter the conformational state of a coenzyme—even in the absence of binding to an apoenzyme, a circumstance which might perhaps be pertinent also to intrinsic and side chain Cotton effects in macromolecules such as proteins and nucleic acids. Much still remains to be understood concerning the interpretation of spectropolarimetric observations in such systems. Undoubtedly any such understanding must eventually depend upon a proper assignment of the spectrum of the coenzyme in a number of known environments which can then be correlated with the circular dichroism spectra. This is done below for the vitamin B_{12} compounds. Subsequently, this information can be used to interpret the

changes in circular dichroism on interaction of the coenzyme with proteins and with substrates.

Electronic Transitions in Corrinoid Complexes. ABSORPTION SPECTRA. No completely satisfactory spectral assignment of a molecule as complex as vitamin B_{12} can be made at the present time for there are usually more bands in the absorption spectra and circular dichroism than predicted by theory which considers only transitions of the π electrons. We shall, therefore, make no attempt at discussing weak absorption bands. Despite this complexity the lowest energy strong band in all the spectra is readily assigned. This is associated with the excitation of an electron from the highest occupied to the lowest unoccupied π orbital ($\psi 7$–$\psi 8$) as discussed by Kuhn *et al.* (1965), Offenhartz (1965), and Day (1967). The assignment is based both on the energy of the transition in the corrin compounds and on its polarization along the C-10 axis of the molecule as has been demonstrated in the nirrin complex (B. Anex, private communication). The band shows extensive vibrational structure and the first two components, presumably the 0–0 and 0–1, are commonly called the α and β bands, respectively. The exact energies and intensities of these bands are dependent on the central metal ion and its axial ligands. (This assignment makes the band at $\sim$470 mμ (β_2) in the *abnormal* spectrum of Figure 4D, identical in character with that seen at 520 mμ (β_1) in the *normal* spectrum of Figure 4A. For this reason both bands are labeled β in the spectra.)

The spectra at higher energies differ markedly from compound to compound. The latest theoretical treatment (Day, 1967) shows that the one-electron transitions ($\psi 7$–$\psi 9$ and $\psi 6$–$\psi 8$) which have the same symmetry if the chromophore belongs to a point group, C_{2v}, should give rise to two bands in the absorption spectrum whose relative intensities will depend on the degree of interaction. Their energies should be nearly twice that of the $\alpha\beta$ bands. All theoretical treatments agree that the intense γ_1 band in the normal spectrum (Figure 4A) is one of these bands, while the other is weak and difficult to assign. Both bands should be polarized *perpendicular* to the C-10 axis. In the nirrin complex (anomalous spectrum), the first strong band of this polarization (the second strong band in the spectrum) has been found in the 300-mμ region. The second strong band is, therefore, assigned in all the spectra as the more allowed member of the above pair of transitions and is labeled γ_1 in the normal compounds and γ_2 in the anomalous compounds (Figure 4A-D).

Following these assignments the strong bands in nirrin, 300 (γ_2) and 440 mμ (β_2), correspond with the strong bands (1) in B_{12r} at 315 (γ_2) and 470 mμ (β_2), (2) in the metal-free complex at 329 (γ_1) and 497 mμ (β_1), (3) in the groups 1 and 3 complexes 350–400 (γ_1) and 520–580 (β_1), and (4) in the anomalous spectra H_2O–carbanion 320 (γ_1) and 470 (β_2). In complete accord with these assignments the separation between the two bands remains very constant at 1000 $\pm$ 100 cm^{-1}. This then leaves a group of complexes, see ethylcobalamin in Figure 2, which have strong bands both at 320 and 380 and broad absorption from 450 to 550 mμ. The most plausible explanation of these spectroscopic observations is that these compounds exist in solution in two main conformations, one showing maxima at 320 and 470 mμ while the other shows maxima at 380 and 540 mμ. In keeping with this conclusion the latter compounds are the very ones which show solvent-dependent changes in absorption spectra. The general proposition that different conformers are present in the compounds is supported by the circular dichroism measurements. First, however, the relationship between the circular dichroism and the absorption spectra needs to be examined.

Relationship between the Circular Dichroism and Absorption Spectra of Corrinoid Complexes. (Figures 4 and 8 should be used as a guide to this discussion.) For those complexes with a normal spectrum the circular dichroism in the region of the $\alpha_1\beta_1$ bands shows an approximate correspondence to the $\alpha_1\beta_1$ system in the absorption spectra. The fact that an exact correspondence is not observed is presumably due to overlapping bands, possibly of differing sign, and will be particularly prevalent in the corrinoids whose spectra have many bands lying close together.

In regions 2 and 3 the association of bands in the circular dichroism with bands in the absorption spectrum is not always easy even for the simple compounds although all such correlations can be made immediately in the spectra of the metal-free compound. It could well be that the *d–d* transitions of the cobalt(III) are mixing with the π–π^* ligand transitions causing anomalies in the circular dichroism both in the number of bands and their intensities (Figure 8). The energy of one band in the circular dichroism in this region is very closely correlated with the energy of band D in the absorption spectrum, for each group of compounds studied (Figures 5 and 6). It is, therefore, also labeled D in the circular dichroism spectra (Figure 8). Furthermore, band M in the circular dichroism correlates well with band E in the absorption spectra of compounds in both groups 2 and 3. Bands E and M can be found in difficult cases with the help of Figure 7. Using these facts the γ band or its vibrational component in the absorption spectra must correlate with bands N and O in the circular dichroism. In those spectra where band O cannot be readily separated from N use can be made of the correlation between $\Delta\epsilon$ of bands O and D (Figure 9). Thus, although a complete connection between absorption and circular dichroism spectra has not been achieved, analyses of series of compounds have made it possible to go a considerable way toward this objective.

The correlation of the circular dichroism with the spectrum in the cases of compounds with anomalous spectra is also not altogether obvious (Figures 4D and 8D). Outside the main region of absorption (Figure 10) there is a strong circular dichroism at long wavelengths. Only one other region of large $\Delta\epsilon$, which is at slightly smaller energies than the γ_2 bands, is observed and it is here assumed to be associated with γ_2 bands in the same general way that O and N are associated with γ_1 in the normal compounds.

Conformational and/or Electronic Changes in Corrin. Considering all these series of compounds together there

are three striking changes as the spectra become increasingly anomalous. (1) In the $\alpha\beta$ region intensity at first moves from the α to the β band as the band profile moves to longer wavelengths. Further intensity shift, however, is associated with a movement of the β band to shorter wavelengths. Throughout the whole series of changes in spectra the circular dichroism ($\Delta\epsilon$) *increases* and moves to *longer* wavelengths. Finally, spectra are obtained with little absorption but very considerable circular dichroism beyond 525 mμ. (2) In the region between 400 and 500 mμ there is a series of compounds in which the circular dichroism first changes sign as the spectra move to longer wavelengths and become more anomalous. However, eventually the absorption bands (D and E) and the circular dichroism (D and M) of this region virtually disappear, *i.e.*, in the most anomalous spectra. (3) In the region 300–400 mμ a clear-cut spectrum with one γ_1 band around 360 mμ in the simplest spectrum is replaced at first by a complex series of at least three bands extending from around 300 to 400 mμ, the γ_1 band moving out to longer wavelengths. The circular dichroism is correspondingly complex. However, in the extreme cases of "anomalous" compounds, the spectrum and the circular dichroism become simple again with a γ_2 band at the much shorter wavelength of 310 mμ and a single large circular dichroism peak at slightly longer wavelengths, *e.g.*, B_{12r}. Taken together with the assignments based on both experimental polarization data and theoretical arguments the most probable explanation for all these changes is that superimposed upon gradual changes of electron density in the corrin ring there is a change of geometry in the chromophore. Such a conclusion is in agreement with X-ray studies of the flexibility of the corrin ring system in different B_{12} derivatives (Hodgkin *et al.*, 1962). It is important to know if the two effects (electronic and conformational) are separable in any of the measurements made here.

An inversion of the circular dichroism occurs on lowering temperature and at a similar series of temperatures with different axial ligands (Figure 10). These data show that an equilibrium exists between two molecular forms with ΔH = approximately 2–3 kcal. It is difficult to avoid terming this a conformational change. It does not correspond with any noticeable change in absorption spectrum and there would, therefore, seem to be little change in electronic structure of the ring. On the other hand, any change in energy of the absorption spectra of a conjugated chain system such as corrin must be linked to change in the electron density in the different parts of the ring. Change of axial ligand (Y) as in the series of compounds in Figure 9, produces gross changes in spectrum and, therefore, must alter this electron density. (This alteration must be relayed through the ligands and the metal.) Change of axial ligand alters the electron density on the metal, then on the chelated nitrogens of the ring and finally on the carbon atoms of the conjugated chain. Now the metal-nitrogen distances will adjust themselves to the electron density donated from the axial ligands and these, in turn, will cause small alterations in bond lengths and angles in the ring itself. There will thus be a slightly different conformer for each different system of ligands, X and Y. Electronic changes may not be possible without conformational changes in these flexible ring systems though it is possible to have a gross conformational change without an appreciable change in the electronic structure as seen in the absorption spectrum.

The systematization of the properties of the corrinoid compounds developed here has obvious relevance to the future investigation of the interaction between vitamin B_{12} compounds and proteins. It is already clear that the spectrum of the coenzyme bound to one enzyme is more anomalous than the spectrum of the coenzyme itself (Weissbach and Taylor, 1966). A detailed study of the circular dichroism of this complex might well establish a parallel between the conformation in the enzyme and that observed say in the acid form of the coenzyme which does have a very similar spectrum. In turn, this would suggest that the bezimidazole is not bound to the cobalt in the enzyme–coenzyme complex. The circular dichroism of B_{12} derivatives will obviously be a powerful tool, too, in following interaction of the enzyme–coenzyme complex with substrates.

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A Sedimentation Equilibrium Study of the Association of Purine in Aqueous Solutions*

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ABSTRACT: The association of purine in aqueous solution has been studied by the sedimentation equilibrium technique. The results are in qualitative agreement with the conclusion of Ts'o *et al.* (Ts'o, P. O. P., Melvin, I. S., and Olson, A. C. (1963), *J. Am. Chem. Soc. 85*, 1289) that a simple reversible polymerization is involved, with a constant free-energy increment for the addition of each successive purine molecule. However, for quantitative analysis of the equilibrium, the thermodynamic nonideality of monomer and polymers must be taken into account; when such corrections have been made, the apparent equilibrium constants become invariant over the entire concentration range. Studies at a series of temperatures have provided values for the enthalpy and entropy changes in the process. The results obtained for ΔG° and ΔH° are somewhat larger than those found previously (Ts'o, P. O. P., Melvin, I. S., and Olson, A. C. (1963), *J. Am. Chem. Soc. 85*, 1289; Gill, S. J., Downing, M., and Sheats, G. F. (1967), *Biochemistry 6*, 272).

Several years ago, Ts'o and collaborators demonstrated that purine (and a number of similar compounds as well) was capable of forming "stacked" aggregates in aqueous solution (Ts'o *et al.*, 1963; Chan *et al.*, 1964). These results lent substance to the earlier suggestion of Sturtevant *et al.* (1958) that hydrogen bonding alone probably could not account for the stability of DNA. Further confirmation for the importance of stacking interactions came from the demonstration that a number of synthetic polynucleotides could form single-strand helices in aqueous solution (see, for example, Witz and Luzzati, 1965; Holcomb and Tinoco, 1965; Van Holde *et al.*, 1965; Brahms *et al.*, 1966; Leng and Felsenfeld, 1966; Poland *et al.*, 1966).

The purine association, which may be regarded as the prototype for such processes, was studied by Ts'o *et al.* by vapor pressure osmometry and nuclear magnetic resonance spectrometry. It was concluded that the reaction was probably a simple polymerization, with equal equilibrium constants for successive additions of monomers to the stack. More recently, Gill *et al.* (1967) have used heat of dilution measurements to obtain the enthalpy and entropy changes for the reaction at 25°.

Since these association processes are of such fundamental importance to molecular biology, we have begun a series of physicochemical and optical studies of such systems. As a first step, we felt that a critical reexamination of the purine association, using the more powerful method of sedimentation equilibrium, would be worthwhile. Such measurements should provide a more stringent test of the association mechanism, and also allow nonideality of the solutions, which might be expected to be appreciable at high concentrations, to be taken into account. We present here the results of that study.

Experimental Section

Reagents. The purine used in all experiments was A grade, purchased from Calbiochem. According to the manufacturer's specifications, it was chromatographically homogeneous and contained 46.71% nitrogen (theory 46.66%). All solutions were prepared by weight.

Partial Specific Volume. Apparent partial specific volumes were determined at 25.00°, using pycnometers fabricated by fusing 1-mm i.d. capillaries to 25-ml volumetric flasks. Results at purine concentrations of 5.63 and 9.56 g/l. were 0.702 and 0.701 ml/g, respectively. Since the difference is within anticipated experimental uncertainty, the partial specific volume has been taken to be 0.701 ml/g.

Sedimentation Equilibrium. A Beckman-Spinco Model

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