987. *The Chemistry of Vitamin* B_{12}. *Part I. The Valency and Spectrum of the Coenzyme.*

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The magnetic susceptibilities of dicyanocobalamin and 5,6-dimethylbenzimidazolylcobamide coenzyme (DBC) in both red and yellow forms have been measured; the compounds are diamagnetic. Comparison of the absorption spectra of solutions of B_{12} and DBC with the reflection spectra of the crystalline solids shows that the same chromophoric group exists in the solid and in solution, and comparison of the absorption spectra of cyano-, ethynyl-, vinyl-, and methyl-cobalamins and DBC shows that the spectra of B_{12} and DBC are related, these two compounds representing the end-members of a sequence.

The experiments described in this Paper are aimed at (1) determining the valency of cobalt in 5,6-dimethylbenzimidazolylcobamide coenzyme (DBC), (2) establishing that the structures of B_{12} and DBC, which are known for the solids,[1,2] also exist in solution, and (3) finding the relationship, if any, between the spectra of B_{12} and DBC (Fig. 3). The absorption bands of B_{12} at 547 and 522 mμ are termed the α- and the β-band, respectively, and the intense band at 360·5 mμ the γ-band.[3] The measurement of the magnetic susceptibility of DBC has been reported briefly.[3]

Experimental

Reagents.—Samples of cobalamins were given by Dr. E. Lester Smith and Professor D. C. Hodgkin. Other cobalamins were prepared in solution as described below. AnalaR reagents were used wherever possible.

Magnetic Susceptibilities.—Magnetic susceptibilities were measured by the Quincke method, the apparatus being designed to give "null-point" readings and to use small quantities

[1] Hodgkin, *Fortschr. Chem. org. Naturstoffe*, 1958, **15**, 167.
[2] Lenhert and Hodgkin, in "Vitamin B_{12} and Intrinsic Factor," ed. Heinrich, Ferdinand Enke, Stuttgart, 1962, p. 105.
[3] Hill, Pratt, and Williams, *J. Theoret. Biol.*, 1962, **3**, 423.

(2—5 ml.) of dilute solutions (~0·01M). The essential features of the apparatus (Fig. 1) are a glass tube (int. diam. 5 mm.), one end of which A lies between the poles of an electromagnet (field ~10,000 gauss). The end B dips into a small beaker of liquid resting on a table C. The meniscus is brought to the top of tube A by turning the micrometer screw D, and a beam of light so focused that it is reflected from the meniscus on to a photo-tube, the current from

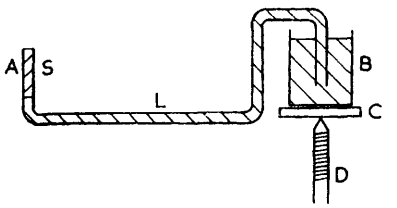

FIG. 1. Part of the apparatus used for magnetic susceptibility measurements.

which is read on a galvanometer. On application of a magnetic field the meniscus moves and can be returned to its original position, as indicated by the galvanometer, by turning the screw D, *i.e.*, by changing the hydrostatic pressure in the glass tube. The magnetic susceptibility is, therefore, related to the reading of the micrometer screw D. The method is equally successful when the tube is completely filled with one liquid or when a small volume of sample S floats on a second inert and immiscible liquid L. Chlorobenzene was used as the inert supporting liquid (s.g. 1·1; b. p. 132°). The apparatus was calibrated with a series of nickel chloride solutions (0·01—0·1N) in 0·01N-hydrochloric acid. The cobalamins were then studied as follows.

2 ml. of a 0·01M-solution of B_{12a} in water were treated with solid potassium cyanide to give a 0·1N-solution. The colour changed from red to violet immediately, but the solution was left for 30 min. to ensure complete formation of the dicyanide. The magnetic susceptibilities of both B_{12a} and dicyanocobalamin were measured.

Solutions of DBC are red above, and yellow below, pH ~3·5; [3,4] the magnetic susceptibilities of both forms were measured. 2 ml. of 0·009M-DBC in water were acidified with 2 drops of concentrated hydrochloric acid to pH <2, giving the yellow form; the solution was then made alkaline (pH >10) with 4 drops of 10M-potassium hydroxide, to give the red form. The total change in volume was less than 5%.

Absorption Spectra.—Visible and ultraviolet spectra were measured with Beckman DK ratio-recording and Unicam S.P. 600 spectrophotometers, in 1- cm. cells.

Photolysis.—Solutions to be photolysed were contained in a 1-cm. cell placed 15—60 cm. from a 230 v 125 w Osram mercury lamp.

Reflection Spectra.—Crystalline B_{12} and DBC were well ground with 5—10 volumes of synthetic zeolite (Linde molecular sieve) as inert diluent. The reflection spectra were measured with a Unicam S.P. 600 spectrophotometer fitted with a reflection-spectrum attachment. Synthetic zeolite was the reference solid.

RESULTS

The magnetic susceptibilities of B_{12a}, dicyanocobalamin, and the red and yellow forms of DBC are given in Table 1; no correction has been made for the change in volume on addition of reagents. All the compounds are diamagnetic.

The reflection spectra of B_{12} and DBC from 300 to 700 mμ are shown in Fig. 2.

TABLE 1.

Magnetic susceptibilities.

Experimental conditions	pH	Unpaired spins/Co (±0·1)
0·01M-B_{12a} in water	~7	0·03
0·01M-B_{12a} in 0·1N-KCN	>10	0·00
0·009M-DBC + HCl (yellow form)	<2	0·03
0·009M-DBC + KOH (red form)	>10	0·03

Effect of Solvent on the Spectra of B_{12} and DBC.—The absorption spectra of B_{12} and DBC in water, dimethyl sulphoxide, and pyridine are shown in Fig. 3, and the positions of the bands

[4] Ladd, Hogenkamp, and Barker, *J. Biol. Chem.*, 1961, **236**, 2114.

TABLE 2.

The effect of solvent on the spectrum of B_{12} and DBC.

(Positions of the main absorption bands in mμ, inflections in parentheses).

B_{12} in H_2O	277	(288)	~305	322	360·5	520	552
Me_2SO	277	(289)	~305	323	360	517	548
pyridine	*	*	309	325	363	516	547
DBC in H_2O	(289)	~305	318	339	375	(~490)	522
Me_2SO	(290)	~304	(~315)	338	375·5	(~490)	519
pyridine ...	*	308	(~315)	(338)	375	485	~510

* Solvent absorbs.

listed in Table 2. The concentrations have been adjusted in order to give similar optical densities of the γ-band in the case of B_{12} and of the 375 mμ band in the case of DBC. Molar extinction coefficients were not determined for the different solutions.

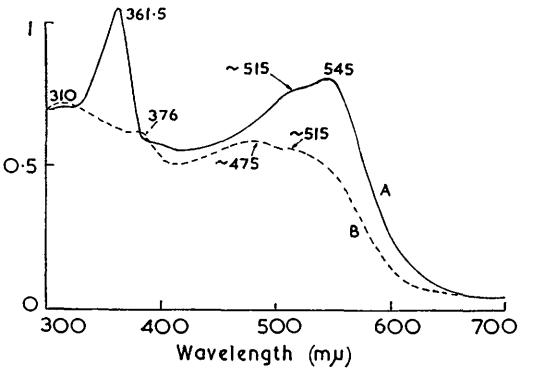

FIG. 2. The reflection spectra of (A) crystalline vitamin B_{12}, and (B) crystalline DBC.

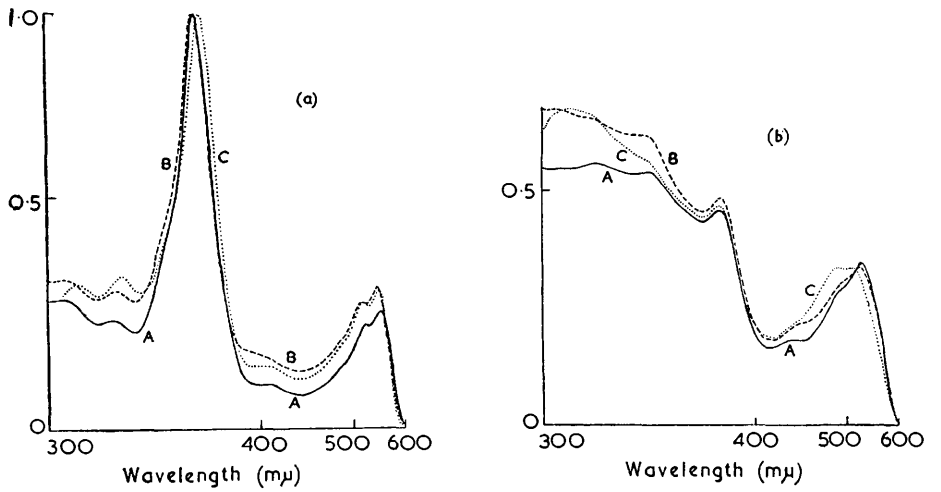

FIG. 3. The absorption spectra in (A) water, (B) dimethyl sulphoxide, and (C) pyridine, of (a) vitamin B_{12}, and (b) DBC.

Determination of Molar Extinction Coefficients.—Using the well-crystallised air-dried B_{12}, in which the asymmetric unit was found to have a molecular weight of 1676,[1] the following values were obtained for the molar extinction coefficients of B_{12} (in acetate buffer pH 3·5 and phosphate buffer pH 10·0): $\varepsilon_\alpha = 0{\cdot}83 \times 10^4$; $\varepsilon_\gamma = 2{\cdot}75 \times 10^4$. George, Irvine, and Glauser [5] report $\varepsilon_\gamma = 2{\cdot}96 \times 104$. The conversion of B_{12} in phosphate buffer at pH 10 into dicyanocobalamin by the addition of solid potassium cyanide gave the following values of the molar

[5] George, Irvine, and Glauser, *Ann. New York Acad. Sci.*, 1960, **88**, 393.

extinction coefficients for the dicyanide: $\varepsilon_\alpha = 0{\cdot}96 \times 10^4$; $\varepsilon_\gamma = 3{\cdot}04 \times 10^4$. The dicyanide has been used as the standard for the determination of the extinction coefficients of other corrinoids, since it has the highest ε_γ of all corrinoids so far studied and most corrinoids can be converted into the dicyanide by treatment with potassium cyanide either in the dark (B_{12}, DBC) or in the presence of light (ethynyl-, vinyl-, and methyl-cobalamin).

TABLE 3.

The spectra of cobalamins with carbon-ligands.

Position of main absorption bands are in mμ. Molar extinction coefficients $\times 10^{-4}$ in parentheses; only the first decimal figure is significant. B_{12} in phosphate buffer pH 10; other cobalamins in water.

Cyano-(B_{12})	304 (0·87)	322 (0·75)	360·5 (2·75)	547 (0·83)
Ethynyl-		~339 (1·21)	367 (1·78)	548 (0·88)
Vinyl-		338 (1·37)	372 (1·31)	522 (0·87)
Methyl-	316 (1·31)	340 (1·40)	374 (1·16)	520 (0·91)
C′$_5$-Deoxyadenosyl-(DBC)	318 (1·32)	339 (1·32)	375 (1·09)	522 (0·82)

Absorption Spectra of Cobalamins with Various Carbon Ligands.—The absorption spectra of B_{12} and DBC in aqueous solution are given in Fig. 3. That of methylcobalamin is similar to DBC, whilst ethynyl- and vinyl-cobalamin are intermediate between cyano- and methyl-cobalamin, see Table 3.

DISCUSSION

Vitamins B_{12} and B_{12a} are diamagnetic (see ref. 6); our results on B_{12a} are in agreement. The violet complex formed on treating B_{12} or B_{12a} with excess of alkaline cyanide has not yet been isolated in crystalline form, but its identification as dicyanocobalamin follows from the facts that its formation from B_{12} requires one cyanide,[5] the benzimidazole is displaced from the cobalt,[7] and the complex acquires a negative charge.[8] As expected, it is diamagnetic (Table 1).

Evidence on the valency of cobalt in the coenzymes, including DBC, is conflicting and has already been summarised.[3] *X*-Ray analysis shows that the structures of B_{12} and DBC differ only in that cyanide has been replaced by deoxyadenosine,[2] which can be considered as a carbanion. This does not establish the valency of the cobalt and the overall charge of DBC since an additional proton or hydroxide in the water of crystallisation would not be detected. B_{12} is, however, uncharged in solution [8] and so is DBC over the pH range 5—10 [4] where its solution spectrum is similar to the reflection spectrum of the solid. The *X*-ray analysis and electrophoretic behaviour alone, therefore, establish the constitution of DBC. Our magnetic measurements confirm it. Other workers have also found DBC to be diamagnetic.[9–11] And though the absence of an electron spin resonance signal [12] cannot be taken as positive evidence for the diamagnetic state of cobalt, it does support the magnetic evidence. Since it is usual to consider ligands as anions, DBC can be regarded as a diamagnetic complex of trivalent cobalt with a carbanion as ligand.

DBC and methylcobalamin can be synthesised by analogous reactions, and they show similar spectra and chemical properties; [13] it is, therefore, safe to conclude that methylcobalamin is also a diamagnetic cobaltic complex. Similar arguments apply to vinyl- and ethynyl-cobalamin.* *X*-Ray analysis has not excluded the possibility that the ring system of DBC may differ from that of B_{12} by the presence of additional hydrogen atoms.[2] It

* *Added in proof.* Electron spin resonance and electrophoretic studies have now shown that ethynyl- and vinyl-cobalamin are both cobaltic complexes containing a carbanion.

[6] Smith, "Vitamin B_{12}," Methuen, London, 1960.
[7] Cooley, Ellis, Petrow, Beaven, Holiday, and Johnson, *J. Pharm. Pharmacol.*, 1951, **3**, 271.
[8] Smith, Ball, and Ireland, *Biochem. J.*, 1952, **52**, 395.
[9] Ehrenburg and Hedbom, personal communication.
[10] Pawełkiewicz, personal communication.
[11] Cunningham, unpublished work quoted in ref. 12.
[12] Hogenkamp, Barker, and Mason, *Arch. Biochem. Biophys.*, 1963, **100**, 353.
[13] Johnson, Mervyn, Shaw, and Smith, *J.*, 1963, 4146.

has recently been shown, however, that methylcobalamin is produced by the reaction of methyl iodide with B_{12s} [formal oxidation state Co(I)] when the latter is prepared by the controlled-potential reduction of B_{12a} and no additional electrons are available to reduce the ring:[14] $Co(I) + CH_3I = Co(III)CH_3^- + I^-$.

There exists, therefore, a series of cobaltic cobalamins which can be used to study the effect on the spectrum of varying virtually only one parameter, namely the electronegativity of the carbon atom attached to the obalt, which falls in the order: cyanide > ethynyl > vinyl > methyl $\simeq$ C_5'-deoxyadenosyl.

The solvent may affect the spectrum of corrinoids in at least two ways, either by co-ordination to the metal or by interaction with the conjugated system, as is known to occur in the case of aromatic hydrocarbons and heterocycles, cyanine dyes, and magnesium chlorophyll (see, for example, refs. 15—18). Fig. 3 and Table 2 show that the spectra of B_{12} and DBC do depend on the nature of the solvent, the effect being greater in the latter. But the range of solvents studied is too small to permit any conclusions as to the nature of the solvent effect.

The reflection spectrum of crystalline B_{12} and its absorption spectrum in solution are very similar both in general shape and in the position of the bands. The reflection spectrum of DBC resembles the absorption spectrum in pyridine rather than that in water, in showing increased absorption around 310 and 480 mμ; but the reflection spectrum of the solid, and all the absorption spectra in solution, show the characteristic band at ~375 mμ. The differences between the spectra of DBC as solid and in aqueous solution, therefore, lie within the range which can be ascribed to a solvent effect. It can be concluded that neither in B_{12} nor in DBC is there any major difference in the structure of the conjugated chromophore (the corrin ring) between the solid, where the structures have been established by *X*-ray analysis,[1,2] and the dissolved state. The reflection and absorption spectra also show that the same chromophore can give rise to such markedly different spectra as those of B_{12} and DBC.

The spectra of cobalamins containing a carbon ligand are summarised in Table 3. Johnson *et al.*,[13] have presented some data on the spectra of alkylcobalamins, and our results are in general agreement, though their molar extinction coefficients are in many cases about 15% lower than ours, presumably owing to the use of a different basis for calculation. The data in Table 3 show that as the ligand is varied in the order cyanide, ethynyl, vinyl, methyl, which corresponds to a decreasing electronegativity of the carbon atom, there is a progressive change in the spectrum over the region 300—400 mμ; the γ-band moves to longer wavelength and falls in intensity, while other bands rise in intensity at shorter wavelengths. B_{12} and DBC, therefore, represent the end-members of a series of corrinoids in which the spectrum is gradually changing, and the low band at 375 mμ in DBC can be related to the intense band at 360·5 mμ in B_{12}. There are a number of bands in the 450—550 mμ region in DBC, and we have not yet been able to establish whether the band at 522 mμ in DBC corresponds to either the α- or the β-band in B_{12}.

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South Parks Road, Oxford. [*Received, December 11th*, 1963.]

[14] Hill, Pratt and Williams, *Chem. and Ind.*, 1964, 197.
[15] Roe, *Spectrochim. Acta*, 1957, **11**, 515.
[16] Badger and Pearce, *Spectrochim. Acta*, 1951, **4**, 280.
[17] McRae, *Spectrochim. Acta*, 1958, **12**, 192.
[18] Rabinowitch, " Photosynthesis," Interscience, New York, 1951, Vol. II, 1, pp. 637—649.