## 515. *The Chemistry of Vitamin* $B_{12}$. *Part III.* The Proton Magnetic Resonance Spectra of Some Cobalamins*

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The proton magnetic resonance spectra of some cobalamins have been obtained, using trifluoroacetic acid and deuterated dimethyl sulphoxide as solvents. The chemical shifts of the 5,6-dimethylbenziminazole hydrogens, in the cobalamins, are dependent on whether the benziminazole is protonated, co-ordinated, or nonco-ordinated and unprotonated. The chemical shift of the methene hydrogen at C-10 depends on the ligand attached to the cobalt. The possibility of protonation of the corrin ring in acidic solutions is discussed.

There has been considerable interest in the proton magnetic resonance (p.m.r.) spectra of the porphyrins [1] and other macrocyclic conjugated polyenes.[2] It was found, for example, that hydrogens bound directly to, and on the outside of, the conjugated ring showed a

* Part II, J. M. Pratt, *J.*, 1964, 5154.

[1] R. J. Abraham, A. H. Jackson, and G. W. Kenner, *J.*, 1961, 3468.

[2] L. M. Jackman, F. Sondheimer, Y. Amiel, D. A. Ben-Efraim, Y. Gaoni, R. Wolovsky, and A. A. Bothner-By, *J. Amer. Chem. Soc.*, 1962, **84**, 4307.

large chemical shift to low field (−1 to +1 τ). If the hydrogen was on the inside of the ring, then it had a chemical shift to a correspondingly high field (10 to 15 τ). This behaviour has been explained [3] by postulating a ring current, induced by the applied field, which gives rise to a magnetic field deshielding those hydrogens outside the ring, as in benzene, and shielding those inside the ring. Indeed, the presence of such a ring current has been proposed [2] as a criterion of aromaticity in macrocyclic polyenes. The cobalamins

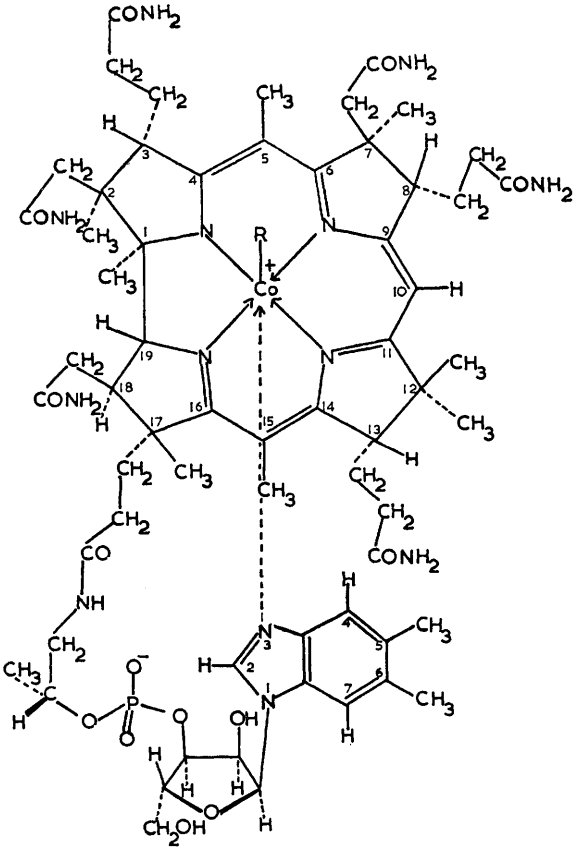

FIGURE 1

lack such a cyclic conjugated system, unless conjugation is continued through the metal, and so the methene hydrogen at C-10 (Figure 1) should not show so large a shift to low field.

Although the structure of the cobalamins in the solid state has been determined,[4] their properties in solution, such as the occurrence of red and yellow species, are less well understood. In particular, we wish to study the effect of the axial ligands on the electronic structure and conformation of the ring. The interaction between axial ligands and conjugated square–planar ligands has been termed [5] perpendicular conjugation. A study of the p.m.r. of the cobalamins was undertaken to observe the effect of the axial ligands on the chemical shift of the methene hydrogen, and to see if there was any striking difference between the red and the yellow forms of the cobalamins.

[3] R. J. Abraham, *Mol. Phys.*, 1961, **4**, 145.

[4] See J. G. White, *Proc. Roy. Soc.*, 1962, *A*, **266**, 440; D. Crowfoot Hodgkin, J. Lindsey, M. MacKay, and K. N. Trueblood, *ibid.*, p. 475; D. Crowfoot Hodgkin, J. Lindsey, R. A. Sparkes, K. N. Trueblood, and J. G. White, *ibid.*, p. 494.

[5] J. A. Elvidge and A. B. P. Lever, *J.*, 1961, 1257; R. J. P. Williams, *Chem. Rev.*, 1956, **56**, 299.

## Experimental

We are very grateful to Dr. E. Lester Smith, F.R.S., and Glaxo Laboratories, for the gift of the cobalamins,[6] and to Dr. Barbara Offenhartz for a sample of 5,6-dimethyl-1-α-D-ribofuranosylbenziminazole (ribazole).

*Measurements.*—The spectra were obtained using a Perkin-Elmer model R 10 Spectrometer at 60 Mc./sec., and 35°. The resonances were not easily saturated, which may be due to the temperature-independent paramagnetism of the cobalt(III). The normal operating conditions were as follows: R.F. level, $1{\cdot}6 \times 10^3$ microvolts, sensitivity 8, and a sweep rate of 48 c./sec. per min. Tetramethylsilane (TMS) was used as an internal standard and the spectra were presented on precalibrated charts in τ values.

*Solvents.*—B.D.H. trifluoroacetic acid (TFA) was the preferred solvent since the cobalamins were moderately soluble (0·025—0·05M) without giving very viscous solutions. Any water present exchanged rapidly with the proton of the solvent giving a combined resonance at −1 τ. The amide hydrogens, of which there are 13, exchange slowly and showed only a broad resonance, which did not obscure the other resonances, in the region 0—4 τ. The solutions in Merck deuterated dimethyl sulphoxide (d-DMSO) were much more viscous, and the amide hydrogens gave prominent resonances which tended to obscure the interesting region of the spectrum, 0—4 τ.

In TFA, the presence of the broad underlying absorption due to the amide hydrogens made electronic integration of the spectra difficult. Therefore, the integration was performed by measuring the areas, using the resonance at 2·4 τ, due to the hydrogens at C-4 and C-7 of the benziminazole, as an internal standard. In the cobinamides, which lack the nucleotide, the single resonance at 3·45 τ in TFA solutions, was assumed to be due to one hydrogen since such an assumption gave a value of 60 ± 5 hydrogens in the region from 6—9 τ, which is as expected.

*Stability of the Compounds in Trifluoroacetic Acid.*—The absorption spectra of the cobalamins in trifluoroacetic acid were identical with those of the corresponding cobinamides indicating the removal of the benziminazole from the co-ordination sphere of the cobalt. An absorption band at 285 mμ was present, due to the protonated benziminazole.

The reaction with TFA was examined by the following method. Samples of concentrated solutions (0·025M) of the cobalamins in TFA were taken at various times and diluted with water. The pH of the solutions was adjusted to a value higher than the p$K$ of the change from cobalamin to cobalamin with the benziminazole free and protonated, so that the cobalamin, if present, would be reformed. Examination of the absorption spectra revealed that after 1 hr., the normal duration of a p.m.r. experiment, negligible change had occurred. However, after 24 hr. the benziminazole side-chain had been removed since the spectra were identical to the corresponding cobinamides, rather than the cobalamins. P.m.r. examination of solutions of the cobalamins in TFA showed little alteration after 24 hr., and none in the region 0—4 τ. Therefore p.m.r. spectroscopy cannot readily distinguish a cobalamin from the corresponding cobinamide plus nucleotide in TFA solutions.

Although the spectra obtained were not entirely satisfactory they are highly reproducible and of as good resolution as those obtained by others working with these compounds.[7] The high molecular weight, low solubility, high viscosity, and correspondingly slow rotation and low correlation time, are expected to affect the spectra adversely. Even so, the main features of the spectra were easily recognised, though, of course, no fine structure due to spin–spin interaction could be identified.

## Results and Discussion

*Proton Magnetic Resonance Spectra of the Benziminazoles.*—The chemical shifts of the hydrogens present in some 5,6-dimethylbenziminazoles are given in Table 1. The resonances of all the hydrogens are solvent-dependent, being shifted to low field in the protonated form. This is particularly marked for the 2-proton. Similar shifts to low field on protonation have been observed with other heterocyclic compounds such as pyridine [8] and the porphyrins.[9]

[6] A. W. Johnson, L. Mervyn, N. Shaw, and E. Lester Smith, *J.*, 1963, 4146.
[7] R. Bonnett, Discussion on Corrin, Royal Society, London, June 1964.
[8] I. C. Smith and W. G. Schneider, *Canad. J. Chem.*, 1961, **39**, 1158.
[9] J. Ellis, A. H. Jackson, G. W. Kenner, and J. Lee, *Tetrahedron Letters*, 1961, **23**.

TABLE 1

Proton magnetic resonance spectra of some 5,6-dimethylbenziminazoles ($\tau$ values)

| Compound | 2-H | 4-H and 7-H | 5,6-Dimethyl | Others | Solvent |
|---|---|---|---|---|---|
| 5,6-Dimethylbenziminazole ..... | 1·0 | 2·2 | 7·35 | | TFA |
| 1,5,6-Trimethylbenziminazole ... | 1·21 | 2·4 | 7·45 | 1-$CH_3$ (5·8) | TFA |
| Ribazole ..... | 0·85 | 2·2 | 7·4 | CH(4) 4·9—$CH_2$(1) 6·0 | TFA |
| 1,5,6-Trimethylbenziminazole ... | 2·0 | 2·59, 2·70 | 7·68 | 1-$CH_3$ (6·22) | d-DMSO |

It is already known [10] that, in water, the benziminazole group of the cobalamins is removed from the co-ordination sphere of the cobalt by acid. Thus, the electronic spectra of the resultant solutions, at wavelengths greater than 300 mμ, are identical with the spectra of the corresponding cobinamides, which lack the nucleotide. The removal of the benziminazole from the co-ordination sphere of the cobalt in acid is also indicated [11] in the electronic absorption spectra by the movement of a band due to the 5,6-dimethylbenziminazole from 288 mμ, in the co-ordinated or unprotonated forms, to 284 mμ, in the protonated form. P.m.r. spectroscopy provides a way of following all three changes in the state of the benziminazole, whereas the electronic absorption does not always distinguish between the bound and free but unprotonated states. Both the electronic absorption spectra and the circular dichroism [12] show that all cobalamins in TFA have lost the benziminazole from the co-ordination sphere of the cobalt, and the former technique shows that, as expected, the benziminazole is protonated. This is also shown by the p.m.r. spectra since the 2-proton of the 5,6-dimethylbenziminazole has a chemical shift of 0·85 $\tau$, as shown in Table 2 (cf. Table 1). Methylcobalamin in d-DMSO, which has the benziminazole bound

TABLE 2

Proton magnetic resonance spectra of the cobalamins in TFA; chemical shifts ($\tau$ values) and estimated number of protons

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ribazole | 0·85 (1·0) | 2·2 (2·0) | | | 4·9—6·0 (6·0) | 7·4 (6·0) | |
| Cyanocobalamin | 0·79 (1·1) | 2·35 (2·0) | 3·2 | 3·45 (2·0) | 4·67—5·7 (~9) | 6·37, 7·47, 8·0—8·55 (~70) | |
| Cyanocobinamide | | | 3·45 (1·0) | | 4·55—5·7 (~4) | 6·37, 7·5, 8·1—8·6 (~60) | |
| " Chlorinated " cyanocobalamin | 0·8 (1·0) | 2·35 (2·0) | 3·3 (1·1) | | 4·2—5·7 (~10) | 6·35, 7·45, 8·0—8·5 (~60) | |
| " Aquocobalamin " | 0·8 (0·9) | 2·38 (2·0) | 3·2 | 3·29 (1·8) | 4·2—5·75 (~9) | 6·25, 7·48, 8·0—8·5 (~60) | |
| Methylcobalamin | 0·8 (1·0) | 2·35 (2·0) | 2·95 (1·1) | 3·25 (1·0) | 4·7—5·8 (~12) | 6·38, 7·3, 7·5, 8·0—8·8 (~65) | 9·96 (2·9) |
| Vinylcobalamin | 0·8 (1·1) | 2·35 (2·0) | 2·75 (0·9) | 3·25 (0·8) | 4·75—5·73 (~11) | 6·3, 7·55, 8·0—9·0 (~70) | 10·2 (1·0) |

to the cobalt, as shown by the absorption spectrum, has a broad band at 2—4 $\tau$, with the benziminazole resonances indistinguishable from the thirteen amide resonances. On addition of sodium cyanide, to give cyanomethylcobalamin, the benziminazole is replaced by cyanide, and is therefore free. It is not protonated, as must be the case in this aprotic solvent, but this is confirmed by the chemical shift of the 2-proton which has a value of 1·7 $\tau$. The two hydrogens at C-4 and C-7 in the benziminazole give a prominent resonance at 2·6 $\tau$ (cf. Table 1). The increase in sharpness is probably due to the release of the side-chain from the co-ordination sphere and the relevant hydrogens probably have a much longer correlation time. The resonance due to the 5,6-dimethyl group at 7·45 $\tau$ also becomes much more prominent.

*The Valence of the Cobalt.*—The p.m.r. spectra in TFA are strikingly similar in view of

[10] P. George, D. H. Irvine, and S. C. Glauser, *Ann. New York, Acad. Sci.*, 1960, **88**, 393.
[11] G. H. Beavan and E. A. Johnson, *Nature*, 1955, **176**, 1264.
[12] H. A. O. Hill, W. R. Jackson, and R. J. P. Williams, to be published.

the marked difference in colour of the solutions, *e.g.*, methylcobalamin gives a yellow solution whereas the cyanocobalamin solution is red, yet the p.m.r. spectra are quite similar (Figure 2). Yellow cobalamins have spectra similar to that of $B_{12r}$, which contains [13] cobalt(II), and have also frequently been presumed to contain low-spin, $d^7$, cobalt(II). The observation of the nuclear magnetic resonance spectra in the usual proton range dismisses the possibility of the presence of any unpaired electrons in the ground state of the molecule.

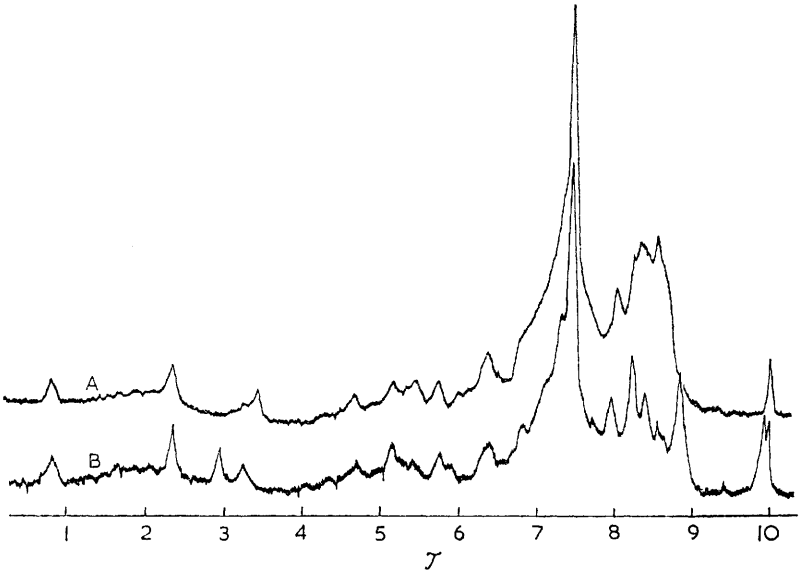


FIGURE 2. Proton magnetic resonance spectra of (A) cyanocobalmin (0·025M), and (B) methylcobalamin (0·025M), in TFA

*The Methene Hydrogen.*—An estimated p.m.r. spectrum based on literature values [14] for all the individual types of hydrogen in the cobalamins, predicts the overall shape observed. Thus, the methyl groups attached to saturated carbons (21 hydrogens), and those methylene hydrogens which are not α to amide groups (8 hydrogens), will have resonances in the region 8—9 τ. Methyl groups attached to unsaturated carbons (12 hydrogens), methylene groups α to amide groups (14 hydrogens), together with the four tertiary hydrogens at C-3, C-8, C-13, and C-18, will have chemical shifts in the region 7—8 τ. (The 5,6-dimethyl group of the benziminazole gives a strikingly prominent resonance in acid solutions at 7·45 τ, and this resonance is, of course, absent in the cobinamide spectra.) Apart from the methene hydrogen, in cyanocobinamide four hydrogens, that at C-19 and those of the aminopropanol should have a resonance in the low-field region, 4—7 τ in TFA. It is noted that there is absorption in this region corresponding to 4·0 ± 0·5 hydrogens. Few of the literature values were from results obtained using TFA as solvent. It would be plausible to assume that only those hydrogens which interact with the solvent by hydrogen-bonding, will be shifted to lower field. It is still comparatively easy to assign the methene hydrogen at C-10 in d-DMSO solution, because of the information available [15] from measurements made on synthetic corrin compounds, which have been prepared by Eschenmoser. The methene hydrogens in a dicyanocobalt(III) corrin, lacking methyl groups at C-5 and C-15, have a chemical shift of 4·5 τ in deuterated acetone solution. Dicyanocobinamide in d-DMSO shows a single proton resonance at 4·35 τ.

[13] H. P. C. Hogenkamp, H. A. Barker, and H. S. Mason, *Arch. Biochem. Biophys.*, 1963, **100**, 353; H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Chem. and Ind.*, 1964, 197.

[14] (*a*) N. S. Bhacca, L. F. Johnson, and J. M. Shoolery, "High Resolution N.M.R. Spectra Catalog," Varian Associates, Palo Alto, California, 1962; (*b*) K. Nukada, O. Tamamoto, T. Suzuki, M. Takeuchi, and M. Ohnishi, *Analyt. Chem.*, 1963, **35**, 1892.

[15] A. Eschenmoser, Discussion on Corrin, Royal Society, London, June 1964.

This is in the polyene region, *e.g.*, comparable hydrogens in polymethine dyes absorb [16] in this region, and thus, as expected, there is no ring current in the corrin system. Surprisingly, dicyanocobalamin in d-DMSO has two resonances in this region, at 3·8 and 4·3 τ. The latter is due to the methene hydrogen but the former cannot be assigned with confidence though it is probably due to the nucleotide. Similarly, cyanomethylcobalamin has two resonances, at 3·8 and 4·5 τ. The shift of the latter to higher field than in the dicyanocobalamin could be caused by increased charge on C-10, and so may constitute evidence for perpendicular conjugation. It is interesting to note that Eschenmoser [15] observed a shift of the methene hydrogens in a synthetic corrin from 3·9 τ, when the axial ligands were both water, to 4·5 τ in the dicyano-form.

In TFA, the situation is rather complicated because there are two resonances in the region 2·7—3·5 τ, as shown in Figure 2 and Table 2. One of these, which is broad, is present in methylcobalamin, " aquocobalamin " (added as such to TFA, but presumably the water is replaced by TFA), and cyanocobalamin, at 3·2—3·3 τ. The other varies from 3·45 τ in cyanocobalamin to 2·75 τ in vinylcobalamin. The resonance which varies with the axial ligand we assign to the methene hydrogen and note that it is absent in the chlorinated cyanocobalamin, which is consistent with the assumption [17] that chlorination and other electrophilic substitutions [18] take place at C-10. The p.m.r. spectrum of cyanocobinamide has a single resonance in this region at 3·45 τ. Bonnett *et al.*[17] found that the hexacarboxylic acid (8-aminocobyrinic acid *c*-lactam) [19] has a resonance at ~3·5 τ, which is assigned to the 10-proton. It is absent in deuterotrifluoroacetic acid. Since the 3·2—3·3 τ resonance is absent in both these compounds, it must be associated with the nucleotide, presumably analogous to the 3·8 τ resonance in the d-DMSO solutions mentioned above. Since the synthetic nucleoside showed no resonance in this region it would be of great interest to have a sample of the nucleotide. It is possible that the rest of the side-chain, and the phosphate group in particular, affects the shielding of the ribose hydrogens, shifting one of them to low field.

Why does the methene hydrogen have a chemical shift at lower field in methylcobalamin than in cyanocobalamin in TFA? From the known [20] donor properties of the methyl group it might be expected that a shift would have taken place to higher field. However, it is also known that proton chemical shifts are very sensitive to the presence of paramagnetic states, and perhaps in the methylcobalamin, there is present a low-lying paramagnetic state. Hydrogen-bonding with the corrin ring, which presumably has a higher electron-density in methylcobalamin then in cyanocobalamin, could also account for the shift. Another possibility is that the change [21] in shape of the corrin ring with change in axial ligand will give rise to a change in the state of hybridisation at C-10.

These observations were made to test a working hypothesis [22] that gross changes in the visible absorption spectra of the cobalamins from the usual red to the yellow of, for example, the coenzyme in dilute acid or of $B_{12}$ in concentrated sulphuric acid involved protonation of the ring. The fact that the p.m.r. absorption of the methene hydrogen in methylcobalamin does not change significantly from the red to the yellow forms excludes the possibility that C-10 is protonated in this compound. The present work is not able to decide whether protonation at C-5 or C-15 occurs, and the yellow solutions in concentrated sulphuric acid have not been studied. However, the p.m.r. spectrum of the yellow nirrin complex [23] excludes protonation at C-5, C-10, or C-15 as a necessary condition for colour change. One

[16] S. Dahne and J. Ranft, *Z. phys. Chem.* (*Leipzig*), 1963, **224**, 65.
[17] R. Bonnett, J. R. Cannon, V. M. Clark, A. W. Johnson, L. F. J. Parker, E. Lester Smith, and A. Todd, *J.*, 1957, 1158.
[18] Wagner, Discussion on Corrin, Royal Society, London, June 1964.
[19] See R. Bonnett, *Chem. Rev.*, 1963, **63**, 573, for a comprehensive review.
[20] J. Chatt and R. G. Hayter, *J.*, 1961, 772.
[21] D. Crowfoot Hodgkin, personal communication.
[22] J. A. Hill, J. M. Pratt, and R. J. P. Williams, *J. Theoret. Biol.*, 1962, **3**, 423.
[23] A. Eschenmoser, E. Bertele, H. Boos, J. D. Dunitz, F. Elsinger, I. Felner, H. P. Gribi, H. Gschwend, E. F. Meyer, M. Pesaro, and R. Scheffold, *Angew. Chem.*, 1964, **76**, 393.

other possibility is that the marked spectral changes are caused by changes in the conformation of the corrin ring which may be connected with the effective charge on the metal.

*Axial-ligand Resonances.*—The three hydrogens of the methyl group attached to the cobalt in methylcobalamin have a chemical shift, in TFA solution, of 9·96 $\tau$. If the shift is related [24] to the electronegativity of the metal atom, then the electronegativiy of cobalt in this compound is similar to that of silicon. This is consistent with the estimation of the electronegativity of cobalt given by Allred.[25]

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South Parks Road, Oxford. [*Received, July 29th,* 1964.]

[24] A. L. Allred and E. G. Rochow, *J. Inorg. Nuclear Chem.*, 1958, **5**, 269.
[25] A. L. Allred, *J. Inorg. Nuclear Chem.*, 1961, **17**, 215.