Carbon-13 Nuclear Magnetic Resonance Studies of Adenosylcobalamin and Alkylcorrinoids, Selectively Enriched with Carbon-13†

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ABSTRACT: The carbon-13 nuclear magnetic resonance spectra of a series of alkylcorrinoids, selectively enriched with ^{13}C in the alkyl ligand, were recorded at 25.2 MHz and 25°. The nature of the axial ligands markedly affects the chemical shift of the labeled alkyl moiety (trans effect) as well as the ^{13}C resonances of selected carbon atoms of the corrin ring (cis effect). Although a number of factors appear to influence the trans effect on the chemical shift of the alkyl ligand (important among them being electric field effects), the cis effect appears to be dominated by changes in charge density (at the methine bridge carbon atoms, C-5, C-10, C-15) and by steric effects (at the methyl groups at C-1, C-5, and C-15) accompanying axial ligation. Spin–lattice relaxation times of several organocorrinoids, selectively labeled with ^{13}C in the ligands attached to cobalt, were also measured. The T_1 values of the methylene carbons of [5′-^{13}C]adenosylcobalamin and [2-^{13}C]carboxymethylcobalamin are very similar to that of the methine bridge carbon atom C-10 of the corrin ring, indicating that rotation about the carbon–cobalt bond of these two corrinoids is severely restricted. On the other hand, internal rotation about the carbon–cobalt bond of methylcobalamin is rapid.

In earlier publications we have demonstrated that the carbon-13 chemical shifts of alkylcorrinoids selectively enriched with carbon-13 in the alkyl moiety are extremely sensitive to the nature of the trans axial ligand (Y in Figure 1, Needham et al., 1973; Walker et al., 1974a). Preparatory to the study of the interaction between adenosylcobalamin and several enzymes, we have extended that work to an investigation of the chemical shift and spin–lattice relaxation time parameters of a wider variety of carbon-13 enriched corrinoids, including chemical shift data for certain of the natural abundance carbon-13 resonances of the corrin ring. The results of the work of other investigators suggested that these additional ^{13}C nuclear magnetic resonance (NMR) parameters should also be a sensitive function of the state of axial ligation of the cobalt(III) center.

Thus, axial ligands have a pronounced effect on the physical and chemical properties of the equatorial corrin ring (the cis effect) (Pratt, 1972), e.g., strongly influencing not only the proton shift of C-10–H (Figure 1) but also the sensitivity of C-10 to electrophilic attack. Since the carbon-13 methine resonances of the macrocyclic ring (C-5, C-10, C-15; Figure 1) occur at intermediate field, well-resolved from the resonances of the other unsaturated carbon resonances (Doddrell and Allerhand, 1971; Strouse et al., 1972; Matwiyoff and Burnham, 1973; Scott et al., 1974), the chemical shifts of these methine bridge carbon atoms should also provide a sensitive probe for cis effects in the corrinoids. Similarly, the carbon-13 resonances of the C-1 methyl group attached to ring A as well as those attached to C-5 and C-15 are readily assigned and, being adjacent to the sterically crowded lower coordination site, should exhibit chemical shifts which are dependent on the nature of the axial ligand.

With respect to the use of carbon-13 spin–lattice relaxation times (T_1) to provide information about the structure and dynamics of large molecules in solution, there exists already a rich literature (Oldfield and Allerhand, 1975). For example, T_1 measurements of ribonuclease A, gramicidin S, oxytocin, lysine-vasopressin, and thyrotropin releasing factor have provided information about the rates of reorientation of these molecules in solution as well as about the rates of internal rotation of the side chains (Allerhand et al., 1971a; Allerhand and Komoroski, 1973; Deslauriers et al., 1974a,b). More recently Villafranca and Viola (1974) estimated the T_1 values of all the carbons of methyl α-D-glucopyranoside in the presence of Mn(II) concanavalin A and were able to determine the preferred orientation of the carbohydrate when bound to the protein.

As background information for our ^{13}C NMR study of the interaction between adenosylcobalamin and several enzymes which use this corrinoid as a coenzyme, ^{13}C chemical shift and T_1 data are required as a function of the state of axial ligation of the organocorrinoids, and of the chemical nature and conformation of the substituents of the corrin ring. To that end we have synthesized [5′-^{13}C]adenosylcobalamin and several other alkylcorrinoids, selectively enriched with carbon-13 (Walker et al., 1974b; Needham et al., 1973). This report presents ^{13}C NMR chemical shift and T_1 measurements of [5′-^{13}C]adenosylcobalamin and several alkylcorrinoids enriched with carbon-13 in the alkyl ligand. The effect of modifications on the periphery of the corrin ring on the chemical shift and relaxation parameters of the alkyl ligands, as well as the effect of the alkyl ligands on the chemical shifts of the resonances of the methine bridge atoms and the methyl groups attached to ring A,

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have been investigated. In the course of these ^{13}C NMR studies, we have also determined a number of pK values for the protonation of the alkylcorrinoids and they are reported, and discussed briefly, here.

Experimental Section

Materials

Cyanocobalamin and hydroxycobalamin hydrochloride were purchased from Sigma Chemical Co. Borane–tetrahydrofuran complex was obtained from Aldrich Chemical Co. [^{13}C]Methyl iodide and [1-^{13}C]- and [2-^{13}C]bromoacetic acid were gifts from Dr. D. G. Ott of LASL. [1-^{13}C]-2-Bromoethanol and [2-^{13}C]-2-bromoethanol were prepared from the [1-^{13}C]- and [2-^{13}C]bromoacetic acids by reduction with diborane in tetrahydrofuran. [5′-^{13}C]Adenosylcobalamin was prepared by the procedure of Walker et al. (1974b). Methylaquocobinamide, methylpyridinecobinamide, methylcyanocobinamide, and dicyanocobinamide were prepared as described before (Needham et al., 1973).

Methods

The purity of the corrinoids was established by spectral analysis using a Cary Model 15 spectrophotometer and by descending paper chromotography in three solvent systems (Dolphin, 1971). Pulse ^{13}C (25.2 MHz) nuclear magnetic resonance spectra were obtained at 25° using a Varian XL-100-15 spectrometer locked to the resonance (15.4 MHz) of internal D_2O and interfaced to a Data General Corp. Nova 1210 computer. Peak positions were determined by computer examination of the final Fourier transformed spectrum. Chemical shifts were measured with respect to a tetramethylsilane external standard. Spin–lattice relaxation times were determined from PRFT spectra obtained by means of a $180° - t - 90°$ pulse sequence, with a relaxation time of $5T_1$ between sequences (Allerhand et al., 1971b). The T_1 values were calculated from a nonlinear least-squares fit of the intensities and the reported T_1 values have standard deviations of less than 10%. Since the measurements were made with D_2O solutions, the following relationships were used to calculate the pK_a values: pD = pH (meter reading) + 0.40; for the protonation of the 5,6-dimethylbenzimidazole ligand: $pK_D - pK_H = 0.243 + 0.0417pK_H$; for the ionization of the carboxyl moieties: $pK_D - pK_H = 0.49$ (Li et al., 1961).

Syntheses of Analogs of Cyanocobalamin. Cyano-13-epicobalamin was prepared by the method of Bonnett et al. (1971), modified as follows. After incubating 1 g of dry cyanocobalamin in 20 ml of trifluoroacetic acid at room temperature for 2 hr, the reaction mixture was evaporated to dryness; the residue was triturated with ether and dried again. The orange residue was dissolved in water, acidified to pH 1.5 with HCl, and passed through a 3 × 45 cm column of SP-Sephadex to remove cobinamides and 13-epicobinamides which are retained on the column. The pass-through containing cyanocobalamin and cyano-13-epicobalamin was adjusted to pH 10 with NaOH and passed through a 3.5 × 2.8 cm column of AG 1-X2 (200–400 mesh), acetate form, to remove carboxylic acid derivatives. The pass-through was concentrated to approximately 50 ml, reduced with sodium borohydride, and converted to the methylcorrinoids as described by Dolphin (1971). The methylcorrinoids were desalted by phenol extraction (Hogenkamp and Pailes, 1968), mixed with several volumes of 0.1 M sodium citrate–phosphate buffer (pH 2.5), and applied to a 5 × 77 cm column of SP-Sephadex. The column was then eluted with the same buffer to yield homogeneous methyl-13-epicobalamin and methylcobalamin. Photolysis of these two corrinoids in the presence of NaCN yields the dicyanocorrinoids and after acidification cyano-13-epicobalamin and cyanocobalamin which were crystallized from aqueous acetone.

Cyanocobalamin lactam was prepared by the procedure of Bonnett et al. (1957). The desalted solution was acidified to pH 3 and applied to a 3 × 45 cm column of SP-Sephadex which was then eluted with water. The water wash was adjusted to pH 10 with NaOH and applied to a 3.5 × 28 cm column of AG 1-X2 (200–400 mesh) acetate form and the column eluted with water. The water wash was concentrated and cyanocobalamin lactam was crystallized from aqueous acetone.

Cyanocobalamin lactone was also synthesized by the procedure of Bonnett et al. (1957). The reaction mixture was desalted by phenol extraction and the aqueous solution was acidified to pH 3; cobinamides were removed on SP-Sephadex as described above. The solution was then adjusted to pH 10 with NaOH and applied to a 5 × 60 cm column of AG 1-X2 (200–400 mesh) acetate form. The column was washed with water and eluted with 0.04 N sodium acetate buffer (pH 4.67). The desired lactone eluted as a prominent red peak well separated from other corrinoids; it was desalted and crystallized from water–acetone–ether.

Cyanocobalamin-*b*, -*d*-, and -*e*-carboxylic acids were prepared as described before (Yamada and Hogenkamp, 1972). Cobinamides were removed on SP-Sephadex as described above. The water wash was then adjusted to pH 10 with NaOH and applied to a 5 × 60 cm column of AG 1-X2 (200–400 mesh) acetate form which was washed with water. Elution with 0.04 N sodium acetate buffer (pH 4.67) gave two well-resolved peaks. The first peak contained cyanocobalamin-*b*-carboxylic acid, which was desalted and crystallized from aqueous acetone. The second peak contained both cyanocobalamin-*d*- and -*e*-carboxylic acids, which were desalted; the aqueous solution was then adjusted to pH 10 and applied to a 5 × 175 cm column of the same resin and eluted with the same buffer. After eluting with approximately 18 l. of buffer, both *d*- and *e*-carboxylic acids emerged in well-separated peaks. Both acids were desalted and crystallized from aqueous acetone.

Cyanoaquo-3,5,6-trimethylbenzimidazolylcobamide was synthesized by the method of Friedrich and Bernhauer (1956). After completion of the reaction, the reaction mixture was acidified to pH 3 with HCl, desalted, concentrated, and applied to a 2.5 × 40 cm column of SP-Sephadex. The column was eluted with a 0.0–0.2 M sodium chloride gradient in 0.025 M sodium phosphate buffer (pH 8.0). The desired cobamide which eluted in a prominent yellow peak was desalted and isolated as a glass.

Synthesis of [^{13}C]Methylcorrinoids. These methylcorrinoids were prepared by published procedures (Hogenkamp et al., 1965; Hogenkamp and Pailes, 1968); [^{13}C]methylcobalamin lactam was crystallized from aqueous acetone; [^{13}C]methylcobalamin lactone, from water–acetone–ether. The [^{13}C]methylcobalamincarboxylic acids and [^{13}C]methyl-3,5,6-trimethylbenzimidazolylcobinamide were isolated as glasses.

Synthesis of [1-^{13}C]- and [2-^{13}C]Carboxymethylcorrinoids. These corrinoids were prepared as described before (Hogenkamp et al., 1965). With exception of the carboxymethyl derivatives of 3,5,6-trimethylbenzimidazolylcobam-

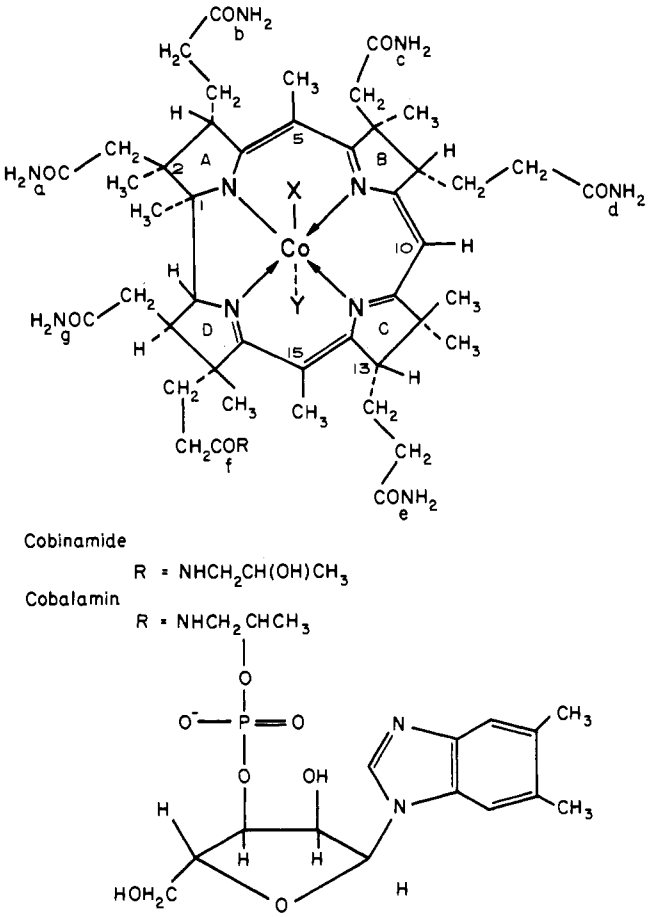

FIGURE 1: Structure of cobalamins and cobinamides.

ide, the carboxymethylcorrinoids were purified by chromatography on Dowex 50-X2 (200–400 mesh) (pH 3.0); the carboxymethyl-13-epicobalamins were eluted with 0.1 *M* sodium acetate buffer (pH 6.4) while the carboxymethyl derivatives of cobalamin lactam, cobalamin lactone, and the cobalamincarboxylic acids were eluted with 0.1 *M* sodium acetate buffer (pH 5.5). The carboxymethylcobalamincarboxylic acids were isolated as glasses while the other carboxymethylcorrinoids were crystallized from aqueous acetone. The carboxymethyl derivatives of 3,5,6-trimethylbenzimidazolylcobamide and cobinamide were purified by chromatography on a 2.5 × 40 cm column of SP-Sephadex. The column was eluted with a linear gradient of 0.0 *M* → 0.2 *M* sodium chloride in 0.025 *M* sodium phosphate buffer (pH 8.0). The desired corrinoids were desalted and isolated as glasses.

Synthesis of [1-^{13}C]- and [2-^{13}C]Hydroxyethylcobalamin. These cobalamins were prepared by reacting [1-^{13}C]-2-bromoethanol and [2-^{13}C]-2-bromoethanol with cob(I)-alamin as described before (Hogenkamp et al., 1965). The cobalamins were purified on Dowex 1-X2, 200–400 mesh, acetate form, and the desired products were crystallized from aqueous acetone.

Results

Effect of the Axial Ligands on the Chemical Shifts of Selected Carbons of the Corrin Ring. The effect of the axial ligands on the ^{13}C resonances of the methine carbons (C_5, C_{10}, and C_{15}, Figure 1) is summarized in Table I. The data clearly demonstrate that the substitution of a weak field ligand by a strong one is accompanied by an upfield

Table I: Carbon-13 Chemical Shifts of the Methine Carbon Atoms of Some Corrinoids.[a]

Corrinoid	C_5	C_{15}	C_{10}[b]	
Methylcobalamin				
"Base on"	106.6	104.7	95.1	(4.11)
"Base off"	108.4	106.9	96.4	(3.14)
Adenosylcobalamin				
"Base on"	107.4	106.0	96.0	
"Base off"	109.4	108.0	98.5	
Methylaquocobinamide	108.3	107.3	93.0	(3.20)
Methylpyridinecobinamide	106.8	105.9	91.9	
Methylcyanocobinamide	104.6	104.1	91.4	
Cyanoaquocobinamide	107.5	105.7	94.2	(3.47; 3.53)
Dicyanocobinamide	106.3	104.0	91.8	(4.13)
Cyanocobalamin	108.8	105.4	96.0	
Dicyanocobalamin	106.3	104.2	92.2	

[a] Ppm (±0.1) from external tetramethylsilane. [b] Numbers in parentheses are the C_{10}–H proton chemical shift with respect to internal Me_4Si.

Table II: Carbon-13 Chemical Shifts of the Methyl Groups Attached to C-1, C-5, and C-15.[a]

Corrinoid	Methyl Group Attached to C_1	C_5	C_{15}
Methylcobalamin			
"Base on"	21.7	18.2	18.0
"Base off"	24.4	19.1	17.7
Adenosylcobalamin			
"Base on"	23.0	18.1	17.6
"Base off"	25.0	19.1	17.6
Cyanocobalamin	21.2	18.1	17.3
Carboxymethylcobalamin			
"Base off"	24.6	19.1	17.5
Ethoxycarbonylmethylcobalamin			
"Base off"	24.8	19.6	17.6
Methylaquocobinamide	24.3	19.3	17.8

[a] Ppm (±0.1) from external tetramethylsilane.

shift of all three methine carbon resonances. For instance, protonation of the 5,6-dimethylbenzimidazole moiety and its displacement at the cobalt atom by water at low pH in the alkylcobalamins causes a downfield shift of the methine resonances. Similarly the displacement of a weak ligand such as water by a stronger one, such as pyridine or cyanide in the methylcobinamide series, is accompanied by an upfield shift of these three resonances. The effect of the axial ligands on the carbon-13 resonances of the methyl groups attached to C-1, C-5, and C-15 of the corrin nucleus is presented in Table II.

Effect of pH on the ^{13}C Chemical Shifts of the Alkyl Ligands. As shown before (Needham et al., 1973) protonation of the 5,6-dimethylbenzimidazole ligand and its displacement by water at low pH are accompanied by an upfield shift of the ^{13}C resonances of the trans organo ligand. This trans effect on the chemical shift of a series of [^{13}C]methylcorrinoids and [2-^{13}C]carboxymethylcorrinoids is demonstrated in Tables III and IV, while a typical titration curve of a [2-^{13}C]carboxymethylcorrinoid is presented in Figure 2. The second titration in the carboxymethylcorrinoids represents the ionization of the carboxyl function (Walker et al., 1974a). It is evident from these data that only in the 13-epicorrinoids is the pK_a of the "base off" → "base on" conversion significantly lowered; even such drastic changes as lactam and lactone formation do not appre-

Table III: Carbon-13 Chemical Shifts of the Methyl Resonances of [^{13}C]Methylcorrinoids as a Function of pH.

Corrinoid	"Base on" δ (ppm)[a]	"Base off" δ (ppm)	pK_a
Cobalamin	7.84	0.20	2.6
13-Epicobalamin	8.39	0.15	2.1
Cobalamin *b*-acid	7.77	−0.06	2.8
Cobalamin *d*-acid	7.82	0.13	2.6
Cobalamin *e*-acid	7.81	0.18	2.5
Cobalamin lactam	9.00	2.11	2.4
Cobalamin lactone	8.42	0.20	2.5
Trimethylbenzimidazolylcobamide		0.32	

[a] Ppm (±0.05) from external tetramethylsilane.

Table IV: Carbon-13 Chemical Shifts[a] of the Methylene Resonances of [2-^{13}C] Carboxymethylcorrinoids as a Function of pH.

Corrinoid	pH 0.2	pH 5	pH 11	pK_1	pK_2
Cobalamin	1.0	13.4	21.2	2.4	7.0
13-Epicobalamin	0.5	13.7	21.5	1.8	7.3
Cobalamin *b*-acid	0.8	13.3	20.6	2.4	7.4
Cobalamin *d*-acid	0.9	13.1	21.1	2.4	7.2
Cobalamin *e*-acid	0.7	13.2	21.2	2.4	7.2
Cobalamin lactam	3.8	14.6	22.4	2.3	7.2
Cobalamin lactone	1.2	13.1	21.4	2.3	7.5
Trimethylbenzimidazolylcobamide	1.0		11.7		6.1
Cobinamide	0.9		11.3		5.9

[a] Ppm (±0.05) from external tetramethylsilane.

ciably alter the pK_a value. As expected, the pK_a of the carboxyl function of the carboxymethylcorrinoids is affected by the trans ligand. Displacement of the 5,6-dimethylbenzimidazole ligand by water in carboxymethyltrimethylbenzimidazolylcobamide or in carboxymethylcobinamide lowers the pK_a of the carboxyl group more than a pH unit. Although the pK_a's of the 13-epicorrinoids are much lower than those of the corresponding corrinoids, the ^{13}C-chemical shifts of the organo ligands are very similar in both series of compounds. However, the alkylcobalamin-γ-lactams show significantly different chemical shifts. Hydrolysis of the γ-lactones to the corresponding hydroxy acids at high pH does affect neither the chemical shifts of the organo ligands nor the pK_a of the carboxyl function of carboxymethylcobalamin-γ-lactone.

Most of the alkyl resonances of the alkylcorrinoids are quite narrow (5–10 Hz full width at half-maximum intensity) in the "base on" or the "base off" forms; however, in the pH range where the 5,6-dimethylbenzimidazole ligand is titrated the alkyl resonances are extremely broadened. For instance [2-^{13}C]carboxymethylepicobalamin shows resonances at 0.5 ppm and at 13.7 ppm in the "base off" and the "base on" form, respectively. The width of these lines is approximately 7 Hz, while at the pK_a the width of the resonance centered on 6.9 ppm is 107 Hz. This broadening phenomenon is dependent on the pK_a value of the corrinoid. It is most pronounced in the cobalamins with low pK_a's such as carboxymethylepicobalamin (pK_a = 1.8) and methylepicobalamin (pK_a = 2.1), while the line broadening of the methylene resonance of [5-^{13}C]adenosylcobalamin (pK_a = 3.5) is only a few hertz. The line broadening reflects the chemical exchange between the "base on" and "base off" forms of the corrinoids. However, a mathematical treatment assuming a rapid two site exchange reaction appears to be inadequate. The exchange mechanism is currently under further investigation.

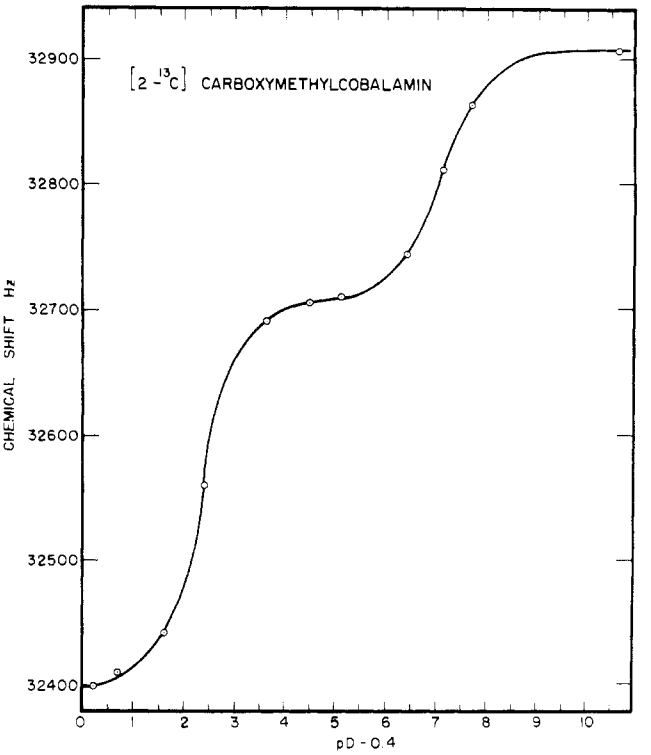

FIGURE 2: pH dependence of the ^{13}C shift of [2-^{13}C]carboxymethylcobalamin.

Relaxation Times (T_1) of Organocorrinoids. NT_1 values of a series of organocorrinoids labeled with ^{13}C in the organo ligand are presented in Table V; included in this table is the NT_1 value of C-10 of the corrin ring determined by Doddrell and Allerhand (1971).

The T_1 values for the "base off" forms of the [^{13}C]methylcorrinoids are consistently shorter than those of the "base on" forms; in contrast, the T_1 values for the "base on" and "base off" forms of carboxymethylcorrinoids and of [5-^{13}C]adenosylcobalamin are very similar. As shown in Tables VI and VII, the T_1 values of both the methylene and carboxyl carbons of carboxymethylcobalamin are not significantly altered by either protonation of the lower 5,6-dimethylbenzimidazole ligand or by changes in the periphery of the corrin ring.

Discussion

A. Chemical Shifts

1. Chemical Shifts of Selected Carbon Nuclei of the Corrin Ring. The chemical shifts of the methine bridge carbons (C-5, C-10, C-15, Figure 1) of the corrin ring for a variety of cobalamins and cobinamides are summarized in Table I. The assignments were made by comparing the spectra with those of cyanocobalamin and dicyanocobalamin specifically enriched with ^{13}C (Scott et al., 1974) and with those of structurally related coproporphyrin III (Matwiyoff and Burnham, 1973) and chlorophylls *a* and *b* (Matwiyoff and Burnham, 1973; Strouse et al., 1972) selectively and uniformly enriched with carbon-13. Like the methine bridge ^{13}C nuclei of the latter structures the C-5, C-10, and C-15 resonances exhibit shifts which are 30–40 ppm upfield of those for benzene and conjugated olefins. As discussed

Table V: Spin–Lattice Relaxation Times of Organocorrinoids, Specifically Enriched in Carbon-13.

Corrinoid	NT_1 (sec)
[^{13}C] Methylcobalamin	
"Base on"	1.58
"Base off"	0.99
[^{13}C] Methyltrimethylbenzimidazolylcobamide	1.12
[1,2-^{13}C] Ethylcobalamin	
CH_3	1.38
CH_2	0.46
[1-^{13}C] Hydroxyethylcobalamin	0.64
[2-^{13}C] Hydroxyethylcobalamin	0.32
[1-^{13}C] Carboxymethylcobalamin	3.00
[2-^{13}C] Carboxymethylcobalamin	0.18
[5′-^{13}C] Adenosylcobalamin	
"Base on"	0.13
"Base off"	0.15
Cyanocobalamin C-10 of the corrin ring	0.11[a]

[a] Doddrell and Allerhand (1971).

Table VI: Chemical Shifts and Spin–Lattice Relaxation Times of Carboxymethylcorrinoids.

Corrinoid	δ (ppm)		NT_1 (sec)	
	CH_2	COO^-	CH_2	COO^-
Cobalamin	21.2	187.5	0.21	3.7
Epicobalamin	21.5	187.6	0.16	3.7
Cobalamin lactam	22.4	186.8	0.20	4.1
Cobalamin lactone	21.4	187.5	0.19	3.3
Trimethylbenzimidazolylcobamide	11.6	184.9	0.21	2.5

Table VII: Chemical Shifts and Spin–Lattice Relaxation Times of Carboxymethylcobalamins as a Function of pH.

	pH 0.4		pH 5.0		pH 11	
	δ (ppm)	NT_1 (sec)	δ (ppm)	NT_1 (sec)	δ (ppm)	NT_1 (sec)
$^{13}CH_2$	1.0	0.14	13.4	0.18	21.2	0.20
$^{13}COOH$	182.9	3.5	185.3	2.9	187.5	3.7

before (Matwiyoff and Burnham, 1973), the origin of these high-field shifts may reside in ring current effects or, more likely, in high charge densities at the methine bridges. In this context it is interesting to note that each of the methine resonances shifts upfield upon substitution of a weak field ligand by a strong field one in the sixth coordination position of cobalt (ligand Y in Figure 1).

In particular the following trends are evident. In the methylcobinamide series, the methine resonances shift to high field in the order water < pyridine < cyanide, which is the order of increasing ligand field or donor strengths (Pratt, 1972). A similar correlation of high-field shifts with increasing donor strengths is noted with the cyanocorrinoids (water < 5,6-dimethylbenzimidazole < cyanide), and with the alkylcobalamins [water (pH $\leq$2) < 5,6-dimethylbenzimidazole (pH $\geq$6)]. A shift to high field also occurs upon substituting cyanide by an alkyl ligand in the upper coordination position (X in Figure 1). These observations suggest that the substantial charge readjustment which occurs upon axial ligation at cobalt and which is reflected in the large ^{13}C shifts of the alkyl ligands (vide supra) is communicated to the corrin ring system. Specifically, the substitution of a weak by a strong field ligand at an axial coordination position reduces the electron demand by the cobalt atom from the corrin ring, resulting in an increased charge density and high-field shifts at the methine bridge carbon atoms.

This model for the effect of axial ligation on the corrin ring system (the cis effect) has been invoked before by Williams and coworkers [Pratt, 1972; Firth et al., 1968; Hill et al., 1965a, 1968]. Thus the resonances of the C-10 proton of cobalamins and cobinamides shift to high field as the donor strength of the axial ligands increases. This high field proton shift is correlated with a shift of the α and β vibrational components of the first allowed electronic transition to lower energies (Hill et al., 1968). It is interesting in this regard to note that the ^{13}C shifts of the methine carbon atoms of the methylcorrinoids are also correlated with the energy of the "β band," the resonances shifting to high field in the order (wavelength of the β band in parentheses) water (463 nm) < pyridine (516 nm) < 5,6-dimethylbenzimidazole (525 nm) < cyanide (574 nm). We have previously reported that the ^{13}C chemical shifts and the ^{13}C–H coupling constants of [^{13}C]methylcobalamins and [^{13}C]methylcobinamides are linearly correlated with the energy of the β band (Needham et al., 1973). This band has been assigned to a π–π^* transition between the highest energy filled and the lowest energy unfilled π orbitals. Molecular orbital calculations indicate a strong participation of the four nitrogen atoms and of the C-5, C-10, and C-15 atoms in the former orbitals (Day, 1967a,b; Johansen and Ingraham, 1969; Schrauzer et al., 1970), the carbon atoms bearing a net negative charge. In the π^* orbital, however, the charge is reduced and positive. Since the calculated orbital energies and atomic charge distributions are a sensitive function of the valence state ionization potentials of the nitrogen atoms, the molecular orbital calculations account qualitatively very well for the trends in the electronic transition energies and for the coupling of axial ligation effects to events at the methine bridge carbon atoms. For example, the enhanced charge densities at C-10 are probably responsible for the facile electrophilic substitution and deuterium exchange at that position (Hill et al., 1965a, 1968; Bonnet et al., 1957).

Two other sets of ^{13}C resonances are strongly influenced by axial ligation. The chemical shifts of the methyl groups bonded to C-1 of ring A and to methine bridge atoms C-5 and C-15 (Figure 1) of a series of alkyl- and cyanocorrinoids are summarized in Table II. The configuration of the C-1 methyl group is syn-periplanar with respect to the C-2 methyl group and a steric interaction (the γ effect) should shift its ^{13}C resonance upfield (Dalling and Grant, 1972). This steric compression should be greatest when a bulky strong field ligand, like the 5,6-dimethylbenzimidazole moiety, occupies the lower coordination site (Y, Figure 1). Indeed, in the crystal structure of adenosylcobalamin the plane of the benzimidazole ligand is tilted appreciably from the normal by contact with the C-1 methyl group (Lenhert, 1968). The data listed in Table II are consistent with a reduction in steric compression at the C-1 methyl group when a compact weak field ligand like water replaces 5,6-dimethylbenzimidazole (in the "base off" form at pH <1). In addition, the data suggest that this steric compression is more severe for cyanocobalamin ("base on" form) than for ade-

nosylcobalamin ("base on" form, pH 6.5). This suggestion is consistent with the structural data, the cobalt–benzimidazole coordinate bond of the coenzyme being 0.16 Å longer than that of cyanocobalamin (Lenhert, 1968; Brink-Shoemaker et al., 1964); and with the relative pK_a values for the protonation of the 5,6-dimethylbenzimidazole moiety (pK_a of cyanocobalamin = 0.1 *vs.* 3.5 for adenosylcobalamin).

The resonances of the methyl groups attached to the methine bridge carbon atoms C-5 and C-15 have been assigned to those occurring in the 17.5–19.5-ppm region (Scott et al., 1974), although a distinction between them has not been made. As indicated in Table II, we have tentatively assigned the low field member of the pair to the C-5 methyl group because it exhibits the larger shift when water is substituted as the axial ligand for 5,6-dimethylbenzimidazole. The crystal structures of the "base on" forms of the corrins (Lenhert, 1968) indicate that strong steric interactions occur between the benzimidazole moiety and the corrin nucleus between C-5 and C-6. This results in a marked ruffling of the corrin ring and a large displacement of the C-5 methyl group from its mean plane. These steric compressions should be relaxed in the "base-off" forms, leading to downfield shifts for the C-5 methyl resonance.

2. Chemical Shifts of the Carbon Nuclei in the Alkyl Moiety Attached to Cobalt. The chemical shifts of the ^{13}C nuclei in the alkyl moieties attached to cobalt (Tables III–VI) are strongly dependent on the nature of the trans group Y and, in some cases, on the nature of the peripheral substituents on the corrin ring. As noted before (Needham et al., 1973; Walker et al., 1974a), it is difficult to ascribe these large shifts to a single effect. However, the large number of derivatives studied here permits some useful speculation. One interesting aspect of these chemical shift data which has been noted in the previous studies (Needham et al., 1973; Walker et al., 1974a) is that the substitution of a weak field ligand (e.g., water) by a strong field one (e.g., dimethylbenzimidazole) leads to substantial downfield shifts contrary to expectations based on simple inductive effects. A consideration of the chemical shifts of the carboxymethylcorrinoids suggests that one origin of these "anomalous" shifts may reside in electric field effects at a highly polarizable carbon–cobalt bond. Inspection of Tables IV and VII reveals that the chemical shift differences of the carboxyl (δ_{COOH}) and methylene (δ_{CH_2}) carbon atoms of the carboxymethylcorrinoids in the conjugate acid and base forms are approximately 2 and 8 ppm, respectively, and in each case the resonance of the conjugate base occurs at *low field*. Corresponding values of these chemical shift parameters for simpler acetic acid derivatives, XCH_2COOH, are: X = NH_3^+, δ_{COOH} 1.5, δ_{CH_2} 2.6; X = H, δ_{COOH} 3.1; δ_{CH_2} 5.2; and X = Br, δ_{COOH} 2.2, δ_{CH_2} 6.8. Again the resonances of the conjugate bases occur at lower field than those for the corresponding parent acids. These latter observations, and the dependence of the effect on the polarizability of the C–X bond [$\delta_{CH_2}(Br) > \delta_{CH_2}(H)$], led Sternlicht and coworkers (Horsley and Sternlicht, 1968; Horsley et al., 1970) to propose that these "anomalous" changes in the ^{13}C shieldings upon ionization of the carboxylic acid arise from a $C^{\delta(+)}$–$H^{\delta(-)}$ bond polarization produced in the electric field of the negatively charged carboxylate group. This model for electric field effects on chemical shifts has been extended by Batchelor et al. (1974) to mono- and polyunsaturated fatty acid derivatives. We suggest that these electric field effects are important also for the carboxymethylcorrinoids, the large values of the δ_{CH_2} parameter resulting from a highly polarizable carbon–cobalt bond.

Interesting in this regard is a comparison of the δ_{CH_2} parameter for the carboxymethyltrimethylbenzimidazolylcobamide (10.7 ppm), carboxymethylcobalamins (~8 ppm), and carboxymethylcobinamide (10.4 ppm) (Table IV), which suggests that the last two corrinoids have a larger carbon–cobalt bond polarizability. The last two corrinoids contain water as the trans ligand Y, whereas the cobalamins contain the stronger field ligand, dimethylbenzimidazole.

Schrauzer and coworkers (1970) have suggested, based on molecular orbital calculations, that the increased charge density transmitted to the cobalt atom by a strong field ligand should stabilize the carbon–cobalt bond, presumably via more effective overlap between the carbon sp^3 orbitals and the cobalt 4s and $3d_{z^2}$ orbitals localized in the axial bonds. Accompanying such improved overlap should be a diminished Co–CH_2 bond polarizability and a shift of electron density from carbon to cobalt. The latter effect could provide a qualitative rationalization for the apparently "anomalous" downfield ^{13}C shifts experienced at the Co–^{13}C moiety when a strong field ligand displaces a weak field ligand (Table III and Needham et al., 1973). However, in apparent contrast to this simple model the dissociation constants of the carboxyl groups (pK_2 in Table IV) of the carboxymethylcorrinoids suggest that the H_2O–Co moiety is more electrophilic than the dimethylbenzimidazole–Co moiety, the cobalamins in the "base on" form above pH 5 exhibiting lower carboxyl group acidities than the cobamide and cobinamide. If a stronger field ligand stabilizes the carbon–cobalt bond, as suggested by Schrauzer and coworkers (1970), with a concomitant shift of the electron density from carbon to cobalt, through-bond inductive effects should have resulted in a higher acidity for the dimethylbenzimidazole–Co–CH_2–COOH system.

The correlation between the chemical shift of the ^{13}C nucleus in the alkyl ligand and the strength of the interaction between the cobalt atom and the trans ligand Y suggest that the structural detail noted for crystalline preparations of the corrinoids is preserved in aqueous solutions. For example, in the cobalamins the propionamide side chain at C-13 (Figure 1) projects on the same side of the corrin plane as the nucleotide attached to C-17. In this configuration, important steric interactions occur between the methylene carbons of the propionamide side chain and the dimethylbenzimidazole moiety, and between the amide terminus and the ribose moiety (Lenhert, 1968). These steric interactions are relieved in the 13-epicobalamins in which the propionamide side chain at C-13 is on the opposite side of the corrin plane (Stoeckli-Evans et al., 1972). The release of this strain should result in a stronger Co–dimethylbenzimidazole bond and in our studies this is reflected indirectly in decreased pK_a values ("base on" $\rightleftarrows$ "base off") of methyl-13-epicobalamin and carboxymethyl-13-epicobalamin relative to pK_a values of the corresponding cobalamins as well as in a downfield shift of [^{13}C]methylepicobalamin relative to that [^{13}C]methylcobalamin (Tables III and IV). In contrast the chemical shift values for "base on" forms of carboxymethylcobalamin and carboxymethylepicobalamin are nearly identical (Table IV) suggesting steric interaction between the carboxymethyl group and the *e*-propionamide side chain which are on the same side of the corrin plane in the epicobalamin. Such interaction is also suggested by the upfield shift exhibited by the "base off" form of [2-^{13}C]carboxymethylepicobalamin relative to [2-^{13}C]carboxymethyl-

cobalamin (Table IV). In contrast the "base off" forms of [^{13}C]methylcobalamin and [^{13}C]methylepicobalamin have nearly identical chemical shifts (Table III). For the latter methylcorrinoid, inspection of molecular models shows negligible steric interaction between the Co–CH_3 group and the *e*-propionamide side chain at C-13. Finally we note that the pK_a and "base off" chemical shift values of the cobalamin *b*- and *d*-acids compared to those for methylcobalamin (Table III) suggest that the structural assignments of these two acid isomers should be reversed. Inspection of scaled molecular models based on crystal structure data (Lenhert, 1968) reveals important steric interactions between the dimethylbenzimidazole moiety and the carbonyl function of propionamide-*d*, whereas the corresponding group of propionamide-*b* shows no important interactions with the nucleotide and its side chain in the lower coordination site. In the studies of Bernhauer et al. (1968), who prepared a variety of these acids by hydrolysis and separated them by ion-exchange chromatography, an unambiguous identification of the *b*- and *d*-acids could not be made.

B. Spin–Lattice Relaxation Times

Allerhand and coworkers (1971b) have demonstrated that the relaxation of protonated carbons of large molecules is dominated by ^{13}C–^{1}H dipolar interactions with the directly bonded hydrogens. In case of molecules undergoing isotropic rotational reorientation accompanied by intramolecular reorientation, the relaxation rates are governed by two correlation times, τ_R and τ_G, τ_R is the correlation time for the isotropic rotational motion of the entire molecule while τ_G is the correlation time for the internal motion of a particular group of the molecule. CPK models of the corrinoids show that these molecules can be considered as isotropic rotors. The temperature dependence of the T_1 values measured for certain of the corrinoids (T_1 increases with increasing temperature), as well as the large nuclear Overhauser effects observed, demonstrates that the extreme narrowing limit applies to the T_1 data and, in the absence of intramolecular reorientation, $1/T_1$ satisfies the well-known expression (Kuhlman et al., 1970)

$$1/T_1 = \hbar^2\gamma_H^2\gamma_c^2 I r^{-6}\tau_R$$

A very good estimate of τ_R of the corrinoids ($\tau_R = 4 \times 10^{-10}$ sec) can be obtained from the T_1 value (0.11 sec) of the C-10 methine bridge carbon atom. This carbon atom is rigidly locked into the corrin nucleus and, thus, no internal motion is possible. In case of the corrinoids listed in Table V, internal rotation about the carbon–cobalt bond should lengthen the NT_1 values. If this internal motion is much faster than the overall reorientation of the molecule ($\tau_G \ll \tau_R$), NT_1 should approach $9NT_1$ for a group with tetrahedral angles ($\theta = 109° 28'$) (Allerhand et al., 1971b). It is evident from the data presented in Table V that rotation about the carbon–cobalt bond of methylcobalamin and about the carbon–carbon bond of ethylcobalamin is rapid and unrestricted ($NT_1 \geq 9 \times 0.11$ sec; $\tau_G \ll 10^{-10}$ sec.). The NT_1 values of the [^{13}C]methylcorrinoids, in which the 5,6-dimethylbenzimidazole ligand is not coordinated to cobalt ($NT_1 = 0.99$ sec for "base off" methylcobalamin and $NT_1 = 1.12$ sec for methyltrimethylbenzimidazolylcobamide), are considerably shorter than the NT_1 value of methylcobalamin (1.58 sec), indicating that in these forms the 5,6-dimethylbenzimidazole nucleotide extends into the solvent, resulting in a larger molecular radius (r) and consequently in longer τ_R and shorter T_1 values.

In contrast, the NT_1 values of [5′-^{13}C]adenosylcobalamin are very short (0.13 and 0.15 sec in the "base on" and "base off" forms, respectively) indicating that rotation about the carbon–cobalt bond is severely restricted and that the correlation time for relaxation is approximately τ_R. These results are in accord with the X-ray data which demonstrated that in the crystalline form the adenine moiety of adenosylcobalamin is fixed over ring C of the corrin nucleus by several intra- and intermolecular hydrogen bonds (Lenhert, 1968). Similarly, the oxygen atom of the five-membered ribose ring is in contact with the corrin ring between C-14 and C-15 and rotation of this bulky moiety about the Co–C bond would be hindered by the methylene groups of the acetamide side chains and by the methyl substituents. A β-coupled rotation of the methylene group itself about the Co–CH_2–C axis is prevented by strong interactions of the methylene hydrogens with the ring oxygen and the 3′-hydrogen of the ribofuranose moiety.

The NT_1 values of the resonances of [2-^{13}C]carboxymethylcorrinoids are also very short. Although the adenosyl moiety of adenosylcobalamin is much more bulky than the carboxymethyl group, the NT_1 values of [5′-^{13}C]adenosylcobalamin and [2-^{13}C]carboxymethylcobalamin are similar, suggesting that the carboxymethyl group is severely restricted in rotation. We feel that this constraint is probably due to hydrogen bonding between the carboxyl function and the acetamide side chains on the periphery of the corrin ring. Earlier, strong hydrogen bondings between these groups are suggested by the unusually high pK_a value of the carboxyl moiety of carboxymethylcobalamin (Walker et al., 1974a).

Finally we note that the relative NT_1 values of the methyl-, ethyl-, and hydroxyethylcobalamins are in the order expected for the effect of increased steric interactions upon the restriction of internal rotation for the Co–alkyl moiety.

References

Allerhand, A. Doddrell, D. Glushko, V., Cochran, D. W., Wenkert, E., Lawson, P. J., and Gurd, F. R. N. (1971a), *J. Am. Chem. Soc. 93,* 544.

Allerhand, A., Doddrell, D., and Komoroski, R. A. (1971b), *J. Chem. Phys. 55,* 189.

Allerhand, A., and Komoroski, R. A. (1973), *J. Am. Chem. Soc. 95,* 8228.

Batchelor, J. G., Cushley, R. J., and Prestegard, J. H. (1974), *J. Org. Chem. 39,* 1698.

Bernhauer, K., Vogelmann, H., and Wagner, F. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349,* 1281.

Bonnett, R., Cannon, J. R., Clark, V. M., Johnson, A. W., Parker, L. F. J., Smith, E. L., and Todd, A. (1957), *J. Chem. Soc.,* 1158.

Bonnett, R., Godfrey, J. M., and Math, V. B. (1971), *J. Chem. Soc. C,* 3736.

Brink-Shoemaker, C., Cruickshank, D. W. J., Hodgkin, D. C., Kamper, M. J., and Pilling, D. (1964), *Proc. R. Soc., London, Ser. A 278,* 1.

Dalling, D. K., and Grant, D. M. (1972), *J. Am. Chem. Soc. 94,* 5318.

Day, P. (1967a), *Theor. Chim. Acta 7,* 328.

Day, P. (1967b), *Coord. Chem. Rev. 2,* 109.

Deslauriers, R., McGregor, W. H., Sarantakis, D., and Smith, I. C. P. (1974b), *Biochemistry 13,* 3443.

Deslauriers, R., Smith, I. C. P., and Walter, R. (1974a), *J. Am. Chem. Soc. 96,* 2289.

Doddrell, D., and Allerhand, A. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1083.
Dolphin, D. (1971), *Methods Enzymol. 18,* 34.
Firth, R. A. Hill, H. A. O., Pratt, J. H., Thorp, R. G., and Williams, R. J. P. (1968), *J. Chem. Soc. A,* 2428.
Friedrich, W., and Bernhauer, K. (1956), *Chem. Ber. 89,* 2030.
Hill, H. A. O., Mann, E. B. Pratt, J. M., and Williams, R. J. P. (1968), *J. Chem. Soc. A,* 564.
Hill, H. A. O., Pratt, J. M., and Williams, R. J. P. (1965a), *J. Chem. Soc.,* 2859.
Hill, H. A. O., Pratt, J. M., and Williams, R. J. P. (1965b), *Proc. R. Soc. London, Ser. A 288,* 352.
Hogenkamp, H. P. C. (1966), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 25,* 1623.
Hogenkamp, H. P. C., and Pailes, W. H. (1968), *Biochem. Prep. 12,* 124.
Hogenkamp, H. P. C., Rush, J. E., and Swenson, C. A. (1965), *J. Biol. Chem. 240,* 3641.
Horsley, W. J., and Sternlicht, H. (1968), *J. Am. Chem. Soc. 90,* 3738.
Horsley, W. J., Sternlicht, H., and Cohen, J. S. (1970), *J. Am. Chem. Soc. 92,* 680.
Johansen, H., and Ingraham, L. L. (1969), *J. Theor. Biol. 23,* 191.
Kuhlman, K. F., Grant, D. M., and Harris, R. K. (1970), *J. Chem. Phys. 52,* 3439.
Lenhert, P. G. (1968), *Proc. R. Soc. London, Ser. A 303,* 45.
Li, N. C., Tang, P., and Mathur, R. (1961), *J. Phys. Chem. 65,* 1074.
Matwiyoff, N. A., and Burnham, B. F. (1973), *Ann. N.Y. Acad. Sci. 206,* 365.
Needham, T. E., Matwiyoff, N. A., Walker, T. E., and Hogenkamp, H. P. C. (1973), *J. Am. Chem. Soc. 95,* 5019.
Oldfield, G., and Allerhand, A. (1975), *J. Am. Chem. Soc. 97,* 221.
Pratt, J. M. (1972), Inorganic Chemistry of Vitamin B_{12}, New York, N.Y., Academic Press.
Schrauzer, G. N., Lee, L. P., and Sibert, J. W. (1970), *J. Am. Chem. Soc. 92,* 2997.
Scott, A. I., Townsend, C. A., Olcada, K., Kajiwara, M., Cushley, R. J., and Whitman, P. J. (1974), *J. Am. Chem. Soc. 96,* 8069.
Stoeckli-Evans, H., Edmond, E., and Hodgkin, D. C. (1972), *J. Chem. Soc., Perkin Trans. 1,* 605.
Strouse, C. E., Kollman, V. H., and Matwiyoff, N. A. (1972), *Biochem. Biophys. Res. Commun. 46,* 328.
Tkachuck, R. D., Grant, M. E., and Hogenkamp, H. P. C. (1974), *Biochemistry 13,* 2645.
Villafranca, J. J., and Viola, R. E. (1974), *Arch. Biochem. Biophys. 169,* 465.
Walker, T. E., Hogenkamp, H. P. C., Needham, T. E., and Matwiyoff, N. A. (1974a), *J. Chem. Soc., Chem. Commun.,* 85.
Walker, T. E. Hogenkamp, H. P. C., Needham, T. E., and Matwiyoff, N. A. (1974b), *Biochemistry 13,* 2650.
Yamada, R., and Hogenkamp, H. P. C. (1972), *J. Biol. Chem. 247,* 6266.