ciated with the ion pair will be part of the broad continuum of frequencies mentioned in the early portion of this paper, as suggested by Zundel.[9] This suggests therefore that the sharp feature near 3610 cm^{-1} is associated with OH^-, which is not strongly vibrationally coupled with its waters of hydration and which does not exchange protons rapidly with the surrounding aqueous medium. Admittedly the existence of such a species is hard to imagine in a water solution, except perhaps at high concentrations when all the water is strongly associated with an ion, making proton transfer to at least some OH^- species difficult. Alternatively one might postulate that the ion pair is a species such as I or II in which the depolarization ratio of the 100-cm^{-1} mode is coincidentally large. In this event one can postulate an ion pair in which the proton in the NaOH fragment is strongly bonded and hence does not exchange rapidly with its surroundings. The sharp 3610-cm^{-1} band would then be the OH stretching frequency of the ion pair. We prefer the first option (structure IV), leaving the question of the source of the 3610-cm^{-1} band unsettled for the moment.

In view of the uncertainty concerning the OH stretching region of the spectrum, no further remarks will be made concerning it save to point out that the sharp decrease with concentration of base in the intensity of the "free" water band at 3420 cm^{-1} in the 5-7 M range may signal the transition in ion conductance mechanism proposed by Lown and Thirsk.[5] The latter calculate that for KOH the transition from proton transfer to hydrodynamic ion conductance mechanism is virtually complete by 7 M, beyond which concentration, they suggest, an increasing fraction of the KOH molecules is closely associated and would not significantly perturb any more water molecules. This would imply that beyond 7 M the portion of the spectrum of aqueous KOH solution due to "free" water should not decrease as rapidly with additional KOH as it did below that concentration, as observed.

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Carbon-13 Nuclear Magnetic Resonance Studies of the Monocarboxylic Acids of Cyanocobalamin. Assignments of the *b*-, *d*-, and *e*-Monocarboxylic Acids[1a]

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Abstract: Analysis of the carbon-13 nuclear magnetic resonance spectra of cyanocobalamin, cyanoepicobalamin, cyanocobalamin lactone, cyanocobalamin lactam, and the three monocarboxylic acids derived from cyanocobalamin has led to the revision of the identity of these three monocarboxylic acids. The observed chemical-shift changes identify the peak I, II, and III acids as the *d*-, *b*-, and *e*-monocarboxylic acids of cyanocobalamin, respectively, and demonstrate that the earlier preparations designated CMS_1 or E_2, CMS_2 or E_1, and CMS_3 are *Coα*-(α-5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,c,d,e,g*-pentamide, *Coα*-(α-5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,b,c,d,g*-pentamide, and *Coα*-(α-5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,b,c,e,g*-pentamide, respectively.

Mild acid hydrolysis of cyanocobalamin yields a mixture of mono- and dicarboxylic acids and one tricarboxylic acid.[2] These acids are derived from the propionamide side chains *b*, *d*, and *e* which are more susceptible to hydrolysis than the amide groups on the acetamide side chains *a*, *c*, and *g* (Figure 1). The structural assignments of the monocarboxylic acids were based on their physical and chemical properties, while the predominant monocarboxylic acid designated E_2 by Armitage and co-workers[2a] and CMS_1 by Bernhauer et al.[2b] was also investigated by X-ray and neutron diffraction analysis.[3]

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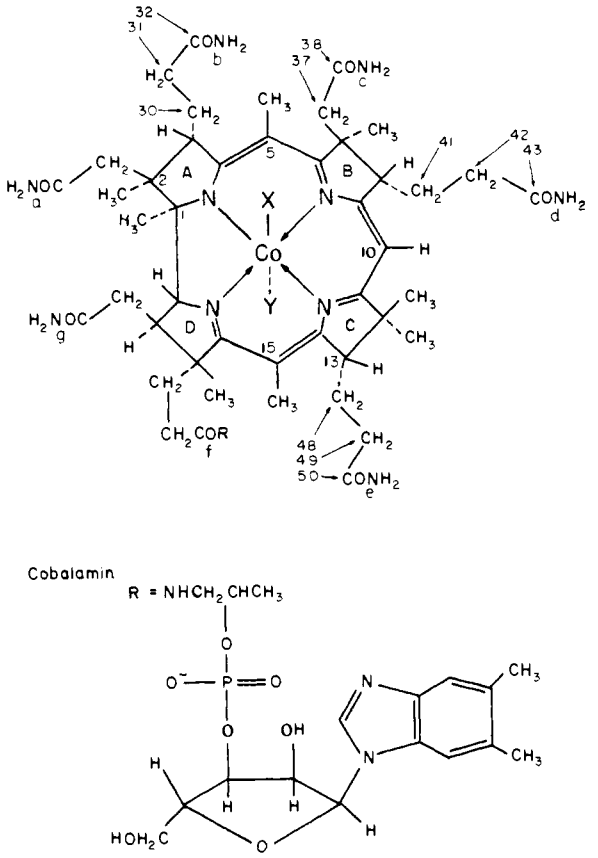

Figure 1. Structure of cyanocobalamin, X = CN, Y = dimethylbenzimidazole. The relevant atomic positions have been numbered.

The latter analysis identified this monocarboxylic acid as *Coα*-(α-5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,b,c,d,g*-pentamide (cyanocobalamin-*e*-carboxylic acid).

However, our carbon-13 nuclear magnetic resonance studies of the *Co*-methyl derivatives of the three cobalamin monocarboxylic acids suggested that the assignments of the *b* and *d* acids should be reversed.[4] Furthermore, Professor Hodgkin pointed out at a recent symposium that the identity of the E_2 monocarboxylic acid was not firmly established and that the assignments of the *e*- and *b*-monocarboxylic acids also could be reversed.[5] The correct assignment of these three monocarboxylic acids is essential because they have been widely used in studies concerning the interaction of intrinsic factor with corrinoids, modified on the periphery of the corrin nucleus,[6a] and more recently in studies concerning the transport of vitamin B_{12}[6b] and the participation of the propionamide side chains in enzyme-coenzyme interaction.[6c]

These recent developments have prompted us to reinvestigate the assignments of the three monocarboxylic acids of cyanocobalamin. In order to provide an unambiguous identification of these acids we have studied the effect of specific modifications of the periphery of the corrin ring on the chemical shifts of the methylene carbons of the propionamide side chains. The specific modifications involve the inversion of the *e*-propionamide side chain (cyano-13-epicobalamin) and modification of the *c*-acetamide side chain (cyanocobalamin lactam and cyanocobalamin lactone). The sites of these modifications have been unambiguously established by X-ray crystallography and by chemical analysis.[7]

Experimental Section

Methods. The purity of the corrinoids was established by spectral analysis using a Cary Model 15 spectrophotometer and by descending paper chromatography in three solvent systems.[8] Paper ionophoresis was carried out using a Savant Instrument, Inc., apparatus as described before.[9] Pulse Fourier transform ^{13}C (25.2 MHz) nuclear magnetic resonance spectra were obtained at 25 °C using a Varian XL-100-15 spectrometer locked to the resonance (15.4 MHz) of D_2O (10% contained in the solvent water) and interfaced to a Supernova computer. The data acquisition time was 1 s with no pulse delay and the spectra were obtained under conditions of simultaneous broadband proton noise decoupling. Peak positions were determined by computer examination of the final Fourier transformed spectrum and the chemical shifts were measured with respect to tetramethylsilane external standard.

Synthesis of Analogues of Cyanocobalamin. Cyanocobalamin monocarboxylic acids were prepared as described before[10] but the isolation procedure was modified as follows. After cyanocobalamin (6 g, 4.16 mmol) was incubated in 600 mL of 1 M HCl at 37°C for 4 h, the reaction mixture was neutralized with NaOH and desalted by phenol extraction.[11] The aqueous solution was then concentrated to approximately 500 mL and applied to a column of AG 1 × 2 (acetate form, 200–400 mesh, 2.5 × 25 cm). The column was washed with water to remove unreacted cyanocobalamin and then treated with 0.04 M sodium acetate, pH 4.67.The eluent, containing the monocarboxylic acids, was applied directly onto a second column of AG 1 × 2 (acetate form, 200–400 mesh, 5 × 80 cm; equilibrated in 0.04 M sodium acetate, pH 4.67). The second column was developed with the same buffer until the second red zone was approximately halfway down the column. The eluting buffer was then changed to 0.04 M sodium acetate, pH 5.3. The first monocarboxylic acid was collected, desalted, and crystallized from aqueous acetone. The other two unresolved monocarboxylic acids were recycled onto the same column. After three cycles through this column they were well separated and were collected as homogeneous preparations. They were desalted and crystallized from aqueous acetone. Yields: I, 297 μmol, 7.1%; II, 631 μmol, 15.1%; III, 377 μmol, 9.0%. The neutral fraction containing unreacted cyanocobalamin was passed through a 5 × 5 cm column of SP-Sephadex to remove cobinamides. The eluate was desalted and the cyanocobalamin crystallized from aqueous acetone, yield 1.78 mmol, 42.7%.

Characterization of the Monocarboxylic Acids. As expected, each of the monocarboxylic acids is negatively charged in neutral and alkaline buffer solutions while they are neutral in acidic solution. The pK_a values of the three monocarboxylic acids were estimated from their relative ionophoretic mobilities over the pH range from 2.0 to 9.0, pK_a of I, 5.10 ± 0.11; II, 4.52 ± 0.07; III, 4.62 ± 0.07. Cyanocobalamin monocarboxylic acid I is very insoluble in acidic aqueous solutions and consequently no ^{13}C NMR spectra of the protonated monocarboxylic acid I could be obtained. The monocarboxylic acids were homogeneous on paper chromatography in three solvent systems.

The purity of the cobalamins was also monitored by high-performance liquid chromatography by the method of Frenkel et al.[12] Retention times (min) were as follows: cyanoepicobalamin, 2.7; cyanocobalamin lactam, 4.0; cyanocobalamin, 5.4; cyanocobalamin monocarboxylic acid II, 6.5; cyanocobalamin lactone, 7.1; cyanocobalamin monocarboxylic acid I, 7.5; cyanocobalamin monocarboxylic acid III, 7.8. The cyanocobalamin lactam preparation showed a small amount of a contaminant with a retention time of cyanocobalamin.

Results and Discussion

In revising the structural assignments of the three monocarboxylic acids of cyanocobalamins by carbon-13 nuclear magnetic resonance spectroscopy, we have made the following assumptions, which are listed in decreasing order of reliability: (1) the structures of cyanocobalamin lactam[7] and cyano-13-epicobalamin[7a] determined by X-ray crystallography are correct; (2) the structure of cyanocobalamin lactone is similar to that of the lactam in the closure of the rings between the acetamide side chain *c* and C-8 of ring B;[7c] (3) the assignments of the ^{13}C resonances of the α and β carbon atoms of the propionamide side chains of cyanocobalamin are correct, being derived from the ^{13}C NMR spectra of cyanocobalamin biosynthetically enriched with [2-^{13}C]δ-aminolevulinic acid and [8-^{13}C]porphobilinogen;[13] (4) a structural perturbation of the corrinoid, e.g., conversion of an amide to an acid or of

Table I. ^{13}C Chemical Shifts[a] of the C_α and C_β Carbon Atoms of the Propionyl Side Chains of Derivatives of Cyanocobalamin

derivative	α carbons			β carbons		
	C-31	C-49	C-42	C-48	C-41	C-30
cyanocobalamin (CNCbl)	35.9	35.6	32.3[b]	29.0	27.0	27.0
CNCbl lactone	35.9	35.4	30.2	28.9	29.8	27.0
CNCbl lactam	35.9	35.4	30.8[c]	28.9	30.3[c]	26.9
CN epi Cbl	35.9	32.5	32.3[b]	27.0	26.6	27.0
peak I CNCbl (C-43) acid, pH >7	35.9	35.5	34.8	28.9	27.8	27.0
peak II CNCbl (C-32) acid, pH <2	34.6	35.6	32.3[b]	29.0	26.9	26.2
peak II CNCbl (C-32) acid, pH >7	38.8	35.6	32.3[b]	29.0	26.9	27.9
peak III CNCbl (C-50) acid, pH <2	35.9	34.3	32.1[b]	28.3	26.9	26.9
peak III CNCbl (C-50) acid, pH >7	35.9	38.1	32.3[b]	29.6	26.9	26.9

[a] Chemical shifts downfield in parts per million with respect to external Me_4Si. [b] The exact position of this resonance could not be determined because of several overlapping resonances. [c] The assignments of the resonances at 30.8 and 30.3 ppm as C-42 and C-41, respectively, may be reversed.

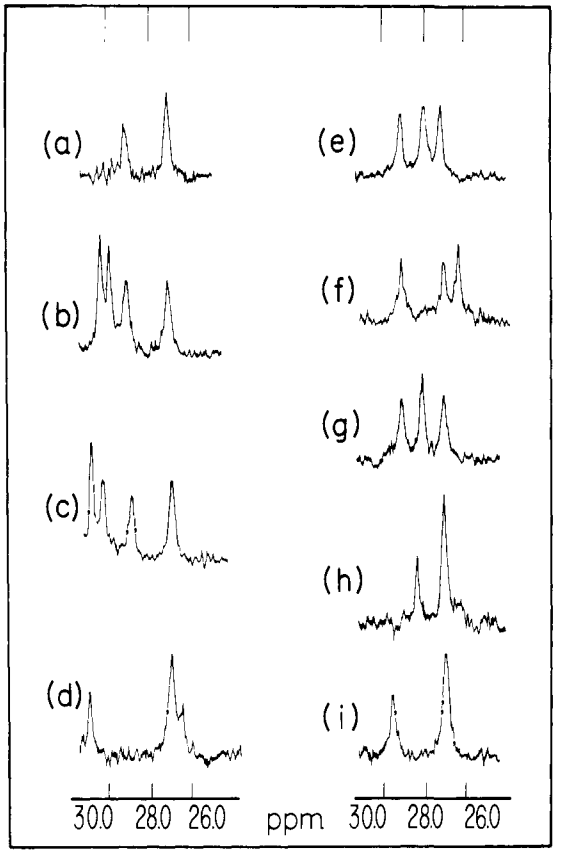

Figure 2. Proton decoupled ^{13}C NMR spectra of the β-methylene carbons of the C-32, C-43, and C-50 propionyl side chains in a series of cyanocobalamin derivatives: (a) cyanocobalamin; (b) cyanocobalamin lactone; (c) cyanocobalamin lactam; (d) cyanoepicobalamin; (e) peak I cyanocobalamin (C-43) acid, pH >7; (f) peak II cyanocobalamin (C-32) acid, pH >2; (g) f, pH >7; (h) peak III cyanocobalamin (C-50) acid, pH >2; (i) h, pH >7.

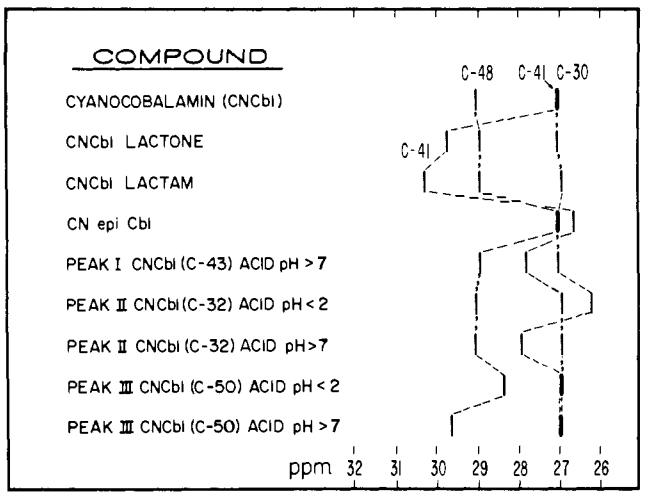

Figure 3. Correlation diagram of the β-methylene carbon ^{13}C NMR resonances of the C-32, C-43, and C-50 propionyl side chains in a series of cyanocobalamin derivatives. The assignments are the corrected ones as discussed in the text.

an acid to its conjugate base, affects most the chemical shift of those ^{13}C atoms which are closest to the perturbed site.

In Figure 2 are summarized the ^{13}C NMR spectra in the region of the chemical shifts of the β carbons of the propionyl side chains for a variety of cyanocobalamin derivatives. It is clear from these spectra that structural perturbations induce large and specific ^{13}C chemical shift changes. These induced shifts, which were used in conjunction with the assumptions listed above, are discussed at greater length below to make the ^{13}C resonance assignments summarized in Table I. In the designation of the revised structural assignments of the monocarboxylic acids we have labeled the acids from their elution position from the anion exchange column, viz., the peak I, II, and III monocarboxylic acids.

Figure 3 contains a correlation diagram which summarizes the effect of structural perturbations on the β-carbon resonances of the propionyl side chains of the cyanocobalamin derivatives critical to the structural assignments. The larger effect of lactone and lactam formation on the chemical shift (~5 ppm) of one of the resonances originally at 27.0 ppm in the parent cyanocobalamin clearly identifies it as the C-41 atom which is α to the perturbed C-8 atom in the B rings of these two derivatives. In other simpler systems, the substitution of a hydrogen atom by an oxygen or nitrogen substituent shifts the ^{13}C resonance of a carbon α to the site of substitution downfield but by a larger amount.[14] For example, the transformation pentanoate → pentanoyl-γ-lactone shifts the C-5 (the carbon α to the modified C-4) resonance 6.8 ppm downfield.[14b] As expected, the β-carbon resonance originally at 29.0 ppm and the other at 27.0 ppm in the parent cyanocobalamin undergo little shift upon lactone or lactam formation in ring B.

In like manner, the inversion of the *e*-propionamide side chain in cyanoepicobalamin is accompanied by a marked upfield shift (~2.0 ppm) of the resonance originally at 29.0 ppm in cyanocobalamin and this resonance is assigned to C-48. As expected, this transformation causes only minor changes in the chemical shifts of the resonances at 27.0 ppm.[15] The upfield shift observed for C-48 in the cyanoepicobalamin is reminiscent of steric compression shifts observed in rigid systems like the cycloalkanes[14a] and may arise from additional steric crowding

Table II. Chemical-Shift Changes for C_α and C_β Carbons of the Cyanocobalamin Propionyl Side Chains and Appropriate Model Compounds[a]

carboxylic acid	β carbon Δ_A[b]	β carbon Δ_B[c]	α carbon Δ_A[b]	α carbon Δ_B[c]
peak II CNCbl (C-32)	1.7	0.9	4.2	2.9
peak I CNCbl (C-43)	*d*	0.8	*d*	2.5[e]
peak III CNCbl (C-50)	1.3	0.6	3.8	2.5
propionic	2.0	0.9	3.6	2.2
butanoic	1.5	0.6	4.0	2.6
pentanoic	1.7	0.7	3.8	2.5

[a] Shift changes (Δ) in parts per million are positive for a downfield shift. [b] Titration shift for the acid to conjugate base. [c] Shift for conversion of an amide to a deprotonated carboxyl. [d] This compound was insoluble at acid pH. [e] This exact shift for C-42 could not be determined (see Table I).

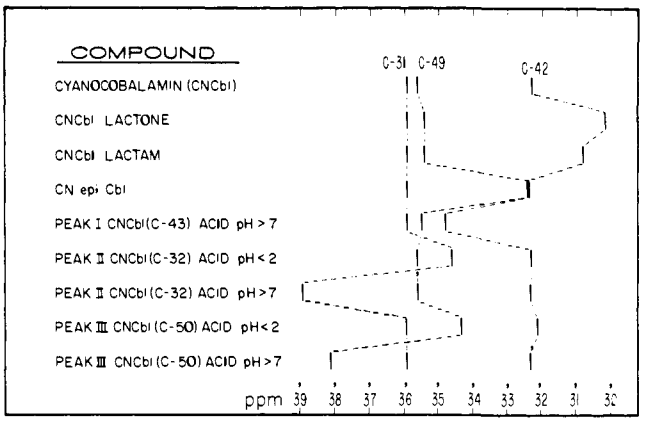

Figure 4. Correlation diagram of the α-methylene carbon ^{13}C NMR resonances of the C-32, C-43, and C-50 propionyl side chains in a series of cyanocobalamin derivatives. The assignments are the corrected ones discussed in the text. The resonance assigned to C-42 for cyanocobalamin is close to several other resonances and the position cannot be stated with certainty.

at the β carbon in the epi isomer. These observations, together with the pattern of chemical shifts observed for the C_β resonances of the peak III acid, allow the unequivocal identification of this acid as the corrinoid with a carboxyl function at C-50: α-(5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,b,c,d,g*-pentamide. Although the chemical-shift patterns of the C_β resonances of the peak I and II acids do not allow such unambiguous structural assignments, this may be accomplished for the latter through a close inspection of the C_α resonances of the propionyl side chains (Figure 4).

Unlike the C_β carbons of the propionyl side chains, which are the only sites in cyanocobalamin biosynthetically labeled by [8-^{13}C]porphobilinogen, the biosynthetic labeling of the propionyl C_α carbons by [2-^{13}C]-δ-aminolevulinic acid is accompanied by the labeling of the three C_α acetyl carbons as

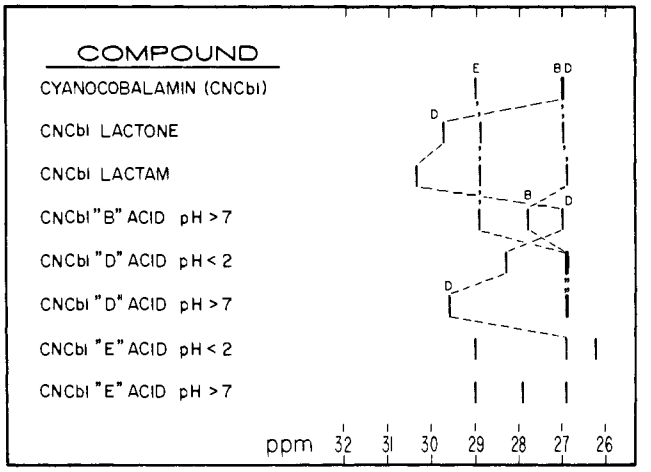

Figure 5. Correlation diagram of the β-methylene carbon ^{13}C NMR resonances of the "*b*", "*d*", and "*e*" propionyl side chains for a series of cyanocobalamin derivatives. The "*b*", "*d*", and "*e*" designations are those originally proposed.[10] As discussed in the text, these assignments do not allow the chemical shifts to be explained in a self-consistent way.

well.[13] The C_α resonances of the former residues were assigned by comparing the ^{13}C chemical shifts of cyanocobalamin and its lactone, lactam, and cyanoepicobalamin derivatives. Only the resonances designated as C-49 and C-42 exhibited large ^{13}C chemical shifts in these derivatives.[16] The C-31 resonance was assigned by difference. The resonance of C-42 (32.3 ppm) is readily assigned by comparison of the lactone and lactam spectra with that for cyanocobalamin—in other simpler derivatives, the substitution of a hydrogen by an oxygen or nitrogen function shifts the ^{13}C resonance of a carbon in an analogous position β to the substitution site by ~2.5 ppm upfield. Since the peak I carboxylic acid exhibits a shift change for C-42, this acid is identified as the corrinoid with a carboxyl at C-43: α-(5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,b,c,e,g*-pentamide. The behavior of the resonance assigned to C-49 (35.6 ppm) is similar to that of C-48, shifting upfield in the cyanoepicobalamin relative to the parent cyanocobalamin. This observation is confirmed by comparing the cyanocobalamin spectrum to that for the peak III acid which on the basis of the propionyl C_β shifts can be designated unambiguously as the corrinoid with a carboxyl function at C-50. These observations, together with the pattern of chemical shifts for the C_α resonances exhibited by the acids, allow the peak II acid to be assigned to the corrinoid with a carboxyl at C-32, α-(5,6-dimethylbenzimidazolyl)-*Coβ*-cyanocobamic acid *a,c,d,e,g*-pentamide.

Further support for these assignments can be obtained by comparing the relative ^{13}C chemical shifts of these cyanocobalamin derivatives with those of appropriate model compounds, specifically, the C_α and C_β chemical shift changes (Δ_A) which accompany the deprotonation of carboxylic acids

Table III. Structure Assignments of the Three Monocarboxylic Acids Derived from Cyanocobalamin

	designation					
	carbon modified	old assignment	column peak no.[a]	Armitage et al.[2a]	Bernhauer et al.[2b]	pK_a[b]
cobamic acid *a,b,c,e,g*-pentamide (cyanocobalamin-*d*-carboxylic acid)	C-43	*b* acid	I		CMS_3	5.10 ± 0.11 (5.05)
cobamic acid *a,c,d,e,g*-pentamide (cyanocobalamin-*b*-carboxylic acid)	C-32	*e* acid	II	E_2	CMS_1	4.52 ± 0.07 (4.65)
cobamic acid *a,b,c,d,g*-pentamide (cyanocobalamin-*e*-carboxylic acid)	C-50	*d* acid	III	E_1	CMS_2	4.62 ± 0.07 (4.60)

[a] Peak designations are given in Experimental Section and in ref 10. [b] pK_a determined by paper electrophoresis; pK_a values determined by potentiometric titration[2a] are given in parentheses.

and those (Δ_B) which accompany the conversion of a propionamide residue to a deprotonated carboxyl. These data, which are summarized in Table II, confirm that the assignments are reasonable.

In contrast to the self-consistency of these data, alternative assignments of the structures of the three carboxylic acids[2b,10] lead to a number of inconsistencies in the ^{13}C NMR data. For example, Figure 5 contains a chemical shift correlation diagram wherein the monocarboxylic acids are identified by the original *b*, *d*, and *e* designations. As in Figure 3, comparison of the cyanocobalamin C_β shifts with those for the lactam and lactone allows the identification of C_β in the *d* side chain. Similarly, the chemical shifts of the *b* acid allow the identification of the *d* and *e* C_β resonances. However, if the *b*, *d*, and *e* structures were correct, it would not be possible to rationalize the following observations in a self-consistent way without invoking "special" structural effects in the cobalamins: (1) in the transformations propionamide → carboxylic acid → conjugate base, the *d* acid C_β resonance shifts downfield in both steps but the *e* acid C_β is unshifted; (2) in the transformation cyanocobalamin → *d* acid, the *e* side chain C_β undergoes a larger shift than the C_β of the *d* acid side chain; (3) in the transformation cyanocobalamin → *e* acid, the C_β resonances of the *b* and *d* side chains shift but that of the *e* acid residue itself does not.

In summary, the ^{13}C chemical shift changes of the α and β carbons of the propionyl side chains of the three monocarboxylic acids derived from cyanocobalamin clearly identify peaks I, II, and III as the *d*-, *b*-, and *e*-monocarboxylic acids of cyanocobalamin, respectively, and thus the E_2 preparation of Armitage et al.[2a] and CMS_1 of Bernhauer and co-workers[2b] is *Co*α-(α-5,6-dimethylbenzimidazolyl)-*Co*β-cyanocobamic acid *a,c,d,e,g*-pentamide (cyanocobalamin-*b*-carboxylic acid). Table III summarizes the revised structure assignments of the three carboxylic acids as well as their earlier designations.

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(1) (a) This work was performed under the auspices of the U.S. Department of Energy and was supported by grants from the National Institutes of Health (GM-23773, H.P.C.H., and RR-00962, N.A.M.) and from the Minnesota Medical Foundation. (b) The University of Minnesota. (c) Los Alamos Scientific Laboratory.

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(16) Of course, the C-37 resonance of the *C*-acetyl residue of the B ring does shift upon the formation of the lactone and lactam,[14b] but that resonance can be distinguished from those of C-31, C-49, and C-42 because its chemical shift (43 ppm in cyanocobalamin) is insensitive to the formation of the peak I–III acids and the cyanoepicobalamin.