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High-performance liquid chromatographic analysis of the (cyanoaquo) stereoisomers of several putative vitamin B_{12} precursors

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ABSTRACT

The cyanoaquo and aquocyano stereoisomers of several putative vitamin B_{12} precursors are reversibly formed and can be separated using analytical high-performance liquid chromatographic methods. The behavior of these stereoisomers varies somewhat depending on the type of column used and the chromatographic conditions employed. Both reversed-phase and ion-exchange columns can be used to observe the reversible formation and separation of the stereoisomers of (H_2O,CN)cobyric acid, cobinamide and the cobinic acid pentaamide-1, -2 and -3 structural isomers. The greatest differences in retention times are seen when the pH of the eluting buffer is less than 4.0 and the buffer contains no KCN.

INTRODUCTION

Cyanocobalamins (vitamin B_{12}), and other cobamides, have been the primary focus of interest in most areas of corrinoid research due to their functions as the important coenzyme partners in many critical enzyme-catalyzed reactions in both eucaryotic and procaryotic organisms [see, for example, reviews such as refs. 1 and 2]. This nexus of interest has resulted in several papers in recent years showing how high-performance liquid chromatography (HPLC) could be used to separate and/or identify cobamides found in mammalian or bacterial materials [3–6].

Cobalamin contains not only the corrin ring and attendant side chains, but the α-side "nucleotide loop" connected to the ring at side chain *f* and is thus known as a "complete" corrinoid. The "incomplete" corrinoids do not have the α-side "nucleotide" loop, but do contain the complete corrin ring and attendant side chains. These compounds are of interest as precursors to the cobamides in microorganisms [7], and may occasionally function as coenzymes or activators in bacteria since Coβ-methylcobyric acid acts as a coenzyme in *Clostridium thermoaceticum* [8], and, not only methylcobalamin, but also diaquocobinamide activates the methylreductase system in extracts of *Methanobacterium bryantii* [9]. In a purely chemical sense, however, the "incomplete" corrinoids have been especially useful in studying the redox and other functions of the corrin ring and complexed cobalt atom [10,11]. Recent publications

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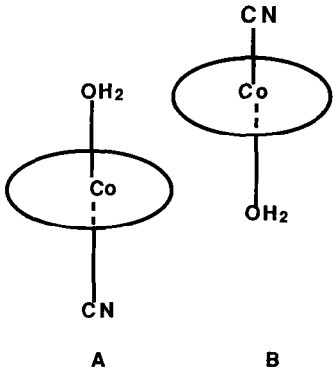

Fig. 1. Abbreviated corrinoid structure showing only the relationship between the CN and H_2O cobalt atom ligands and the corrin ring plane. A = α-cyano,β-aquo; B = α-aquo,β-cyano.

have shown the usefulness of one of these "incomplete" corrinoids, cobinamide, as a dehalogenating agent for environmental contaminants such as lindane and C-1 polychlorinated hydrocarbons [12,13]. In addition, the incomplete corrinoids can serve as models for the "base-off" cobamides, the forms in which many of the biologically active corrins function as coenzymes [14,15].

Structurally, the "incomplete" corrinoids are nearly unique in that they can exhibit stereoisomerism which is not usually seen with the "complete" corrinoids, except at very low pH for cobalamin [16] and in the case of the phenolyl and cresolyl cobamides recently identified in *Sporomosa ovata* [17]. The cyanoaquo-"incomplete" corrinoids may show either [α-cyano,β-aquo] or [α-aquo,β-cyano] structures (see Fig. 1). This fact was reported in a series of papers by Friedrich and co-workers in the late 1960s [18–20]. These authors showed that the stereoisomers of cobyric acid were thermally unstable and yet clearly distinguishable under the right experimental conditions.

^{13}C NMR studies of the aquo^{13}CN- and aquo$^{13}CH_3$-cobinamide stereoisomers have been reported showing small differences in the chemical shifts of the two isomers [10,21]. In addition several reports in the literature have noted the presence of separably identifiable stereoisomers of aquocyano-cobinamide upon HPLC analysis, but none of these reports revealed further investigation of this phenomenon [3,5,6].

In this paper we show how HPLC can be used to distinguish and analyze the cyanoaquo stereoisomers of several different incomplete corrinoids, including cobinamide, cobyric acid, and three isomeric cobinic acid pentaamides, compounds of useful interest in many areas of chemistry.

EXPERIMENTAL

Whatman DE-53 (high-capacity DEAE) and CM-52 ion-exchange celluloses were purchased from Whatman. Dowex AG-1X2 and cyanocobalamin (vitamin B_{12}) were purchased from Sigma. All solvents used for HPLC were of HPLC-grade purity, and water used for preparation of aqueous solvents was distilled, deionized in a Millipore system, filtered through an 0.2-μm membrane, then degassed. All other

solvents and chemicals were of reagent grade. Phenol used for desalting of corrinoids was prepared fresh at 91% (v/v) using liquified reagent-grade crystals and distilled, deionized water. Small quantities of cobyric acid and cobinamide in their cyanoaquo forms and of aquocobalamin to use as standards were kindly supplied by H. C. Friedmann.

Preparation of cobinic acid pentaamide isomers, cobyric acid and cobinamide

Three isomeric cobinic acid pentaamides of known structure were prepared as follows:

A 5-g amount of vitamin B_{12} was incubated for 4 h in 500 ml 1 *M* HCl at 37°C. After incubation, the solution was neutralized with 5 *M* NaOH, desalted by phenol extraction [22], and the resulting corrinoid mixture separated on Dowex AG-1X2 as described by Anton *et al.* [23]. The purified cobamic acid pentaamides-1, -2 and -3 as described by these authors were then individually desalted by phenol extraction, crystallized, and the nucleotide was removed from each by the method of Renz [24]. Purification of the individual cobinic acid pentaamides resulting was by chromatography on DE-53 (acetate) columns (Whatman) equilibrated in 0.05% aqueous HCN; 0.08% acetic acid in 0.05% aq. HCN was the eluting solvent. Yields and cobinic acid pentaamide isomer characteristics are given in the text. To remove contaminating heavy metals, the cobinic acid pentaamides were passed through columns of Chelex 100 (Sigma) as suggested by Bratt and Hogenkamp [25], with 0.1 *M* KCN being used as the eluting solvent, then each isomer was desalted by phenol extraction and dried to a powder or glass. This treatment results in the cyanoaquo form of the incomplete corrinoids as evidenced by a color change from purple to red, and the shift of the γ absorption band in the electronic spectrum from 367 to 352–354 nm. Natural abundance ^{13}C NMR (proton decoupled) was performed on the cobinic acid pentaamide isomers in $^{2}H_2O$ using a Nicolet spectrophotometer, with a 16-μs pulse, 1.0-s pulse delay, tetramethylsilane as external standard and methanol as internal standard to determine precise chemical shift.

Cobyric acid was prepared from vitamin B_{12} as described by Renz [24] with the modifications in final product purification previously published [7]. Cobinamide was available from these preparations as a major side product.

Preparation of the Coβ-adenosyl forms of cobyric acid and of the cobinic acid pentaamides was as previously described [26]. In these preparations, the cyanoaquocobinic acid pentaamide or cyanoaquocobyric acid was dissolved in deoxygenated distilled/deionized water (1 mg/2 ml). Argon or nitrogen gassing of the solution was used to maintain the oxygen-free atmosphere of the solution. A crystal of cobalt nitrate was added as catalyst [27] and the solution was continuously stirred. After sealing the solution in a vial, and continuing gassing via a septum, a 10-fold molar excess of aq.(deoxygenated) $NaBH_4$ was added via syringe. The color of the solution turned dark grey and gas was given off. The solution was transferred to the dark room, where a 1.5 fold molar excess of 5′-iodo-5′-deoxyadenosine (Aldrich) in 1.0 ml deoxygenated water was injected. After stirring an additional 15 min at room temperature in the dark, excess borohydride was destroyed by addition of 2.0 ml 1 *M* acetic acid, and the solution was phenol extracted. Final purification was via preparative high voltage paper electrophoresis (0.05 *M* acetate buffer, pH 4.5). Due to their light-sensitivity, the Coβ-adenosyl corrinoids were stored frozen, desiccated and protected from the light, and were handled only in the dark room in the presence of a red safety lamp.

Analytical HPLC

Analytical HPLC was performed using a Waters system with dual Model 501 high-pressure pumps, an automated gradient controller and a Model 441 fixed-wavelength absorbance detector with a 365-nm filter. A Kipp and Zonen Model BD41 flatbed recorder was used to record absorbance data. Peak area was used to estimate the percent of individual components in mixtures and when corrinoid stereoisomers clearly separated during chromatography. Two different types of columns, ion exchange (Waters $-NH_2$ or Whatman Partisil-5-SAX), and reversed-phase (Waters μBondapak C_{18} or Alltech Adsorbosphere C_{18}), were used. An RCSS CN guard column was employed for the ion-exchange experiments, and an RCSS C_{18} guard column for the reversed-phase experiments.

With ion exchange, pyridine acetate buffers in water–tetrahydrofuran (96:4) [3] at 80 m*M* or less concentration and pH ranges from about 6.0 to 3.5 were used as described in the text, whereas with reversed-phase columns, methanol–aqueous acetic acid [6] or pyridine acetate buffers mixed with 30% or less acetonitrile were employed. All columns used except the Whatman Partisil-5-SAX were 25 cm × 4.6 mm I.D.; the Partisil column was 10 cm × 4.6 mm I.D. Corrinoids were dissolved in HPLC-grade water and filtered via syringe using an 0.2-μm PTFE or nylon membrane before injection. Amounts of incomplete corrinoids injected ranged from 1–10 nmoles in 20 μl HPLC-grade water or HPLC-grade water containing 0.01% KCN, depending on the experiment. A maximum of 15 nmol were used in one experiment when corrinoid isomers were collected from the analytical column.

Additional analytical and preparative techniques

Analytical electrophoresis was performed on paper using buffers and techniques previously described [26]. Determination of the pK_a value for each of the cobinic acid pentaamide isomers was essentially as suggested by Anton *et al.* [23] for the cobamic acids using corrinoid migration on paper electrophoresis in buffers of varying pH with B_{12} as an external standard. To substantiate the electrophoretically determined pK_a values, cobinic acid pentaamide-2 was also titrated by standard analytical means, and the pK_a value determined from the titration plot.

Analytical thin-layer chromatography (TLC) was performed on silica gel 60-coated (0.2 mm thickness) plastic plates (E. Merck, Darmstadt, F.R.G.), and preparative TLC was run on glass-backed plates of the same material from E. Merck, layer thickness 2 mm. Solvents used for both analytical and preparative TLC were those described in ref. 28. Analytical ascending paper chromatography was performed using Whatman No. 1 paper in solvent systems E, H and I as described by Bernhauer *et al.* [29]. Visible and UV absorbance spectra were recorded using a Perkin-Elmer Lambda 3B UV–VIS spectrophotometer with 1-ml quartz cells (10 mm light path) and a PE R100A recorder. Concentrations of incomplete corrinoids in solution were calculated using published molar extinction values [17,30], or, in the case of cobinic acid pentaamide-2, using our own experimentally determined values (Table I).

TABLE I

YIELDS AND PHYSICAL/CHEMICAL CHARACTERISTICS OF SEVERAL COMPLETE AND INCOMPLETE CORRINOID ACIDS PREPARED FROM CYANOCOBALAMIN

	Corrinoid					
	Cobamic acids[a]			Cobinic acids[b]		
	1[c]	2[d]	3[e]	1[c]	2[d]	3[e]
Yield (%)[f]	6.7(7.1)	9.4(15.1)	6.9(9.0)	35	73	51
Overall yield	–	–	–	2.4	6.9	3.5
pK_a[g]	5.10 ± 0.11	4.52 ± 0.07	4.62 ± 0.07	3.30 ± 0.01	3.39 ± 0.01	3.34 ± 0.01

Corrinoid	Electronic spectrum: λ (nm) (ε_M)[h]			
	α	β	γ	UV_{max}
Cobyric acid-dicyano-Coβ-adenosyl	578(8950)	539(7950)	367(28 100)[i]	314(11 150), 260[k](18 200)
	466(10 300)[j]		351(18 050)	315 and 304(17 350), 262(37 800)
Cobinic acid-1[c]-dicyano-Coβ-adenosyl	580[l]	540(6650)	368[l]	311(13 400), 260[k](28 550)
	465(8250)[j]		349[k](15 750)	313(21 500), 262(45 300)
Cobinic acid-2[d]-dicyano-Coβ-adenosyl	579(8300)	539(7000)	367(25 000)	313(13 950), 255[k](36 600)
	460(10 250)[j]		349[k](15 250)	315(26 100), 261(56 000)
Cobinic acid-3[e]-dicyano-Coβ-adenosyl	579[l]	539(6900)	367[l]	311(12 600), 259[k](25 800)
	462(8100)[j]		347[k](11 600)	315 and 303(20 800), 262(39 800)

[a] Prepared per Anton *et al.* [23] by mild acid hydrolysis of cyanocobalamin.
[b] In the cyanoaquo form.
[c] Isomer 1 is the [*a,b,c,e,g*]pentaamide [23].
[d] Isomer 2 is [*a,c,d,e,g*]pentaamide [23].
[e] Isomer 3 is [*a,b,c,d,g*]pentaamide [23].
[f] The yields shown in parentheses are those reported by Anton *et al.* [23].
[g] Cobamic acid pK_a values are as reported in ref. 23.
[h] ε_M = Molar extinction. Values in l/mol · cm. Extinctions are rounded off to the nearest 50.
[i] Very close to those values reported in ref. 30.
[j] No clear maximum above 500 nm.
[k] Shoulder.
[l] Extinctions were determined for cobinic acid-2 by weight; the standard values determined for -2 at the α and γ bands were assumed for -1 and -3 as well and were used in these calculations.

RESULTS AND DISCUSSION

Preparation/characterization of cobinic acid isomers

The yields and certain characteristics of the isomeric cobinic acid pentaamides-1, -2 and -3 are summarized in Table I. The intermediate compounds prepared initially from cyanocobalamin were the cobamic acid pentaamide isomers arising from the hydrolysis of the propionamide groups at corrin ring positions *b, d* and *e* originally described by Anton *et al.* [23]. The location on the corrin ring of each free carboxylic acid in the cobamic series was determined by these authors using ^{13}C NMR; two recent papers substantiate these designations and complete the unabiguous isomer assignments using 1- and 2-dimensional NMR [31,32].

Since the cobinic acids were prepared from the cobamic acids by removal of the α-side nucleotide loop using the mild conditions of Renz [24], we have assumed that the location of the cobinic acid pentaamide free acid group in each case is identical with that of the cobamic acid from which it was derived. Such an assumption seems warranted by two facts. First, Brown and Peck-Siler [10] observed that when [^{13}C]methylcobalamin was converted to [^{13}C]methylcobinamide via the same method of nucleotide removal that we used, no structural changes in the corrinoid except those anticipated by nucleotide removal were identified using NMR. Second, our own preliminary ^{13}C NMR work on the cobinic acid pentaamide isomers [33] has given spectra which can be correlated fairly easily with the spectra of the cobamic acids from which they were derived [23,31,32]. This is especially true in the area of the downfield carbon resonances (carbonyl, imine and pyrrole), where isomeric variations are likely to be seen.

It is interesting to note that the cobinic acid pentaamide-2 analogue prepared by us via the procedure of ref. 23 followed by that of ref. 24, is identical by HPLC, TLC, analytical electrophoresis and analytical ascending chromatography to the only cobinic acid pentaamide we reported previously [7] as derivable in significant amounts from the mild acid hydrolysis of cobinamide. In addition, hydrolysis of the amide group at ring position *b* in cobalamin, to give cobamic acid pentaamide-2 (cobamic acid [*a,c,d,e,g*]pentaamide), was the easiest to accomplish, i.e. gave the highest yield reported by Anton *et al.* [23]. Thus the removal of the amide group at position *b* on the corrin ring when hydrolysis is accomplished under mild conditions using acid appears to be especially facile.

As is shown in Table I, the pK_a values for the cobinic acid pentaamides are from 1–1.5 pH units lower than those for the corresponding cobamic acid pentaamides. The apparent effect of these differences on separation of the cobinic acid pentaamide-1, -2 and -3 structural isomers compared to separation of the cobamic acid pentaamide isomers by HPLC is discussed in the next section.

Fig. 2 illustrates the complete electronic absorption spectrum of the Coβ-adenosyl and dicyano forms of cobinic acid pentaamide-2. In addition, the major absorption bands in the electronic spctra of the Coβ-adenosyl and dicyano forms of cobyric acid and of the three the cobinic acid pentaamide structural isomers are listed on Table I along with the molar extinction coefficient for each major band. As can be seen from this list, the dicyano-forms have the same band locations, but slightly different extinctions, whereas the Coβ-adenosyl forms exhibit somewhat different locations of the β bands, and variation in almost every extinction value.

HPLC analysis of cobinic acid pentaamide isomers

Fig. 3 illustrates a typical data print-out from ion-exchange HPLC of the cyanoaquocobinic acid pentaamide isomers and cobyric acid. Tables II–IV summarize the results when the behavior of the cobinic acid pentaamide isomers on HPLC was compared to the behavior of cyanocobalamin, aquocobalamin, cobinamide, cobyric acid and the cobamic acid pentaamides-1, -2 and -3. Table II shows, for example, that when using an ion-exchange column and in the absence of KCN in the buffer, the incomplete corrinoids in their cyanoaquo-forms all exhibited two components, in a pH-dependent manner, whereas the complete corrinoids uniformly showed but one component at all pH values tested. In those cases where the components were

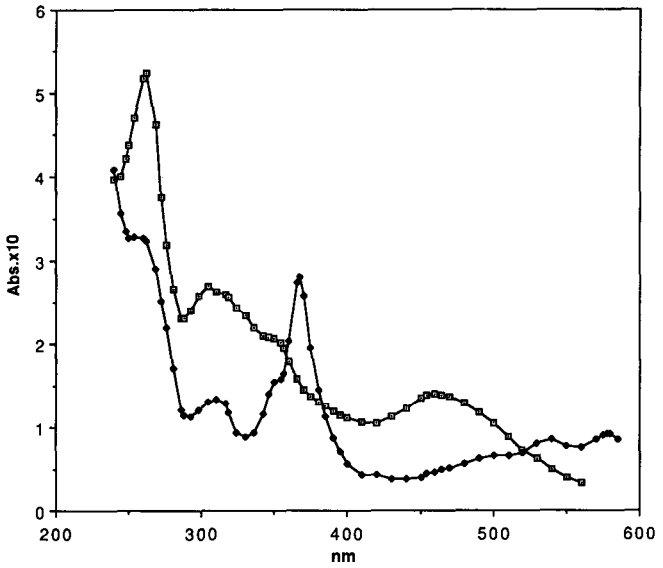

Fig. 2. Electronic absorption spectrum of cobinic acid [*a,c,d,e,g*]pentaamide, 11.2 μM. □ = Coβ-adenosyl- (neutral pH, water); ■ = dicyano- (pH > 10, 0.1 *M* aq. KCN).

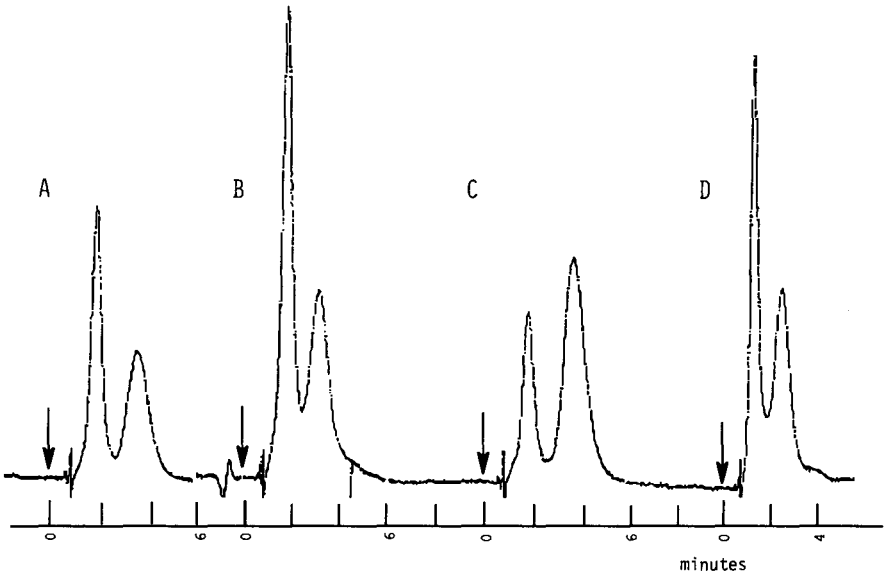

Fig. 3. Typical HPLC behavior of cyanoaquo stereoisomers of the cobinic acid pentaamides and cobyric acid. Whatman Partisil 5-SAX column, 80 m*M* pyridine acetate buffer (in water–tetrahydrofuran, 96:4) pH 3.58; 2.0 ml/min., 365 nm filter on detector, 0.05 a.u.f.s. The arrows indicate injection points in time plot. (A) Cobinic acid[*a,b,c,e,g*]pentaamide (4.9 nmol/20 μl); (B) cobinic acid[*a,c,d,e,g*]pentaamide (6.5 nmol/20 μl); (C) cobinic acid[*a,b,c,d,g*]pentaamide (4.9 nmol/20 μl); (D) cobyric acid (4.6 nmol/20 μl).

TABLE II

ION-EXCHANGE HPLC OF INCOMPLETE CORRINOIDS

Partisil-5-SAX column 10 cm × 4.6 mm I.D.; isocratic; eluting buffer: 80 m*M* pyridine acetate in water–tetrahydrofuran (96:4) at the pH indicated; + KCN = addition of 0.02% KCN (all corrinoids were pre-treated with 0.02% KCN when KCN was used in the buffer); 1.0 ml/min pump rate.

Corrinoid	Retention time (min) (component % of total)					
	Buffer pH 3.62		Buffer pH 4.40		Buffer pH 6.05	
	−KCN	+KCN[a]	−KCN	+KCN[a]	−KCN	+KCN[a]
Cyanocobalamin	3.5(100)	3.6(100)	3.5(100)	3.8(100)	3.8(100)	3.6(100)
Aquocobalamin	2.7(100)	3.6(100)	2.6(100)	–	3.5(100)	–
Cobamic acid-1[b]	–	–	4.6(100)	4.1(100)	–	5.5(100)
-2	–	–	6.0(100)	4.6(100)	–	5.1(100)
-3	–	–	6.9(100)	4.7(100)	–	5.2(100)
Cobinamide	2.6(68)	3.0(100)	2.6(−)[c]	3.0(100)	4.3(−)[c]	3.2(100)
	3.7(32)		3.0(−)		4.7(−)	
Cobinic acid-1[b]						
cyanoaquo	3.2(49)	3.7(100)	4.5(100)	3.8(100)	4.5(100)	4.4(100)
	4.9(51)					
Coβ-adenosyl	6.2[d]					
Cobinic acid-2						
cyanoaquo	3.0(56)	3.5(100)	4.0(100)	3.8(100)	3.1(49)	3.9(100)
	4.3(44)				4.0(51)	
Coβ-adenosyl	5.4[d]					
Cobinic acid-3						
cyanoaquo	3.2(30)	3.7(100)	4.2(−)[c]	3.8(100)	3.9(−)[c]	4.1(100)
	5.0(70)		4.8(−)		4.3(−)	
Coβ-adenosyl	5.9[d]					
Cobyric acid						
cyanoaquo	2.7(52)	3.4(100)	3.4(100)	3.5(100)	3.5(−)[c]	3.7(100)
	3.8(48)				3.8(−)	
Coβ-adenosyl	3.6[d]					

[a] Cobamic acids were in the cyano form; when the buffer contained KCN, each incomplete corrinoid was converted to its dicyano form which appeared as a single peak.

[b] For structural designations and systematic names see Table I.

[c] Components not clearly enough separated to calculate percentages.

[d] Single broad peak; 2.0 ml/min pump rate.

sufficiently separated, the percent of each component was calculated by integrating the area under the curve. For all of the cobinic acid pentaamide isomers, cobinamide and cobyric acid, the best separation of the two stereoisomers in each case occurred when the pH was below 4, but only when KCN was absent from the buffer. In the presence of KCN in the buffer, a single corrinoid form was stabilized and only one component was found for each of the incomplete corrinoids, presumably that of the dicyano-corrinoid, and its retention time was approximately half way between those of the two cyanoaquo stereoisomers. In some experiments, when dicyanocobinic acid pentaamide was injected, and buffer without KCN was used, a small peak of intermediate retention time, along with peaks corresponding to the two cyanoaquo stereoisomers, was observed. This component appeared as a minor constituent, with variations in

TABLE III

REVERSED-PHASE HPLC OF INCOMPLETE CORRINOIDS

An Alltech Adsorbosphere C_{18} 25 cm × 4.6 mm I.D. column was used througout. Isocratic conditions: acetonitrile–80 m*M* pyridine acetate (30:70) in water–tetrahydrofuran (96:4) pH 3.6 buffer (buffer A); 1.0 ml/min. Gradient conditions: 10-min linear gradient, starting at acetonitrile–buffer A (5:95), ending at acetonitrile–buffer A (30:70); 2.0 ml/min.

Corrinoid	Retention time (min) (component % of total)	
	Isocratic	Gradient
Cyanocobalamin	3.1(100)	5.8(100)
Aquocobalamin	6.9(100)[a]	–
Cobamic acids-1, -2 and -3[b]	3.2–3.3(100)[c]	6.9(100)[c]
Cobinamide	5.6(55)	7.4(61)
	6.3(45)	9.9(39)
Cobinic acid-1[b]		
cyanoaquo	5.8(47)	8.1(47)
	6.6(53)	10.6(53)
Coβ-adenosyl	3.9[d]	
Cobinic acid-2		
cyanoaquo	5.7(48)	7.7(51)
	6.3(52)	9.9(49)
Coβ-adenosyl	3.8[d]	
Cobinic acid-3		
cyanoaquo	5.8(25)	8.2(17)
	6.5(75)	10.9(83)
Coβ-adenosyl	3.9[d]	
Cobyric acid		
cyanoaquo	5.4(42)	6.2(60)
	6.0(58)	9.2(40)
Coβ-adenosyl	3.4[d]	

[a] Broad peak.
[b] For structural designations and systematic names see Table I.
[c] All three isomers had indistinguishable retention times under these conditions.
[d] 2.0 ml/min pump rate; very sharp peak.

retention time and amount observed, but seemed to correspond to the dicyano-form. The appearance of this component is in keeping with the observations of Reenstra and Jencks [16] who reported that the dicyano-form of cobalamin (in its "base-off" form at low pH) was the obligate intermediate in the reversible formation of the cyanoaquocobalamin stereoisomers. It should be noted here that our experiments showed this behavior when either ion-exchange or reversed-phase columns were used. It is puzzeling that although Stupperich *et al.* [6] mentioned the problem of multiple peaks when dicyanocobinamide was injected in a reversed-phase column without KCN present in the buffer, Jacobsen *et al.* [5] reported only a single component with a very short retention time under similar conditions. The observation of a single component for dicyanocobinamide in the latter case was probably due to the fortuitous choice of buffer pH and ionic strength, since formation of the cyanoaquo stereoisomers from the dicyano form is rapid and facile under almost all of the large number of experimental conditions we tested unless KCN was present in the buffer.

TABLE IV

REVERSED-PHASE HPLC OF INCOMPLETE CORRINOIDS FOLLOWED BY TLC OR HPLC OF COLLECTED STEREOISOMERS

HPLC conditions: Waters μBondapak C_{18} column; solvent methanol–17 m*M* acetic acid (40:60), isocratic, 1.0 ml/min; fractions were collected directly from the detector outflow and immediately dried under a stream of nitrogen. TLC was run on analytical silica gel plates in 2-propanol–28% NH_4OH–water (7:1:2) [28] using corrinoid solutions reconstituted from the HPLC-derived powders. I and II refer to the original HPLC order of elution: I = faster component, II = slower component.

Corrinoid[b]	Retention time (min) (component % of total)		R_F, TLC	
	Original HPLC	Second HPLC[a]	I	II
Cobinamide	I 7.6(65)	I 7.7(59)	0.57	0.54
		9.4(41)	0.14	0.14
				0.10
	II 9.5(35)	II 8.3(45)		
		10.1(55)		
Cobinic acid-1[c]	I 6.9(53)	I 7.6(54)	0.62	0.62
		10.8(46)	0.28	0.28
			0.095	0.069
	II 8.6(47)	II 8.3(47)		
		10.3(53)		
Cobinic acid-2	I 7.5(58)	I 8.5(60)	0.49	0.59
		11.4(40)	0.40	
			0.22	0.23
	II 10.1(42)	II 9.1(29)	0.034	0.052
		13.3(68)		
Cobinic acid-3	I 9.5(34)	I 10.2(27)	0.54	0.53
		14.2(73)	0.22	0.19
	II 12.8(66)	II 10.0(19)		
		13.3(81)		
Cobyric acid	I 5.7(49)	I 6.5(49)	0.38	0.39
		7.7(51)	0.11	0.13
	II 7.1(51)	II 6.3(46)		
		7.8(54)		

[a] HPLC fractions for reinjections were prepared from the original HPLC-derived powders by addition of HPLC-grade water. Conditions for rerun were exactly the same as in the original HPLC runs.

[b] All incomplete corrinoids were in their cyanoaquo forms.

[c] For cobinic acid pentaamide structural designations see Table I.

Table II also illustrates the problem of separating the three isomeric cobinic acid pentaamides from each other based on their pK_a values using ion-exchange chromatography. Unlike the cobamic acid isomers which have pK_a values differing by 0.1 up to 0.5 pH units (see Table I), and which are separable in mixtures via HPLC on an ion-exchange or a reversed-phase column [3], the cobinic acid pentaamide isomers have pK_a values differing by less than 0.1 pH unit, and mixtures are not clearly separable on an ion-exchange or reversed-phase column, even with changes in buffer pH and/or ionic strength, using isocratic or gradient elutions.

Table III illustrates the behavior of the cyanoquo stereoisomers on a reversed-phase column using an aqueous buffer and an organic solvent mixture for elution. Clear separation of the isomers occurred under both isocratic and gradient conditions,

although the gradient gave a greater difference in retention times for the two stereoisomers for each. In the case of the cobinic acid pentaamide-3, however, the separation was complicated by a large column hold up, which required a 100% methanol wash to remove.

Data are shown in Tables II and III illustrating the behavior of the Coβ-adenosyl forms of the cobinic acid pentaamide isomers as well as of cobyric acid. The Coβ-adenosyl-corrinoids are assumed to be the forms in which the corrins exist in the biosynthetic sequence in producer microorganisms, as well as the form involved in many important metabolic transformations [1,2]. The reversed-phase (C_{18} column) HPLC data have been reported on the behavior of Coβ-adenosylcobinamide [5], and this work was not repeated here. Our data support the assertion that, in contrast to the cyanoaquo forms, the Coβ-adenosyl-forms of the incomplete corrinoids do not isomerize during HPLC, at least under the various experimental conditions we employed (see Tables II and III).

Table IV shows the results of collection of the separate stereoisomers from the HPLC (reversed-phase) column, then reinjection of each on the same column under the same conditions, and TLC analysis of each component. As can be seen from Table IV, reinjection of each stereoisomer again gave two stereoisomers, as one might predict from the thermal instability of the cyanoaquo stereoisomers as described by Friedrich and co-workers [18–20]. The same sort of behavior was seen on TLC, in cyanide-free solvents. In fact, more than two spots were seen in several cases, possibly corresponding to intermediate forms, or the dicyano- or the diaquocorrinoid in each case. It should be noted that the relative concentrations of methanol and 17 m*M* acetic acid used in these experiments differed from those reported by Stupperich *et al.* [6]. Their methanol–acetic acid (24:76) solvent mixture did not give a sharp separation of the cobinic acid pentaamide isomers, however, a 40:60 or 50:50 mixture was serviceable.

To summarize the results illustrated on Table IV, the reinjected solutions showed two components, as expected, but the percent of each was either unchanged or slightly enriched in the component with the longer retention time.

In keeping with the nomenclature system devised by Friedrich and Nordmeyer [18,19] for cobyric acid using low-pressure column chromatography at 3°C, it seems plausible to designate the chromatographically slower component (longer retention time on HPLC) as the α-cyano,β-aquo (Fig. 1A) stereoisomer, and the faster component (shorter retention time) as the α-aquo,β-cyano (Fig. 1B) stereoisomer. Friedrich and coworkers [20,34] as well as others have studied the formation of the Co-methyl corrinoids of cobyric acid and cobinamide. This work has led to confusing results [see especially, discussion in ref. 10]. Some authors have found *only* the Coβ-alkyl form of cobinamide upon reductive alkylation of cobinamide [10,35], whereas others [20,34] have found *mixtures* of the Coβ/Coα-methyl incomplete corrinoids under the same synthetic conditions. Fanchiang *et al.* [36] found that when Coβ-methylcobalamin transfers the methyl group to diaquocobinamide, only the β isomer is formed. This work appears to show that the Coβ-methylcorrinoids are much more thermally stable to isomerization than the cyanoaquo forms, as was shown by Friedrich and co-workers [20,34] who reported that temperatures up to 80°C and the addition of CO gas was required for *complete isomerization* of the Co-methyl (incomplete) corrinoids. This is, of course, in contrast to the isomerization of the cyanoaquo forms, which occurs easily at room temperature.

Component percent composition data in Tables II–IV also illustrate interesting differences in stereoisomer formation among the various incomplete corrinoids tested. Cyanoaquocobinamide exhibits (Table III) an approximately equal mixture of the two stereoisomers with a slight excess of the faster (β-cyano) component during isocratic reversed-phase HPLC using acetonitrile–pyridine acetate buffer at pH 3.61. In contrast, upon isocratic reversed-phase (using methanol–acetic acid) or ion-exchange HPLC at pH 3.61, or reversed-phase HPLC using a gradient, cyanoaquobinamide shows an increase in ratio of β-cyano/β-cyano to 2:1. This is in contrast to the results of Brown and Hakimi [21] who found, using ^{13}C NMR, a 2:1 ratio of α-cyano/β-cyano isomers when $K^{13}CN$ was added to diaquocobinamide, and of Reenstra and Jencks [16] who reported a similar ratio of α- to β-cyano isomers for "base off" cyano-aquocobalamin, but agrees with the results of Stupperich *et al.* [17] who found nearly equal quantities of the cyanoaquo/aquocyano stereoisomers of the phenolyl- and cresolylcobamides in *Sporomosa ovata*. Brown and Peck-Siler [10] attribute the higher percent of the α-cyano isomer to thermodynamic control of product ratio, finding a nearly 100% preponderance of the β-$^{13}CH_3$ cobinamide when the formation of the methylaquo-stereoisomeric pairs is under kinetic control.

The ratio of the two aquocyano stereoisomers for cobinic acid pentaamides-1 and -2, and cobyric acid using both reversed-phase and ion-exchange columns approximates 1:1, however, cobinic acid pentaamide-3 shows a marked predominance of the slower (α-cyano) component with ratios of 2:1 to 4:1, α/β. According to Brown and co-workers [10,21] this shows a greater thermal stability of the α-cyano stereoisomer. This may be due to the interaction of the free –COOH at position *e* with the cyano group to stabilize it, since the *e* carboxyl is on the α-side of the corrin ring. Interestingly, Brown and Peck-Siler [10] found an interaction between α-side benzimidazole B3 –N, and the *e* side chain –NH in the "base off" benzimidazole deprotonated form of cobalamin.

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REFERENCES

1 F. Wagner, *Ann. Rev. Biochem.*, 35 (1966) 405.
2 D. Dolphin (Editor), *B-12, Vol. 2, Biochemistry and Medicine*, Wiley, New York, 1982.
3 M. Binder, J. F. Kolhouse, K. C. VanHorne and R. H. Allen, *Anal. Biochem.*, 125 (1982) 253.
4 W. B. Whitman and R. S. Wolfe, *Anal. Biochem.*, 137 (1984) 261.
5 D. W. Jacobsen, R. Green, E. V. Quadros and Y. D. Montejano, *Anal. Biochem.*, 120 (1982) 394.
6 E. Stupperich, I. Steiner and M. Rühlemann, *Anal. Biochem.*, 155 (1986) 365.
7 S. H. Ford, *Biochim. Biophys. Acta*, 841 (1985) 306.
8 L. Ljungdahl, E. Irion and H. G. Wood, *Biochemistry*, 4 (1965) 2771.
9 W. B. Whitman and R. S. Wolfe, *J. Bacteriol.*, 164 (1985) 165.
10 K. L. Brown and S. Peck-Siler, *Inorg. Chem.*, 27 (1988) 3548.
11 B. P. Hay and R. G. Finke, *J. Am. Chem. Soc.*, 109 (1987) 8012.
12 T. S. Marks, J. D. Allpress and A. Maule, *Appl. Env. Microbiol.*, 55 (1989) 1258.
13 U. E. Krone, R. K. Thauer and H. P. C. Hogenkamp, *Biochemistry*, 28 (1989) 4908.
14 S. Ragsdale, P. A. Lindahl and E. Münck, *J. Biol. Chem.*, 262 (1987) 14289.
15 T. G. Pagano, P. G. Yohannes, B. P. Hay, J. R. Scott, R. G. Finke and L. G. Marzilli, *J. Am. Chem. Soc.*, 111 (1989) 1484.

16 W. W. Reenstra and W. P. Jencks, *J. Am. Chem. Soc.*, 101 (1979) 5780.
17 E. Stupperich, H. J. Eisinger and B. Kräutler, *Eur. J. Biochem.*, 186 (1989) 657.
18 W. Friedrich, *Z. Naturforsch. B.*, 21 (1966) 595.
19 W. Friedrich and J. P. Nordmeyer, *Z. Naturforsch. B*, 23 (1968) 1119.
20 W. Friedrich and R. Messerschmidt, *Z. Naturforsch. B*, 24 (1969) 465.
21 K. L. Brown and J. M. Hakimi, *Inorg. Chem.*, 23 (1984) 1756.
22 H. A. Barker, R. D. Smyth, H. Weissbach, H. Munch-Peterson, J. I. Toohey, J. N. Ladd, G. E. Volcani and R. N. Wilson, *J. Biol. Chem.*, 235 (1960) 181.
23 D. L. Anton, H. P. C. Hogenkamp, T. E. Walker and N. A. Matwiyoff, *J. Am. Chem. Soc.*, 102 (1980) 2215.
24 P. Renz, *Methods Enzymol.*, 18c (1971) 82.
25 G. T. Bratt and H. P. C. Hogenkamp, *Arch. Biochem. Biophys.*, 218 (1982) 225.
26 S. H. Ford and H. C. Friedmann, *Arch. Biochem. Biophys.*, 175 (1976) 121.
27 D. Dolphin, *Methods Enzymol.*, 18c (1971) 43.
28 T. Toraya, E. Krodel, A. S. Mildvan and R. H. Abeles, *Biochemistry*, 18 (1979) 417.
29 K. Bernhauer, B. Becher, G. Gross and G. Wilharm, *Biochem. Z.*, 332 (1960) 562.
30 R. Bonnett, J. M. Godfrey and D. G. Redman, *J. Chem. Soc.*, (1969) 1163.
31 T. G. Pagano and L. G. Marzilli, *Biochemistry*, 28 (1989) 7213.
32 H. M. Marques, D. C. Scooby, M. Victor and K. L. Brown, *Inorg. Chim. Acta*, 162 (1989) 151.
33 S. H. Ford and B. Williams, unpublished results.
34 W. Friedrich and J. P. Nordmeyer, *Z. Naturforsch. B*, 24 (1969) 588.
35 O. Müller and G. Müller, *Biochem. Z.*, 337 (1963) 179.
36 Y. T. Fanchiang, G. T. Bratt and H. P. C. Hogenkamp, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 2698.