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Separation and study of corrinoid cobalt-ligand isomers by high-performance liquid chromatography

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ABSTRACT

Vitamin B_{12} belongs to a group of complex organo-cobalt compounds, the corrinoids. "Complete" corrinoids, such as B_{12}, contain a nucleotide as the cobalt α-(lower)ligand. This nucleotide is also connected to the periphery of the corrin ring. The "incomplete" corrinoids, in contrast, contain simple cobalt α-ligands, such as water or cyanide. Using analytical reversed-phase and anion-exchange high-performance liquid chromatography (HPLC), we have been able to study the behavior of several aquocyano-"incomplete" corrinoids: three isomeric cobinic acid pentaamides, cobinamide, and cobyric acid, all of which exist as thermally unstable isomers, which separate during HPLC. All but one of these corrinoids gave isomer mixtures of 1:1, the exception giving mixtures of 2:1 to 3:1. The separated stereoisomers had different retention times and were collected from analytical columns for further study.

INTRODUCTION

Vitamin B_{12} (cyanocobalamin) is a member of a group of complex organo-cobalt compounds called the corrinoids. Vitamin B_{12} itself is a "complete" corrinoid, having not only the corrin ring, which supplies four bonds to the central cobalt atom, but also a fifth and sixth ligand. These ligands are a cyanide group on the β (upper) side of the corrin ring and a nucleotide loop, which is connected to the periphery of the ring at position *f* (see Fig. 1) on the α (lower) side of the ring. Although most of the biologically important corrinoids are "complete", there is a group of naturally occurring corrins which do not contain the α side nucleotide loop and are therefore

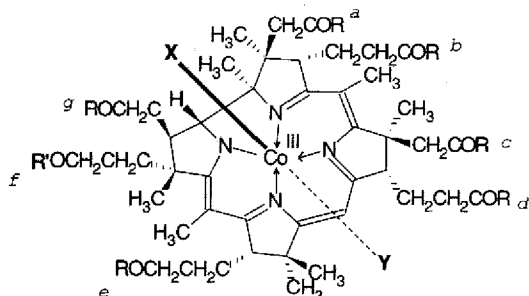

Fig. 1. Structure of the corrin ring.

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called "incomplete." Such corrinoids are of interest mainly as known or suspected cobalamin precursors, although one or two have also been identified as biologically active coenzymes [1]. In addition, study of the cobalt-ligand chemistry and reactivity in incomplete corrinoids has given insight into the mechanism of action of cobalamin-dependent enzymes [2].

The incomplete corrinoids, most of which are six-coordinate, may contain any two of a number of possible cobalt ligands in the α and β (axial, lower and upper, respectively) positions. Although water and/or cyanide are the ligands usually encountered, alkyl groups, carbohydrates, or amino acids can be attached as the sixth ligand under the right conditions [3,4]. When two different fifth and sixth ligands are present in incomplete corrinoids, then two different stereoisomers are possible. Fig. 1 illustrates the corrin ring, with the fifth and sixth ligands designated as X and Y. When these ligands are a water molecule and a cyanide group, the name of the corrinoid incorporates aquocyano as a prefix. The position of each of these ligands [*i.e.* whether α (lower) or β (upper)] is known to shift rather rapidly at room temperature, but the individual stereoisomers can be "frozen out" and separated during chromatography at 3°C, as was demonstrated by Friedrich [5,6] in the case of cobyric acid.

HPLC can be used to separate and identify many of the naturally occurring corrinoids from bacterial or mammalian materials [7,8]. Although the complete corrinoids are the ones usually targeted for identification, the incomplete corrinoids are often encountered in mixtures from natural sources. Since these corrinoids can exist as stereoisomers, they often appear as double peaks during high-performance liquid chromatography (HPLC), leading to possible confusion about purity and identity of corrinoid mixture components. This study was designed to investigate the formation/isomerization of the (aquocyano) incomplete corrinoid stereoisomers during HPLC.

EXPERIMENTAL

All solvents used for HPLC were of HPLC grade. Water for HPLC buffers and solutions was distilled, deionized in a Millipore (Milford, MA, U.S.A.) system, filtered through a 0.2-μm membrane, and then degassed. Other chemicals and solvents were of analytical reagent grade.

High-performance liquid chromatography

HPLC was performed with a Waters system (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.), consisting of an automatic gradient controller, dual Model 501 high-pressure pumps, a Rheodyne injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl injection loop, and a Model 441 fixed-wavelength detector with a 365-nm filter. Peak areas from the data print-out on a Kipp & Zonen (Delft, The Netherlands) BD41 flatbed recorder were used to estimate the percentage of individual components in mixtures and of individual, separated stereoisomers. Both ion-exchange (Waters -NH_2 or Whatman Partisil 5-SAX, Whatman, Clifton, NJ, U.S.A.) and reversed-phase (Waters μBondapak C_{18} or Alltech Adsorbosphere C_{18}, Alltech Assoc., Deerfield, IL, U.S.A.) columns were used. The guard column, inserted between the injector and the analytical column, was RCSS CN (Millipore, Waters Chromatography Division) for ion-exchange and RCSS C_{18} for reversed-phase

chromatography. The Waters -NH_2 and Alltech Adsobosphere C_{18} columns were 250 × 4.6 mm I.D. The Waters μBondapak C_{18} column was 300 × 4.6 mm I.D. and the Whatman Partisil 5-SAX was 100 × 4.6 mm I.D.

Solvents used for ion-exchange HPLC were water–tetrahydrofuran (96:4) pyridine acetate buffers at 80 m*M* or less, pH range 6.0–3.5. Solvents for reversed-phase chromatography were methanol with aqueous acetic acid or acetonitrile with aqueous pyridine acetate buffer. Use of these solvents was as described in the text. Corrinoids were dissolved in HPLC-grade water and syringe-filtered through a 0.2-μm PTFE membrane (Vanguard, Neptune, NJ, U.S.A.) before injection. The amounts of incomplete corrinoid were in the 1–2-nmol range in 20-μl water or 0.01% aqueous KCN.

Corrinoid preparation

Incomplete corrinoids were obtained by chemical degradation of cyanocobalamin (Sigma, St. Louis, MO, U.S.A.). Cobyric acid (Fig. 1: R = NH_2, R′ = OH) was prepared by the Renz method [9] and cobinamide (Fig. 1: R = NH_2, R′ = $NHCH_2CHOHCH_3$) was obtained as a major side-product from that preparation. The cobinic acid pentaamides (isomers 1 or 2 or 3, respectively, Fig. 1: R(*d* or *b* or *e*) = OH, otherwise R = NH_2, R′ = $NHCH_2CHOHCH_3$) were prepared via weak-acid hydrolysis [10], followed by treatment with cerous hydroxide [9]. All of these compounds were purified by Whatman DE-53 (acetate) low-pressure column chromatography with 0.05% aqueous HCN/0.08% aqueous acetic acid. After desalting by phenol extraction, and drying under a nitrogen stream, the aquocyano form was obtained for each of these incomplete corrinoids as a powder or glass. This form of incomplete corrinoids has a characteristic electronic absorption spectrum, with the γ band (the most intense in the spectrum), located at *ca.* 350–354 nm.

Diaquocorrinoids were prepared, with some modifications, following the method of Baldwin *et al.* [11] for diaquocobinamide. These compounds were frozen in sealed vials in aqueous solution and stored under nitrogen to prevent oxidation [11].

Corrinoid standards

Small quantities of cobyric acid and cobinamide, were kindly supplied by H. C. Friedmann (University of Chicago, Chicago, IL, U.S.A.).

Electronic absorption spectrophotometry

This was performed in matched quartz cuvettes, using a Perkin-Elmer (Norwalk, CT, U.S.A.) Lambda 3B UV–VIS spectrophotometer (1 cm light path), and spectra were recorded on a PE R-100A Recorder.

Analytical thin-layer chromatography

This was performed on silica gel 60 plates (0.2 mm thickness) (E. Merck, Darmstadt, F.R.G.) with the solvents described in ref. 12.

RESULTS AND DISCUSSION

Table I shows the results of both ion-exchange and reversed-phase HPLC of both the aquocyano incomplete corrinoids and the diaquo forms. These results illustrate several important characteristics of the aquocyano stereoisomers. First, all of the incomplete corrinoids tested, with only a couple of exceptions, showed approximately equal percentages of the component stereoisomers, when cyanide was absent from the eluting solvent in both ion-exchange and reversed phase chromatography. The appearance of two peaks for each of the aquocyano- forms is as predicted by Friedrich and co-workers [6,13,14] who described the separation of the stereoisomers at 3°C by low-pressure column chromatography, and assigned structures according to their retention times: short retention corresponds to the α-aquo,

TABLE I

ISOCRATIC ION-EXCHANGE (IE) AND REVERSED-PHASE (RP) HPLC OF INCOMPLETE CORRINOID STEREOISOMERS

RP conditions: Alltech Adsorbosphere C_{18} column; 70% 80 m*M* pyridine acetate buffer in 4% aqueous tetrahydrofuran (pH 3.61) and 30% acetonitrile; 1.0 ml/min. IE Conditions: Whatman Partisil-5-SAX Column; 80 m*M* pyridine acetate buffer in 4% aqueous tetrahydrofuran (pH 3.62); 1.0 ml/min.

Corrinoid[a]	Retention time (min) (component % of total)		
	IE		RP, −KCN
	−KCN	+KCN	
(AqCN)Cobinamide $R=NH_2$, $R'=NHCH_2CHOHCH_3$	2.6(68) 3.7(32)	3.0(100)[b]	5.6(55) 6.3(45)
$(Aq)_2$Cobinamide	5.0(100)[c]	3.1(100)[b]	13.6(100)[c]
(AqCN)Cobyric acid $R=NH_2$, $R'=OH$	2.7(52) 3.8(48)	3.4(100)[b]	5.4(42) 6.0(58)
$(Aq)_2$Cobyric acid	3.6(100)[c]	–	–
(AqCN)Cobinic acid-1[d]	3.2(49) 4.9(51)	3.7(100)[b]	5.8(47) 6.6(53)
$(Aq)_2$Cobinic acid-1	5.7(100)[c]	3.6(100)[b]	–
(AqCN)Cobinic acid-2[e]	3.0(56) 4.3(44)	3.5(100)[b]	5.7(48) 6.3(52)
$(Aq)_2$Cobinic acid-2	5.1(100)[c]	–	12.6(100)[c]
(AqCN)Cobinic acid-3[f]	3.2(30) 5.0(70)	3.4(100)[b]	5.8(25) 6.5(75)
$(Aq)_2$Cobinic acid-3	5.1(100)[c]	–	–

[a] (AqCN) = Axial ligands on cobalt are water and cyanide (= aquocyano); $(Aq)_2$ = both axial ligands on cobalt are water (= diaquo).

[b] In KCN both axial ligands on cobalt are cyanide (= dicyano).

[c] Single, *broad* peak centered at this retention time.

[d] Cobinic acid (*a,b,c,e,g*) pentaamide (= *d* OH, *f* = $NHCH_2CHOHCH_3$).

[e] Cobinic acid (*a,c,d,e,g*) pentaamide (= *b* OH, *f* = $NHCH_2CHOHCH_3$).

[f] Cobinic acid (*a,b,c,d,g*) pentaamide (= *e* OH, *f* = $NHCH_2CHOHCH_3$).

β-cyano stereoisomer (Fig. 1: $X=CN$, $Y=OH_2$) and long retention corresponds to the β-aquo, α-cyano isomer (Fig. 1: $X=OH_2$, $Y=CN$).

Second, when cyanide was present in the solvent, all of the incomplete corrinoids showed a single component, presumably the dicyano form. In all cases, this dicyano form had a retention time *in between* the retention times of the two aquocyano stereoisomers. This dicyano form was unstable, unless cyanide was present in the eluting solvent. When dicyanocobinamide was injected under the IE or RP conditions described in Table I, and no cyanide was present in the solvent, the major components formed were the two aquocyano stereoisomers. Only a small amount, if any, of the dicyano form persisted. Our observations in this regard correspond to those in ref. 7,

TABLE II

SEPARATION AND REFORMATION OF CORRINOID STEREOISOMERS ON ION-EXCHANGE HPLC

HPLC Conditions: Whatman Partisil-5-SAX Column, 80 m*M* pyridine acetate in 4% aqueous tetrahydrofuran (pH 5.97 or 3.48, as noted below); 1.0 ml/min. Stereoisomers collected directly from the detector effluent were concentrated under an N_2 stream before reinjection; only the highest part of each peak was collected.

Corrinoid[b]	Retention time (min) (Component % of Total)				
	Cpt.[a]	Original injection, pH 5.97	Reinjection		
			Cpt.	pH 5.97	pH 3.48
Cobinamide	I	3.8(43)	I	4.1(46)	2.9(64)
				6.1(54)	4.7(36)
	II	5.2(57)	II	4.1(32)	3.0(63)
				6.2(68)	4.6(37)
Cobyric acid	I	3.1(52)	I	3.1(31)	2.5(47)
				3.9(30)[c]	3.5(53)
	II	3.9(48)	II	3.1(28)	2.6(74)
				3.9(65)[c]	3.5(26)
Cobinic acid-1[d]	I	3.7(50)	I	3.8(33)	3.0(41)
				4.7(30)[c]	4.6(59)
	II	4.5(50)	II	3.7(16)	3.0(85)
				4.4(50)[c]	4.7(15)
Cobinic acid-2	I	3.3(49)	I	3.3(30)	3.0(56)
				4.1(38)[c]	4.5(44)
	II	4.1(51)	II	3.5(16)	2.9(88)
				4.2(73)[c]	4.6(12)
Cobinic acid-3	I	3.5(75)	I	3.5(34)	2.9(48)
				4.5(33)[c]	4.7(52)
	II	4.5(25)	II	3.5(12)	2.9(87)
				4.4(72)[c]	4.9(13)

[a] Designation for stereoisomers: Cpt. I and Cpt. II.

[b] All corrinoids were in the aquocyano form.

[c] Balance of % accounted for by a slow-moving component, which appears as a broad peak and is probably the diaquo form (see Table I).

[d] For nomenclature of the cobinic acid pentaamides, see Table I.

but contrast with those in ref. 15, where a single component of short retention time was reported when dicyanocobinamide was injected.

Third, the diaquo forms of all of the cobinic acid isomers and cobinamide exhibited longer retention times, whereas diaquocobyric acid showed an intermediate retention time.

Finally, cobinic acid-3 (the *a,b,c,d,g*-pentaamide) exhibited percentages of component stereoisomers that were very different from those of other incomplete corrinoids tested, the faster component being present at 1/2 to 1/3 of the amount of the slower component. According to refs. 16 and 17 this behavior may indicate a greater thermal stability of the α-cyano stereoisomer, or it may be due to the interaction of the corrin ring-peripheral carboxylic acid/carboxylate or amides (Fig. 1, R groups) with the α side cyanide (Fig. 1, Y ligand) [16].

Table II shows the result of collecting the stereoisomers from the analytical ion-exchange column and then reinjecting them at two different pH values. The original injection in this case was at pH 5.97. In all cases, except that of cobinic acid-3, the stereoisomers were formed in a ratio of about 1:1. When each individual component was then reinjected into the same column at the same or different pH, both isomers were again formed, but no longer in 1:1 ratio. At pH 5.97, the component with longer retention time usually predominated, and the diaquo form appeared. Reinjection at pH 3.48 usually showed the opposite: predominance of the component with the shorter retention time. These data clearly show that the stereoisomers were, in fact, thermally unstable, facile reformation of the "other" isomer occurring at room temperature, although the amount of each isomer formed was dependent on solvent pH and corrinoid structure. These results support the assertions in ref. 6 to the effect that the cyanide group can "migrate" from one corrinoid to another at elevated temperatures.

The separated stereoisomers were studied not only by HPLC, but also by thin-layer chromatography. As one would predict, each stereoisomer showed two or three components in thin-layer chromatography with 2-propanol–28% ammonia–water (7:1:2) [12]. R_F values always clustered in 3 areas: 0.5–0.6, 0.15–0.25 and 0.05, corresponding to, respectively, the two aquocyanostereoisomers (fast and slow), and the diaquo form.

Friedrich [6] described differences in the spectra of the separated aquocyano stereoisomers of cobyric acid. Our attempts to discern reproducible differences in the electronic absorption spectrum of each of the separated stereoisomers were unsuccess ful, however, as one would expect in light of the thermal instability of these compounds.

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REFERENCES

1 L. Ljungdahl, E. Irion and H. G. Wood, *Biochemistry*, 4 (1965) 2771.
2 B. P. Hay and R. G. Finke, *J. Am. Chem. Soc.*, 109 (1987) 8012.
3 D. Dolphin, *Methods Enzymol.*, 18c (1971) 41.
4 D. A. Baldwin, E. A. Betterton, S. M. Chemaly and J. M. Pratt, *J. Chem. Soc., Dalton Trans.*, (1985) 1613.

5 W. Friedrich, *Biochem. Z.*, 342 (1965) 143.
6 W. Friedrich, *Z. Naturforsch. B*, 21 (1966) 595.
7 E. Stupperich, I. Steiner and M. Ruhlemann, *Anal. Biochem.*, 155 (1986) 365.
8 D. W. Jacobsen, R. Green and K. L. Brown, *Methods Enzymol.*, 123 (1986) 14.
9 P. Renz, *Methods Enzymol.*, 18c (1971) 82.
10 D. L. Anton, H. P C. Hogenkamp, T. E Walker and N. A. Matriwiyoff, *J. Am. Chem. Soc.*, 102 (1980) 2215.
11 D. A. Baldwin, E. A. Betterton and J. M. Pratt, *J. Chem. Soc., Dalton Trans.*, (1983) 217.
12 T. Toraya, E. Krodel, A. S. Mildvan and R. H. Abeles, *Biochemistry*, 18 (1979) 417.
13 W. Friedrich and J. P. Nordmeyer, *Z. Naturforsch. B*, 23 (1968) 1119.
14 W. Friedrich and R. Messerschmidt, *Z. Naturforsch. B*, 24 (1969) 465.
15 D. W. Jacobsen, R. Green, E. V. Quadros and Y. D. Montejano, *Anal. Biochem.*, 120 (1982) 394.
16 K. L. Brown and S. Peck-Siler, *Inorg. Chem.*, 27 (1988) 3548.
17 K. L. Brown and J. M. Hakimi, *Inorg. Chem.*, 23 (1984) 1756.