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Isolation of Factor III_m Coenzyme and Cobyric Acid Coenzyme plus Other B₁₂ Factors from *Clostridium thermoaceticum**

Eckart Irion and Lars Ljungdahl

ABSTRACT: Corrinoid compounds from *C. thermoaceticum* were obtained by extraction with 0.1% NaCN or with acetone. Eight corrinoids were identified in the cyanide extract and eleven were identified in the acetone extract. The most abundant complete B₁₂ factor is 5-methoxybenzimidazolylcobamide (Factor III_m) (12–17% of the total corrinoid content). It occurs mostly as its Co-5'-deoxyadenosyl derivative but also in minute amounts as hydroxy (aquo) Factor III_m and Co-methyl-Factor III_m. The main corrinoid is cobyrinic

acid (50%), and it is present predominantly as the coenzyme, but the Co-methyl derivative was also found. Other corrinoids present in small amounts are Factor B (cobinamide) coenzyme, Factor B phosphate (cobinamide phosphate) coenzyme, and most likely the di- to pentaamides of Co-5'-deoxyadenosylcobyrinic acid. These incomplete factors may be intermediates in the biosynthesis of complete B₁₂ factors. Two complete factors and several incomplete factors remain to be identified.

A total synthesis of acetate from CO₂ is carried out by *Clostridium thermoaceticum* (Wood, 1952). Poston *et al.* (1964) obtained evidence for a possible role of corrinoids in this synthesis, when they demonstrated that the methyl ligand group of Co-methylcobalamin is incorporated into the methyl group of acetate. We therefore have investigated the corrinoid content of *C. thermoaceticum* and in this paper report the isolation of 15 different corrinoids. Eleven of these have been identified using paper electrophoretic, paper chromato-

graphic, and spectrophotometric methods. Two of these corrinoids, Co-methylcobyrinic acid and Co-(methyl)-5-methoxybenzimidazolylcobamide (Co-methyl-Factor III_m), have been described in a previous paper (Ljungdahl *et al.*, 1965). They showed that the Co-methyl group of these two compounds is formed from CO₂ and that this methyl is the precursor of the methyl of acetate. These Co-methylcorrinoids appear to be the normal intermediates of acetate synthesis by *C. thermoaceticum*. Seven of the identified incomplete factors are suggested to be intermediates in the synthesis of complete B₁₂ factors.

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Methods

Bacterial Culture. *C. thermoaceticum* was grown and harvested as described in the previous paper (Ljungdahl

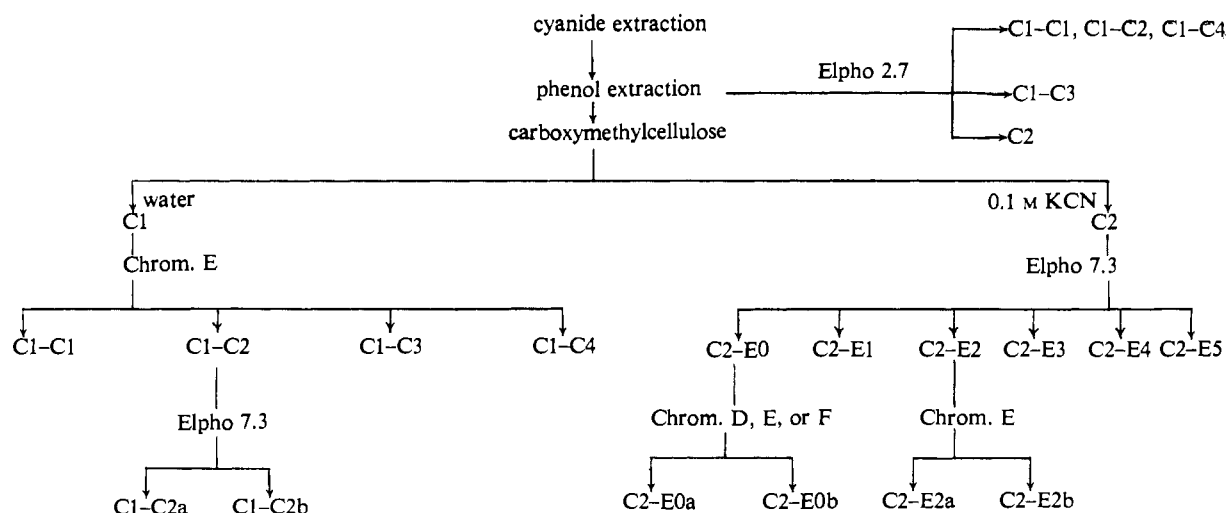


FIGURE 1: Scheme for the separation of cyano corrinoids obtained by extraction of *C. thermoaceticum* with 0.1% NaCN. Chrom = paper chromatography in solvents D, E, or F. Elpho = paper electrophoresis at pH 2.7 (0.5 N acetic acid) or 7.3 (0.02 M phosphate) both containing 0.01% cyanide.

et al., 1965). The cells (about 350–400 g) from 108 l. of broth cultures were used for each extraction.

Chemicals. Crystalline Factor III_m (cyano-5-methoxybenzimidazolylcobamide), crystalline Factor V_{1a} (monocyanomonoaquocobyrinic acid), and cobinamide phosphate (Factor B phosphate) were generous gifts from Professor K. Bernhauer, Technische Hochschule, Stuttgart, Germany. Factor B (cobinamide) was obtained by light cleavage and addition of HCN to a crystalline sample of cobinamide sulfonate (Bernhauer and Wagner, 1963). The cobinamide sulfonate was kindly provided by Dr. O. Wagner, University of Michigan, Ann Arbor, Mich. Benzimidazolylcobamide coenzyme was a gift from Dr. H. A. Barker, University of California, Berkeley, Calif. The Co-alkylcorrinoids were synthesized as indicated in the previous paper (Ljungdahl *et al.*, 1965). All other chemicals are commercially available.

Paper Chromatography and Ion-Exchange Chromatography. Paper chromatography was used both for analytical and for preparative purposes. For analytical chromatography the ascending technique was used with paper 2043a from Schleicher and Schuell but for preparative chromatography the chromatography was descending with Schleicher and Schuell 2043b or Whatman 3MM paper which had been previously washed with water. The following six solvents were used: (A) water-saturated 2-butanol, (B) water-saturated 2-butanol-acetic acid (100:1), (C) 2-butanol-water-25% NH₃ (100:36:14), (D) 2-butanol saturated with 0.01% HCN, (E) 2-butanol saturated with 0.01% HCN-acetic acid (100:1), and (F) 2-butanol saturated with 0.01% HCN-25% NH₃ (100:0.5). The solvents A, B, and C were for coenzyme and alkylcorrinoids, while solvents D, E, and F were used for cyanocorrinoids (Friedrich and Bernhauer, 1959).

Ion-exchange chromatography was carried out on

carboxymethylcellulose (Brown Company, Berlin, N. H.) and Dowex 1-X4. The carboxymethylcellulose was treated as previously described (Ljungdahl *et al.*, 1965). Dowex 1-X4 (chloride), 50–100 mesh, was washed with 1 N HCl and then with water until neutral. The resin was equilibrated with 2 M sodium formate.

Paper Electrophoresis. The paper electrophoresis was performed according to methods summarized by Pawelkiewicz (1962) with an apparatus from E. C. Apparatus Corp., Philadelphia, Pa. Whatman 3MM paper was used in preparative separations and Schleicher and Schuell 2043b paper for analytical investigations. Before use both types of paper were washed with water. The electrophoresis was carried out in the following solutions: 0.5 N acetic acid, 0.02 M phosphate, pH 7.3, and 0.1 M Na₂CO₃. Potassium cyanide, 0.01%, was added to these solutions for the electrophoresis of the cyano forms of the corrinoids. Migration values were corrected for electroosmosis by the use of cyanocobalamin as the uncharged (neutral) reference compound.

Spectrophotometric Methods. Ultraviolet and visible spectra were measured with a Cary Model 14 recording spectrophotometer using a scanning time of 10 A/sec and a 1-cm light path. Quantitative determinations were carried out in a Zeiss PMQII spectrophotometer at the following wavelengths: 361 mμ, ϵ 28.1 $\times 10^3$ M⁻¹ cm⁻¹ for B₁₂ and complete factors in their mono-cyano forms (Friedrich and Bernhauer, 1959); 367 mμ, ϵ 30.8 $\times 10^3$ M⁻¹ cm⁻¹ for incomplete factors in their dicyano forms (Bernhauer *et al.*, 1961); and 367 mμ, ϵ 30 $\times 10^3$ M⁻¹ cm⁻¹ for estimation of the total corrinoid content in mixtures. Corrinoids in their coenzyme or alkyl forms were determined after conversion of an aliquot part to the corresponding cyano forms.

Isolation of Corrinoids by Cyanide Extraction. Cor-

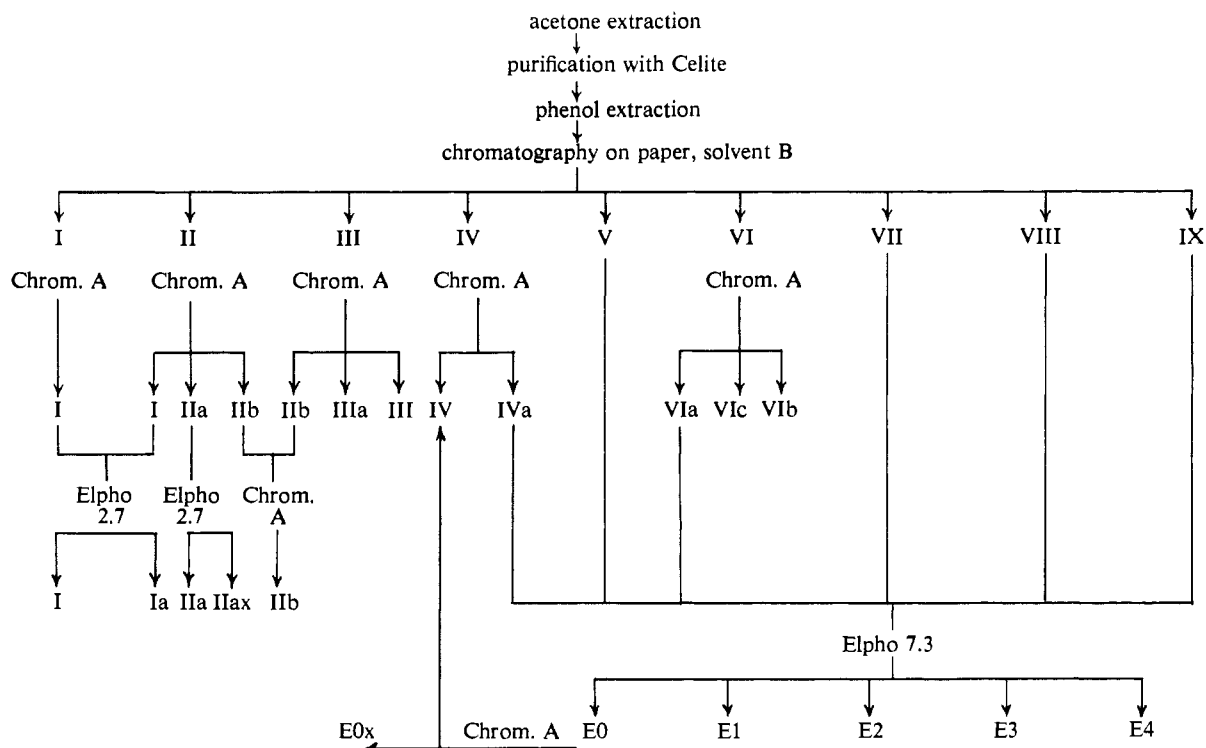


FIGURE 2: Scheme for the separation of the corrinoids in *C. thermoacetica* as obtained by acetone extraction. Chrom A = paper chromatography in solvent A. Elpho = paper electrophoresis at pH 2.7 (0.5 N acetic acid) or 7.3 (0.02 M phosphate).

rinoids from *C. thermoacetica* were isolated in their cyano forms by autoclaving 120 g of wet cells with 0.1 % sodium cyanide at pH 6.0 according to Bernhauer *et al.* (1959). The bacterial debris was recovered by centrifugation and re-extracted twice as above. The corrinoids were extracted from the aqueous solution with phenol-chloroform mixture (3:2). Butanol was added to the phenol-chloroform extract and the corrinoids were again transferred into a water solution. The corrinoids in this water solution were fractionated into 13 components using ion exchange on carboxymethylcellulose, paper chromatography, and paper electrophoresis according to the scheme in Figure 1. Ion-exchange chromatography on carboxymethylcellulose was done on a 18 × 240 mm column. After application of the solution of the corrinoids the column was eluted with water, giving fraction C1 which was followed by elution with 0.1 M KCN to remove the absorbed corrinoids, fraction C2.

Fraction C1 was chromatographed on paper in solvent E yielding four fractions which were labeled according to their rate of migration. Fraction C1-C1 with the fastest migration rate was red and was the main fraction. Fraction C1-C2 was purple and was the second largest. C1-C3 was red and C1-C4 was purple. The color of C1-C4 changed to orange on drying the paper or after acidification. This change is due to loss of a CN group and is a general property of incomplete B₁₂ factors, which lack the nucleotide moiety

or part of it (Friedrich and Bernhauer, 1959; Pawelkiewicz, 1962). These two slow-moving fractions were present only in small amounts. Fractions C1-C1, C1-C3, and C1-C4 could not be separated further by paper chromatography or paper electrophoresis and were considered homogeneous. Fraction C1-C2 separated on paper electrophoresis at pH 7.3 in the presence of KCN into two compounds: C1-C2a was red and neutral, and C1-C2b was purple and the migration indicated that it was a dianionic compound. C1-C2a and C1-C2b could not be separated further by paper chromatography and paper electrophoresis.

Fraction C1-C3 could be obtained better from the water solution of the phenol extraction by paper electrophoresis in 0.5 N acetic acid. At this pH (2.7) the corrinoids were separated into three fractions. The fastest moving fraction had the same migration (+1) as Factor B (monocyanomonoaquocobinamide) and was identical with fraction C2 from the carboxymethylcellulose column. At pH 2.7 Factor B migrates as a monocation (Bonnett, 1963). A small fraction, migration +0.8, was identical with C1-C3, and the neutral fraction contained compounds C1-C1, C1-C2, and C1-C4.

Fraction C2 from the carboxymethylcellulose column was separated by paper electrophoresis in the presence of KCN at pH 7.3 into six purple zones which migrated as neutral to pentaanionic compounds. They were designated on the basis of their migration rate as

TABLE I: Electrophoretic Properties of Corrinoid Compounds Isolated by Acetone Extraction of *C. thermoaceticum*.

Compound	Buffer		
	0.5 N Acetic Acid ^a	0.02 M Phosphate, pH 7.3	0.1 M Na ₂ CO ₃
I. Complete factors:			
IIIa [Factor III _m coenzyme]	+1.2	0 (neutral)	0 (neutral)
VIb and Co-methyl-Factor III _m	+0.45	0 (neutral)	0 (neutral)
IIb and Hydroxy- (aquo-) Factor III _m	+1.0		
IIa	+0.7	0 (neutral)	0 (neutral)
IIax	+1.4		0 (neutral)
II. Incomplete factors:			
III [cobyric acid coenzyme]	+1.4	0 (neutral)	
VIc and Co-methylcobyric acid	+1.0		0 (neutral)
E1 ^b [dicarboxylic acid coenzyme]	+1.4	-1 anionic	
E2 ^b [tricarboxylic acid coenzyme]	+1.4	-2 anionic	
E3 ^b [tetracarboxylic acid coenzyme]	+1.4	-3 anionic	
E4 ^b [pentacarboxylic acid coenzyme]	+1.4	-4 anionic	
IV [cobinamide coenzyme]	+1.4	≈ +0.8 cationic	
I [cobinamide phosphate coenzyme]	+0.65	-1 anionic	
Ia	+1.4	+1 cationic	
III. Reference compounds:			
Co-methylcobalamin	+0.30		0 (neutral)
Co-carboxymethylcobalamin	+0.15		-1 anionic
Benzimidazolylcobamide coenzyme	+1.2	0 (neutral)	0 (neutral)

^a Values relative to the migration of monocyanocobinamide = +1. ^b These acids probably are coenzyme forms of cobyrinic acid diamide (E4) to pentaamide (E1).

C2-E0, neutral, C2-E1, monoanionic, etc., to C2-E5. On drying the paper or by acidification these purple compounds turned orange, which indicated they were incomplete B₁₂ factors. Fractions C2-E1, C2-E3, C2-E4, and C2-E5 after elution from the paper and desalting by phenol extraction were found to be homogeneous, while C2-E0 by chromatography in solvents D, E, or F was separated into C2-E0a and C2-E0b, and fraction C2-E2 when chromatographed in solvent E gave C2-E2a and C2-E2b.

Isolation of Corrinoids by Acetone Extraction. To obtain the corrinoids in their natural coenzyme or alkyl forms cells were extracted with 75% acetone and the corrinoids were purified by absorption on Celite and by phenol extraction as described by Ljungdahl *et al.* (1965). All work was carried out in dim light. The corrinoids in the aqueous solution from the phenol extraction were fractionated as outlined in Figure 2. Nine fractions were obtained by descending chromatography using solvent B and Whatman 3MM paper. The fractions were numbered on the basis of migration from the slowest moving, I, to the fastest, IX. Some of the fractions from the paper chromatograms overlapped partially. They were further separated by

descending paper chromatography in solvent A (fractions I, II, III, IV, and VI), or by paper electrophoresis (fractions V, VII, VIII, and IX). Fraction I gave only one band in the chromatogram and fraction II separated into three bands (I, IIa, and IIb); the slowest moving was identical with fraction I and was combined with it. The combined fraction I was divided up into two compounds, I and Ia, by paper electrophoresis in 0.5 N acetic acid. Compound I had a migration of +0.65 and compound Ia of +1.4 in relation to Factor B (+1).

Fraction IIa was separated into two compounds (IIa migration +0.7 and IIax migration +1.4) by electrophoresis in 0.5 N acetic acid. Fraction III when chromatographed in solvent A separated into three compounds (IIb, IIIa, and III), of which one proved to be identical with fraction IIb; they were combined and rechromatographed in solvent A.

Fraction IV gave a fast moving yellow band (compound IV) and a slower moving (fraction IVa, also yellow) when chromatographed in solvent A. Using the same chromatographic system fraction VI was divided into fraction VIa and compounds VIc and VIb. Compounds VIb and VIc were identified as Co-(methyl)-5-methoxybenzimidazolylcobamide and Co-methylco-

TABLE II: Paper Chromatography of Corrinoid Compounds Isolated by Acetone Extraction of *C. thermoaceticum*.^a

Compound	<i>R_F</i> Value in Solvent		
	A	B	C
I. Complete factors:			
IIIa [Factor III _m coenzyme]	0.11	0.11	0.06
VIb and Co-methyl-Factor III _m	0.22	0.20	0.18
IIb and Hydroxy-(aquo-) Factor III _m	≈ 0.16 ^b	~0.1 ^b	
IIa	0.06	0.07	0.03
IIax	0.07	0.05	0.04
II. Incomplete factors:			
III [cobyric acid coenzyme]	0.09	0.12	0.05
VIc and Co-methylcobyric acid	0.18	0.24	0.13 ^c
E1 ^d [dicarboxylic acid coenzyme]	< 0.07	0.19	< 0.02
E2 ^d [tricarboxylic acid coenzyme]		0.23	
E3 ^d [tetracarboxylic acid coenzyme]		0.30	
E4 ^d [pentacarboxylic acid coenzyme]		0.36	
IV [cobinamide coenzyme]	0.17	0.20	0.13
I [cobinamide phosphate coenzyme]	0.05	0.05	0.02
III. Reference compounds:			
Co-methylcobalamin	0.25	0.23	0.24
Co-carboxymethylcobalamin	0.18	0.19	0.10
Benzimidazolylcobamide coenzyme	0.11	0.11	0.06

^a Ascending chromatography on Schleicher and Schuell paper 2043a, 20–24 hr at room temperature (22–24°).^b Hydroxy (aquo) forms are trailing because of the shifting equilibrium of the hydroxy (aquo) ligand. ^c Partial formation of a red amino complex at higher concentrations. ^d These acids probably are the coenzyme forms of cobyrinic acid diamide (E4) to pentaamide (E1).

byric acid, respectively, as described by Ljungdahl *et al.* (1965).

Fractions IVa, V, VIa, VII, VIII, and IX were all heterogeneous mixtures of corrinoids and were combined. Paper electrophoresis of this mixture at pH 7.3 resulted in the formation of five zones with the migration indicating presence of slightly cationic and of neutral to tetraanionic compounds. The two zones of neutral and cationic compounds were not completely separated and were eluted together, giving fraction E0. The four acidic zones were clearly separated and were eluted separately, giving compounds E1 to E4 having monoanionic to tetraanionic properties. These four compounds were pure, as judged by paper chromatography in different solvents and paper electrophoresis at different pH values. Fraction E0 was separated into E0x and a compound identical with compound IV by chromatography in solvent A.

Anaerobic Photolysis of Compounds III and IIIa. Compounds III and IIIa are the most abundant corrinoids in *C. thermoaceticum*. They were subjected to anaerobic photolysis (Hogenkamp, 1963; Bernhauer and Irion, 1964). Between 0.1 and 0.5 μ mole of the corrinoid in 4 ml of water was exposed under anaerobic conditions for 24 hr to a 200-w incandescent lamp placed 15 cm from the sample. One milliliter of 0.05% HCN was added, and the solution was evaporated under

vacuum to a small volume for paper electrophoresis at pH 2.7 in the presence of cyanide. The paper was examined in ultraviolet light and, in addition to the cyanocorrinoid, a compound was detected which absorbed ultraviolet light. This compound was formed from both compound III and IIIa, and it had the same migration as adenosine and 8,5'-cycloadenosine. It was eluted and investigated by paper chromatography and spectrophotometry.

Cleavage of Coenzyme Corrinoids with KCN. Co-5'-Deoxyadenosylcorrinoids react with KCN in the absence of light yielding adenine and D-erythro-2,3-dihydroxypent-4-enal (Johnson and Shaw, 1960; Toohey *et al.*, 1961; Barker, 1962). All compounds isolated from the acetone extract except VIb, VIc, and IIb yielded adenine when treated with KCN as follows: 0.5 ml of 0.04 M KCN solution was added to 0.05–0.1 μ mole of the corrinoid in 0.5 ml of water. After 5 hr in darkness at room temperature paper electrophoresis was done in 0.5 N acetic acid in the presence of cyanide. The spot corresponding to adenine, detected in ultraviolet light, was eluted and identified as adenine by chromatography and spectrophotometry by comparison with an authentic sample.

Isolation of the Nucleoside from Compound C1–C1. The most abundant complete B₁₂ factor in the cyano extract from *C. thermoaceticum* is compound C1–C1.

TABLE III: Electrophoretic Properties of the Cyanocorrinoids Isolated by Extraction of *C. thermoaceticum* with 0.1% NaCN.

Compound	Buffer		
	0.5 N Acetic Acid ^a + Cyanide	0.02 M Phosphate ^b + Cyanide	0.1 M Na ₂ CO ₃
I. Complete factors:			
C1-C1 and Factor III _m	0 (neutral)	0 (neutral)	0 (neutral)
C1-C2a	0 (neutral)	0 (neutral)	0 (neutral)
C1-C3	+0.8	0 (neutral)	
II. Incomplete factors:			
C2-E1 and cobyrinic acid	+1	-1	
C2-E2a ^c [dicarboxylic acid]	+1	-2	
C2-E3 ^c [tricarboxylic acid]	≈ +0.9	-3	
C2-E4 ^c [tetracarboxylic acid]	≈ +0.8	-4	
C2-E5 ^c [pentacarboxylic acid]	≈ +0.8	-5	
C2-E0a and Factor B	+1	0 (neutral)	
C1-C2b and Factor B phosphate	0 (neutral)	-1.8	
C2-E0b	+1	0 (neutral)	
C2-E2b	+1	-2	
III. Reference compound			
B ₁₂	0 (neutral)	0 (neutral)	0 (neutral)

^a Values relative to the migration of monocyano-cobinamide (Factor B) = +1. ^b Values relative to the migration of dicyanocobyrinic acid (Factor V_{1a}) = -1. ^c These acids probably are cobyrinic acid diamide (C2-E5) to pentaamide (C2-E2a).

The nucleoside from 4.6 μ moles of this compound was obtained by cleavage with ceric hydroxide as described by Friedrich and Bernhauer (1956b). The hydrolysate was fractionated on a Dowex 1-X4 formate column (12 \times 200 mm). The corrinoid (cobinamide) was eluted with 20 ml of water. The column was washed with an additional 75 ml of water, whereafter the nucleoside was eluted in a sharp peak with 0.1 M formic acid. The elution was followed by absorption at 280 m μ . The nucleoside was purified by descending chromatography in solvent E (R_F 0.54-0.58) and was detected by its strong violet-blue fluorescence in ultraviolet light. The nucleoside was crystallized from hot water by slow evaporation.

Results

All isolated compounds were tested for homogeneity and purity by paper chromatography and by paper electrophoresis. The electrophoretic and chromatographic properties of the corrinoids from the cyano extraction and from the acetone extraction are listed in Tables I-IV.

Identification of Compounds C1-C1, IIb, IIIa, and VIb. The identification of compound C1-C1 as 5-methoxybenzimidazolylcobamide (Bernhauer's Factor III_m) is based on the following evidence: (1) C1-C1 cochromatographed in solvents D, E, and F with an authentic

sample of Factor III_m (R_F values, see Table IV); (2) it migrated on paper electrophoresis at pH 2.7, 7.3, and 10.5 the same as Factor III_m (Table III); (3) the spectra of C1-C1 in the monocyano form in water and of the dicyano form in 0.1 N KCN as shown in Figure 3 are identical with the spectra obtained with Factor III_m; and (4) the nucleoside from C1-C1 obtained after hydrolysis with ceric hydroxide gave in acid, neutral, and alkaline solutions the same spectra (Table V) as 5-methoxybenzimidazolyl ribofuranoside phosphate, as reported by Friedrich and Bernhauer (1956a). It should be pointed out that there was no shift of the maximum peak from 290 m μ at pH 8 to 316 m μ at pH 12 as occurs for the corresponding 5-hydroxybenzimidazolyl ribofuranoside found in Factor III (Friedrich and Bernhauer, 1956a; Lezius and Barker, 1965). The isolated nucleoside was remarkably stable toward acid hydrolysis and only a small amount of a sugar, identified as ribose, was formed with 0.5 N HCl at 100° for 30 min. Hydrolysis in 6 N HCl in a sealed tube at 130° gave many products, as found by chromatography. The sugar was completely destroyed, but one of the products had a spectrum similar to 5-hydroxybenzimidazole (Table V).

Compound VIb, identified as Co-(methyl)-5-methoxybenzimidazolylcobamide (Co-methyl-Factor III_m) (Ljungdahl *et al.*, 1965), when cleaved by light gave the spectrum corresponding to the hydroxy (aquo) complex of Factor III_m in water (Figure 3). The same

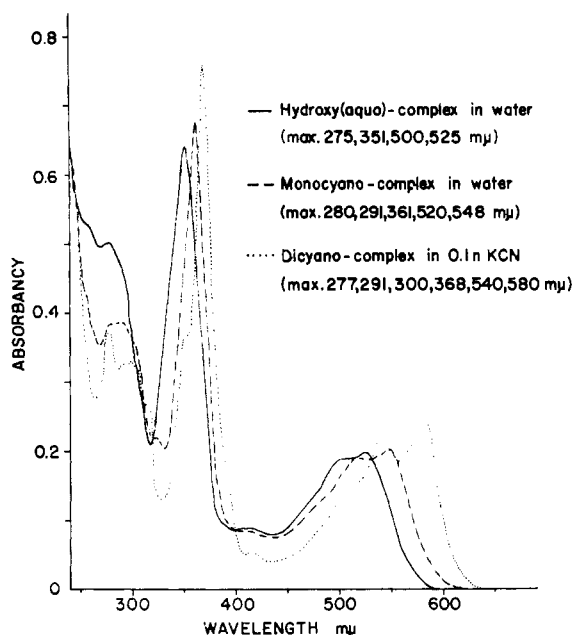


FIGURE 3: Absorption spectra of 5-methoxybenzimidazolylcobamide (compound C1-C1) in the mono-cyano, dicyano, and hydroxy (aquo) complex (compound IIb). The concentration is approximately 2.4×10^{-5} M.

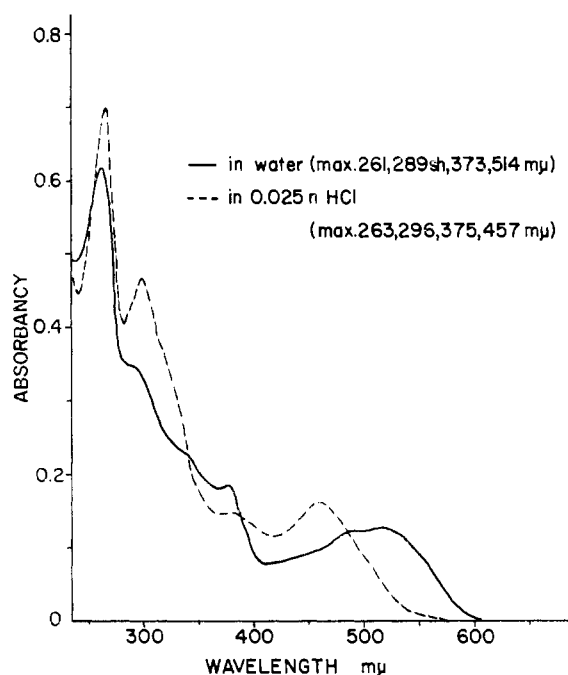


FIGURE 4: Absorption spectra of 5-methoxybenzimidazolylcobamide coenzyme (compound IIIa). The concentration is approximately 1.6×10^{-5} M.

TABLE IV: Paper Chromatography of the Cyanocorinoids Isolated by Extraction of *C. thermoaceticum* with 0.1% NaCN.

Compound	R_F Value in Solvent ^a		
	D	E	F
I. Complete factors:			
C1-C1 and Factor III _m	0.14	0.15	0.19
C1-C2a	0.13	0.10	0.15
C1-C3	0.12	0.11	0.15
II. Incomplete factors:			
C2-E1 and cobyrinic acid	0.11	0.26	0.10
C2-E2a ^b [dicarboxylic acid]	0.07	0.31	
C2-E3 ^b [tricarboxylic acid]	<0.05	0.35	
C2-E4 ^b [tetracarboxylic acid]		0.41	
C2-E5 ^b [pentacarboxylic acid]		0.45	
C2-E0a and Factor B	0.30	0.27	0.27
C1-C2b and Factor B phosphate	0.07	0.09	0.05
C2-E0b	0.08	0.08	0.11
C2-E2b	0.05	0.28	
III. Reference compound:			
B ₁₂	0.21	0.23	0.24

^a Ascending chromatography on Schleicher and Schuell paper 2043a, 20-24 hr at room temperature (22-24°). ^b These acids probably are cobyrinic acid diamide (C2-E5) to pentaamide (C2-E2a).

spectrum also was obtained by photolysis of synthetically prepared Co-methyl-Factor III_m. Compound IIb without any treatment also gives this spectrum, indicating that this compound is hydroxy (aquo) Factor III_m. On addition of HCN the spectrum shifted to that of the monocyno complex of compound C1-C1, and the spectrum of the dicyano complex was formed by the addition of KCN. Cochromatography and coelectrophoresis of compound IIb (Table I and II) with the photolysis product of the synthetically prepared Co-methyl-Factor III_m, which is hydroxy (aquo) Factor III_m, confirmed that compound IIb and this product are identical.

Compound IIIa was identified as Co-(5'-deoxyadenosyl)-5-methoxybenzimidazolylcobamide (Factor III_m coenzyme). Paper chromatograms in solvents A, B, and C and the migration on paper electrophoresis were the same as those of benzimidazolylcobamide coenzyme (Tables I and II). However, the absorption spectra for compound IIIa (Figure 4) in water and 0.025 N HCl differed from the corresponding spectra of this coenzyme (Barker, 1962), especially between 280 and 360 mμ. The high absorption maxima at 261 mμ as well as the ratio of 4.90 between ϵ_{\max} at 261 mμ and ϵ_{\max} 514 mμ indicates the presence of a 5'-deoxyadenosyl moiety, as found in coenzyme B₁₂ factors (Barker, 1962). The shift of ϵ_{\max} 514 mμ in water to ϵ_{\max} 457 mμ in 0.025 N HCl shows that compound IIIa is a complete B₁₂ factor. Similar shifts due to loss of coordination between the cobalt atom and 3 nitrogen of

TABLE V: Ultraviolet Absorption Maxima of Nucleotides, Nucleosides, and Bases from Compound C1-C1, Factor III_m, and Factor III.

Compound	Absorption Max in Water (mμ)			
	pH 1	pH 8		pH 12
Nucleotide from Factor III _m ^a	288	247.5	289.5	247.5 289.5
Nucleoside from C1-C1	287	247	289	247.5 289
Nucleotide from Factor III ^a	289.5	248.5	291.5	316
5-Hydroxybenzimidazole ^b	286	246	286	306
HCl hydrolysis product of nucleoside from C1-C1	285	247	286	290 310 ^c

^a Friedrich and Bernhauer (1956a). ^b Friedrich and Bernhauer (1956c). ^c This is a shoulder, and not a peak.

the benzimidazole base are typical for complete coenzyme factors (Ladd *et al.*, 1961).

Photolysis in the presence of cyanide of compound IIIa followed by purification of the cyanocorrinoid yields compound C1-C1 (Factor III_m), as judged by chromatographic and electrophoretic analysis (Table III and IV) as well as by comparison of spectra. When compound IIIa is cleaved by anaerobic photolysis 8,5'-cycloadenosine is formed, as judged by the spectra in water and in 0.01 N HCl, chromatography in solvents A, B, and C, and electrophoresis according to Bernhauer and Irion (1964). The absorption maxima in water at 264 mμ and in 0.01 N HCl at 260.5 mμ are close to the values for 8,5'-cycloadenosine published by these authors and by Hogenkamp (1963). The formation of 8,5'-cycloadenosine by anaerobic photolysis and adenine by cleavage with KCN in darkness clearly demonstrates that compound IIIa contains the 5'-deoxyadenosyl moiety.

Compound C1-C1 (Factor III_m) thus exists in the following three forms in *C. thermoaceticum*. Compound IIIa is Co-(5'-deoxyadenosyl)-5-methoxybenzimidazolylcobamide (Factor III_m coenzyme), compound IIb is hydroxy- (aquo-) 5-methoxybenzimidazolylcobamide, and compound VIb is Co-(methyl)-5-methoxybenzimidazolylcobamide (Co-methyl-Factor III_m).

Identification of Compounds C2-E1, III, and VIc. Compound C2-E1 was the main corrinoid found in the cyanide extract and compound III the most abundant in the acetone extract. Compounds III and VIc on photolysis in the presence of cyanide yielded the same corrinoid, which after purification had identical spectra and the same migration on electrophoresis and chromatography as compound C2-E1. This cyano compound was found to be identical with a crystalline sample of cobyrinic acid by cochromatography, by coelectrophoresis (Table III and IV), and by comparison of spectra. There are therefore two compounds in the acetone extract which are derivatives of cobyrinic acid. Compound VIc was shown to be Co-methylcobyrinic acid (Ljungdahl *et al.*, 1965). Compound III on anaerobic photolysis gave 8,5'-cycloadenosine and by cleavage with

cyanide in the dark adenine, indicating a 5'-deoxyadenosyl moiety. The spectra of compound III are identical with the spectra of Co-5'-deoxyadenosylcobyrinic acid (Factor V_{1a} coenzyme) as published by Migliacci and Rusconi (1961). We therefore concluded that compound III is the coenzyme form of cobyrinic acid.

Identification of Compounds C2-E2a, C2-E3, C2-E4, C2-E5, E1, E2, E3, and E4. The four yellow compounds E1 to E4 in water have the same spectra as cobyrinic acid coenzyme with absorption maxima at 264, 304, and 456 mμ. These maxima are typical for incomplete coenzyme B₁₂ factors (Bonnett, 1963). The spectrum did not change much after acidification, which again suggests the compounds to be incomplete B₁₂ factors. The high absorption at 264 mμ (ratio 264/456 = 4.72) indicates that these compounds are coenzymes containing a 5'-deoxyadenosyl group, and this was confirmed by cleavage with cyanide in the dark to form adenine. Paper electrophoresis at pH 7.3 (see Table I) revealed the anionic character to be monoanionic (E1) to tetraanionic (E4). This was also shown by paper chromatography (see Table II). In solvent A and C there was almost no migration due to the dissociation of the acid groups, while in the acid solvent B the acids are not or less dissociated, rendering them more hydrophobic and leading to an increased migration dependent on the number of acid groups (Rietz, 1964). By photolysis of the compounds in the presence of cyanide the corrinoids yielded the corresponding cyano compounds: E1 → C2-E2a, E2 → C2-E3, E3 → C2-E4, and E4 → C2-E5, which was shown by cochromatography and coelectrophoresis. The cyano compounds were one unit more anionic than the coenzyme forms due to the loss of the 5'-deoxyadenosyl moiety. The electrophoretic mobility at pH 7.3 (Table III) for C2-E2a therefore corresponds to a dianionic compound, etc., up to C2-E5, which is pentaanionic. These properties indicate that the cyano compounds are homologs of cobyrinic acid (cobyrinic acid a, b, c, d, e, g hexamide) and most likely are cobyrinic acid pentamide (C2-E2a) to diamide (C2-E5) and that compounds E1 to E4 correspond to

TABLE VI: Summary of Corrinoid Compounds in *C. thermoaceticum* as Obtained by Extraction with Acetone and with 0.1% NaCN.

Isolated by Acetone Extraction	% ^a	Isolated by Cyanide Extraction	% ^a
I. Complete factors:			
IIIa [Co-(5'-deoxyadenosyl)-5-methoxybenzimidazolylcobamide, Factor III _m coenzyme]	11-16	C1-C1 [5-methoxybenzimidazolylcobamide, Factor III _m]	12-17
VIb [Co-(methyl)-5-methoxybenzimidazolylcobamide]	0.2-0.4		
IIb [hydroxy-(aquo-) 5-methoxybenzimidazolylcobamide]	0.2-0.4		
IIa [unknown coenzyme]	2.5	C1-C2a	2.5
IIax [unknown coenzyme]	0.5	C1-C3	0.5
II. Incomplete factors:			
III [Co-5'-deoxyadenosyl-cobyric acid]	49.5	C2-E1 [cobyric acid, Factor V _{1a}]	50
VIc [Co-methylcobyric acid]	0.2-0.4		
E1 ^b [dicarboxylic acid coenzyme]	8-10	C2-E2a [dicarboxylic acid]	8-10
E2 ^b [tricarboxylic acid coenzyme]	4-5	C2-E3 [tricarboxylic acid]	4-5
E3 ^b [tetracarboxylic acid coenzyme]	2-3	C2-E4 [tetracarboxylic acid]	2-3
E4 ^b [pentacarboxylic acid coenzyme]	1-2	C2-E5 [pentacarboxylic acid]	1-2
IV [Co-5'-deoxyadenosyl-cobinamide]	4	C2-E0a [cobinamide, Factor B]	4
I [Co-5'-deoxyadenosyl-cobinamide phosphate]	5	C1-C2b [cobinamide phosphate, Factor B phosphate]	5
Ia, E0x, etc. [unknown coenzymes]	6	C1-C4, C2-E0b, C2-E2b, etc.	6

^a Figures in per cent of total corrinoids. One hundred grams of wet cells contains about 25-30 μ moles of corrinoids.

^b Probably coenzyme forms of cobyrinic acid diamide to pentamide and from the cyano extraction the cyano forms of corresponding cobyrinic acid amides.

their coenzyme forms. A detailed study of the cobyrinic acids has been presented by Rietz (1964). However, it is also possible that compounds E1 to E4 and their corresponding cyano forms can be derivatives of the cobin acid amide series.

Identification of Compounds C2-E0a, C1-C2b, IV, and I. Compounds C2-E0a and C1-C2b have spectra similar to the above acidic compounds indicating they are incomplete factors. C2-E0a is neutral at pH 7.3 and C1-C2b is dianionic (Table III). Chromatography in solvents D, E, and F (Table IV) and paper electrophoresis at different pH values (Table III) of C2-E0a in a mixture with an authentic sample of Factor B failed to separate the two. We therefore have concluded that C2-E0a is Factor B (cobinamide). Factor B was also obtained by Ce(OH)₃ hydrolysis of Factor III_m. Compound IV, as judged by spectra and the release of adenine on treatment with cyanide in the dark, contains a 5'-deoxyadenosyl moiety. It is in its cyano form identical with compound C2-E0a and therefore is the

coenzyme of Factor B.

The dianionic C1-C2b was cochromatographed (Table IV) and investigated by electrophoresis (Table III) together with a sample of Factor B phosphate and they were found to be identical. Compound I is the coenzyme form of Factor B phosphate.

Properties of Other Corrinoids. Several corrinoids in the cyano extract remain to be identified. The R_F values and electrophoretic behavior of compounds C1-C2a, C1-C3, C2-E0b, C2-E2b are given in Tables III and IV. Spectra of C1-C2a and C1-C3 show these compounds to be complete factors, and they are obtained in their coenzyme forms from the acetone extraction (compounds IIa and IIax). They exist only in small amounts and are still subjects for identification. Compounds C2-E0b and C2-E2b also apparently exist as coenzymes. These compounds are judged from spectra as incomplete factors and are under investigation.

A summary of the corrinoids found so far in *C.*

thermoaceticum is given in Table VI, which also lists the approximate amounts of the corrinoids. However, some variations of the quantities were observed from different preparations.

Discussion

Clostridia have been considered to preferentially synthesize purine cobamides rather than analogs of benzimidazolycobamides (Bernhauer *et al.*, 1962). However we have found no evidence of the presence of a purine cobamide or its derivatives in *C. thermoaceticum*. The most abundant corrinoids in *C. thermoaceticum* are Co-5'-deoxyadenosylcobyrinic acid and Co-(5'-deoxyadenosyl) - 5 - methoxybenzimidazolycobamide. The latter is the coenzyme of Bernhauer's synthetic Factor III_m (Friedrich and Bernhauer, 1956a). Factor III_m has been postulated to occur in nature (Friedrich and Bernhauer, 1958) but its isolation from biological material has not been reported previously.

C. thermoaceticum contains a relatively large amount of corrinoids of many varieties. Most interesting is the presence of the two Co-methylcorrinoids and possibly also a Co-carboxymethyl derivative, which were described in a previous paper (Ljungdahl *et al.*, 1965) and appear to be intermediates in the synthesis of acetate from CO₂. Except for the alkylcorrinoids and a small amount of hydroxy (aquo) Factor III_m the corrinoids are present in the form of coenzymes. This form is apparently the most common in bacteria (Toohey *et al.*, 1961; Volcani *et al.*, 1961), although Lezius and Barker (1965) isolated the vitamin (cyano) form of Factor III from *Methanobacillus omelianskii*.

Cobyric acid coenzyme constitutes about 50% of the corrinoid content in *C. thermoaceticum* which therefore appears to be a good source for the isolation of this corrinoid. The coenzyme form of cobyrinic acid has previously been isolated from a mutant strain of *Nocardia rugosa* (Migliacci and Rusconi, 1961). This compound, as well as the other coenzyme forms of incomplete factors in *C. thermoaceticum*, have been postulated to be intermediates in the biosynthesis of complete B₁₂ factors. This synthesis starting with cobyrinic acid diamide probably occurs with a 5'-deoxyadenosyl group attached to the cobalt atom of the corrinoids (Bernhauer *et al.*, 1964). The coenzyme form of the cobyrinic acid itself or of its monoamide has not been found in nature nor has it been biosynthesized. Compound E4 seems to correspond to cobyrinic acid diamide coenzyme. From this compound the synthesis of complete B₁₂ factors may occur *via* the following intermediates, which all have been found in their coenzyme forms in *C. thermoaceticum*.

In cobyrinic acid diamide two of the carboxyl groups a, b, c, d, e, or g of cobyrinic acid (Figure 5) are amidated. In reactions 1, 2, 3, and 4 the other carboxyls are amidated with the formation of cobyric acid (cobyric acid a, b, c, d, e, g hexamide), which has one free carboxyl group in position f. In reaction 5 1-amino-propan-2-ol, which is derived from threonine (Krasna *et al.*, 1957), is added to the carboxyl f to form cobin-

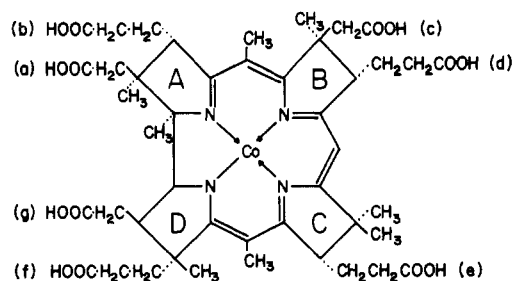
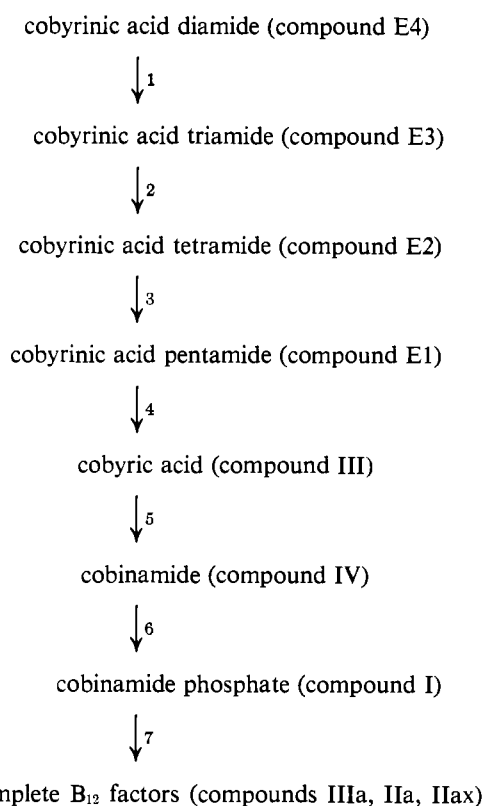
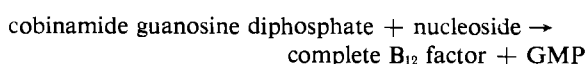
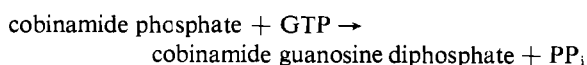


FIGURE 5: Cobyric acid.



amide. In step 6 the hydroxyl group of the amino-propanol is phosphorylated, possibly by adenosine triphosphate. Step 7 involves the addition of ribose and a base, and several pathways have been suggested for this step (Bernhauer *et al.*, 1959, 1962, 1964). Thus Barchielli *et al.* (1960) have isolated cobinamide guanosine diphosphate as an intermediate and postulated following reactions.



Recent findings by Friedman and Harris (1965) suggest that the nucleoside in the second reaction is formed by a specific trans-*N*-glycosidase. The possibility also

exists that the nucleoside reacts directly with cobinamide phosphate forming the complete B₁₂ factor. It is possible that some of the unidentified incomplete corrinoids in *C. thermoaceticum* are intermediates in step 7.

Friedrich and Bernhauer (1958) compared Factor III_m and benzimidazolylcobamide and found them to have similar chromatographic and electrophoretic properties. The coenzyme forms of these factors have now also been compared, and their properties are also much alike as judged by chromatographic and electrophoretic analysis (Tables I and II).

The small amounts of the hydroxy form of Factor III_m may have arisen by photolysis or chemical cleavage of the coenzyme or of Co-methyl-Factor III_m during the isolation. However, methyl and coenzyme forms are stable under the conditions of isolation, as also was pointed out by Lezius and Barker (1965). We therefore assume that the hydroxy (aquo) Factor III_m is formed from either a reduced form corresponding to B_{12r} by oxidation or from unstable Co-formyl or Co-carboxyl derivatives (Müller and Müller, 1963). The latter compounds can perhaps be intermediates in the formation of Co-methyl groups, while B_{12r} may be obtained by a reductive cleavage of Co-carboxymethyl or from other Co-alkylcorrinoids.

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