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Corrinoid Compounds of *Methanobacillus omelianskii*. I. Fractionation of the Corrinoid Compounds and Identification of Factor III and Factor III Coenzyme*

A. G. Lezius† and H. A. Barker

ABSTRACT: The corrinoid compounds from an extract of *M. omelianskii* were separated into ten components by chromatographic methods. 5-Hydroxybenzimidazolylcobamide and its Co-5′-deoxyadenosyl derivative (Factor III coenzyme) are the most abundant corrinoid compounds in this organism, accounting for 31 and 23%, respectively, of the total vitamin B_{12} activity in the bioassay with *Escherichia coli* 113-3.

The coenzyme was synthesized from Factor III and found to be identical with the natural product in spectrum, paper chromatography, paper ionophoresis, and in the glutamate mutase assay. The nature of four other yellow coenzymes is discussed.

The possible participation of a vitamin B_{12} coenzyme in the metabolism of one-carbon compounds of *Methanococcus vannielii* and *Methanobacillus omelianskii* was first suggested by Stadtman (1960) because of the high level of cobamide coenzymes in these organisms. The recent finding of Blaylock and Stadtman (1963) and Wolin *et al.* (1963) that the methyl group of synthetic Co-methyl cobalamin can be converted into methane by cell-free extracts of *Methanosarcina barkeri* and *M. omelianskii* indicates a possible role of the vitamin as a methyl-carrying coenzyme in the methane fermentation. The presence of methyl cobalamin or one of its analogs in bacteria or other organism has not been demonstrated so far, although substantial indirect evidence for the participation of an enzyme-bound methyl cobalamin intermediate in the biosynthesis of methionine has been reported by Foster *et al.* (1964). The present investigation was started with the objective of determining whether a Co-methyl cobamide is present in cells of *M. omelianskii*. We soon found, in agreement with previous reports (Neujahr and Callieri, 1958, 1959), that this organism contains a variety of corrinoid compounds which could not be positively identified by chromato-

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graphic methods. Consequently we have undertaken to isolate and characterize these compounds by both physical and chemical methods. In this paper we report the separation of ten different corrinoid compounds from extracts of a pure culture of *M. omelianskii* and the identification of the two most abundant compounds as Bernhauer's (Friedrich and Bernhauer, 1953, 1954a, 1956a) Factor III (5-hydroxybenzimidazolylcobamide) and its 5′-deoxyadenosyl derivative. The identification of some of the other corrinoid compounds will be reported later.

Experimental Procedure

Growth of the Bacteria. M. omelianskii strain Mb2 isolated by Barker (1940) was routinely cultured in the semisolid ethanol-bicarbonate medium described by Johns and Barker (1960) except that 0.023% thioglycolic acid (Eastman) was used in place of sodium hydrosulfite as a reducing agent. To obtain large quantities of cells, the bacteria were grown in the same medium using a set of ten 20-liter Pyrex bottles; each bottle was inoculated with about 2 liters of an active culture. Each culture vessel was closed with a rubber stopper containing a gas-outlet tube connected through a manifold to a gasometer. The cultures were incubated at 30–35° in the dark until gas production decreased markedly, usually 5–6 days. The bacteria were then harvested in a continuous-flow centrifuge and the cell paste was frozen and stored at −15°. The yield of moist bacteria was between 40 and 50 g/100 liters of medium.

Chemicals. Factor III (5-hydroxybenzimidazolylcyanocobamide) was a generous gift of Professor K. Bernhauer, Technische Hochschule, Stuttgart, Germany. Methyl cobalamin and 2′,3′-isopropylidene-5′-tosyladenosine were gifts of Dr. H. P. C. Hogenkamp, University of Iowa, Iowa City, Iowa. 5-Methoxybenzimidazoleribofuranoside was a gift of Dr. D. Perlman, Squibb Institute for Medical Research, Rahway, N.J. Cobalamin coenzyme was prepared as previously described (Barker *et al.*, 1960b). All other chemicals were commercial preparations.

Ion Exchangers. Dowex 50W-X2, 200–400 mesh, and Dowex 2-X8 (OH^-), 50–100 mesh, were used as described by Barker *et al.* (1960a) and Toohey *et al.* (1961). Dowex 2-X2 (formate), 50–100 mesh, was used instead of Dowex 2(OH^-) in the large-scale isolation. Phosphocellulose (Cellex P, Bio-Rad Laboratories, Richmond, Calif.), exchange capacity 68 meq/g, was prepared for use and equilibrated against 5 mM sodium acetate, *p*H 3.9, as described by Peterson and Sober (1962). By the same general method TEAE-cellulose (Cellex T, Bio-Rad Laboratories), exchange capacity 0.76 meq/g, was washed and equilibrated against 5 mM Tris-HCl buffer, *p*H 9.0. Carboxymethylcellulose (Brown Co., Berlin, N.H.), exchange capacity 0.78 meq/g, was washed several times with water and used in the acid form without previous equilibration.

Microbiological Assay of Vitamin B_{12} Activity. E. coli was used as the test organism and the tube assay was carried out in Burkholder's (1951) medium as modified by Ford *et al.* (1955), except that sodium cyanide was omitted. The cultures were incubated without shaking for 16 hours at 30°. Crystalline cyanocobalamin was used as standard.

Visible and Ultraviolet Spectra. These were measured with a Cary Model 14 recording spectrophotometer using a narrow slit and a scanning time of 25 A/sec. The light path was 1 cm. For the quantitative determination of Factor III coenzyme, the molar extinction coefficient of cobalamin coenzyme at 522 mμ, $\epsilon = 8.0 \times 10^3$ M^{-1} cm^{-1} (Hogenkamp and Barker, 1961), was used.

Paper Ionophoresis. This was carried out with the apparatus described by Crestfield and Allan (1955). The distance between electrodes was 50 cm. Strips of acetylated cellulose "Oxoid" (Oxo Ltd., London, England), 5 × 35 cm, were used as carrier. The strips were dropped into the buffer, spread on the glass surface, and blotted with absorbent paper. The strips were connected to the electrode vessels by Whatman No. 1 paper saturated with buffer. For equilibration of the system, a current of 2000 v was applied for 15 minutes. Then 5–25 μl of the sample was applied. The apparatus was covered with a black cloth for protection from light and the electrophoresis was carried out for 60 minutes at 2000 v, and the strips were dried in air. Faint coenzyme spots were detected more readily after spraying the strip with 1 M KCN solution.

Paper Chromatography and Thin-Layer Chromatography on Cellulose Powder. The ascending technique was used on Whatman No. 1 paper or on a thin layer of cellulose powder on a glass plate prepared as follows: Five g cellulose powder, Whatman standard grade, was suspended in a mixture of 25 ml of acetone and 5 ml of distilled water and blended in a Servall Omni Mixer for 10 minutes at highest speed, the vessel being cooled in an ice bath. The finely dispersed suspension was then applied on glass plates of 5 × 20 and 20 × 20 cm in a layer of 0.25 mm, using the equipment of Research Specialties Inc., Richmond, Calif. The plates were dried in an oven at 80° for 10 minutes and stored in a closed box.

The following solvents were used for chromatography: (I) water-saturated 2-butanol–glacial acetic acid–10% KCN (100:1:0.1); (II) 2-butanol–water–27% NH_3–10% KCN (50:18:7:0.1); (III) water-saturated 2-butanol–10% KCN (100:0.002); (IV) water-saturated 2-butanol–10% KCN–sodium tetraphenylborate (100:0.1:1 g); (V) 2-butanol–water–glacial acetic acid (100:50:3); (VI) 1-butanol–water–glacial acetic acid (100:20:2); (VII) 1-butanol–water–glacial acetic acid (40:50:1), upper phase. Solvents I–IV are recommended by Friedrich and Bernhauer (1959) for separation of B_{12} vitamins. Solvent V was used by Barker *et al.* (1960a) for chromatography of cobamide coenzymes. Solvent VII is reported by Folkers *et al.* (1957) for chromatography of the nucleoside derived from Factor III.

Coenzyme activity was determined by the spectrophotometric assay described by Barker *et al.* (1963). *Bacterial extracts* were prepared for column chromatog-

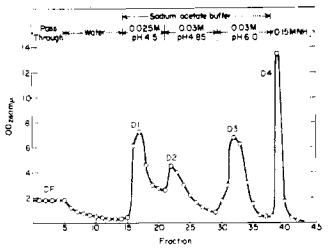

FIGURE 1: Chromatography of a partially purified extract of *M. omelianskii* on a column of Dowex 50W. The details of the preparation and chromatography of the extract are given in the text.

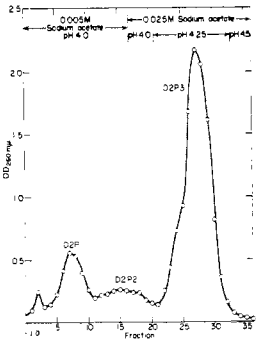

FIGURE 2: Chromatography of fraction D2 of the Dowex 50 column (Figure 1) on a phosphocellulose column. The details are given in the text.

raphy according to the method of Barker *et al.* (1960a) and Toohey *et al.* (1961), except that for the large-scale preparation starting from 550 g of bacteria Dowex 2 (OH^-) was replaced by Dowex 2-X2 (formate), 50–100 mesh. Phenol extractions were performed as described by Barker *et al.* (1960a).

Fractionation of the corrinoids on Dowex 50 was carried out according to Toohey *et al.* (1961) and Barker *et al.* (1960a). Aqueous solution (400 ml) resulting from extraction of 550 g bacteria with hot 80% ethanol, passage through Dowex 2 (formate), and phenol extraction was adjusted to *p*H 3.0 with 6 N HCl and passed into a 0.8 × 12-cm column of Dowex 50W-X2, 200–400 mesh. The column was eluted successively with 120 ml water; 85 ml of 25 mM sodium acetate buffer, *p*H 4.5; 95 ml of 30 mM sodium acetate, *p*H 4.85; 110 ml of 30 mM sodium acetate buffer, *p*H 6.0; and finally 40 ml of 0.15 M ammonia. Fractions of 12 ml were collected, and the absorbance of the fractions at 260 mμ was determined. The elution pattern is shown in Figure 1. The pass through and the washings including fraction 14 were combined and concentrated by phenol extraction. The concentrated solution will be designated DF (Dowex 50 filtrate). Its pink color indicated the presence of corrinoid compounds. The fractions in each peak (Figure 1) were combined, acidified to *p*H 3.0, and concentrated by adsorption on a 0.5 × 1.5-cm column of Dowex 50 and elution with 5–10 ml of 30 mM ammonia. The concentrated solutions were numbered in order of their elution from the Dowex 50 column: D1 (fractions 15–20) was faintly colored, contained only traces of corrinoid compounds, and was not further investigated; D2 (fractions 21–29) was orange colored; D3 (fractions 30–38), the main coenzyme peak, was deep red; and D4 (fractions 39–40) was dark brown.

Purification of Fraction DF. An aliquot of this fraction was spotted on a sheet of Whatman No. 1 paper and developed in the dark by ascending chromatography in solvent III, KCN being omitted. A red zone of R_F 0.1 (cyanocobalamin R_F = 0.2) was cut out and eluted from the paper with water. The eluate exhibited a cyanocobalamin-like spectrum, showing absorbance maxima at 545, 520, and 360 mμ; the region below 340 mμ was obscured by impurities. One-fifth of fraction DF was deluted with 30 mM KCN to 5 ml and converted into the dicyano derivative. The strongly opalescent and viscous solution was further diluted to 10 ml and passed through a 0.5 × 4.0-cm column of Dowex 2 (formate). The purple material was adsorbed on the top of the column, whereas the colorless, opalescent solution passed through. The column was washed with 10 ml of distilled water and then the corrinoid compound was eluted with 0.1 M sodium acetate buffer, *p*H 4.0. The red fractions were combined and concentrated to 0.25 ml by evaporation. Aliquots of this solution were used for thin-layer chromatography (Table VI). Further purification of the corrinoid compound could be achieved by chromatography on a 1.0 × 4-cm TEAE-cellulose column, previously washed with 5 mM Tris-Cl^- buffer, *p*H 9.0, containing 2 mM KCN. The concentrated solution of the dicyanocobamide was applied to the column. On elution with 0.05 M Tris-Cl, *p*H 9.0, containing 2 mM KCN, a yellow zone moved in front of the purple corrinoid zone and separated completely. The purple fractions were concentrated by absorption on a 0.5 × 2.5-cm column of Dowex 2 (formate) and elution with 0.1 M sodium acetate buffer, *p*H 4.

Fraction D2. This fraction was first desalted by phenol extraction. The aqueous solution was then passed into a 0.8 × 5.0-cm column of phosphocellulose at *p*H 3.9 and a red zone formed on the top of the column. The column was eluted first with 5 mM acetate buffer, *p*H 4, until a second yellow-orange zone appeared in the effluent. Then the buffer concentration was raised to 25 mM, and the zone was washed out completely. Now the *p*H of the eluting buffer was increased to 4.25 and a third orange zone moved quickly through the column. Fractions of 2 ml were collected and the absorbance at 260 mμ of the fractions was measured. The elution pattern is shown in Figure 2. Fractions belonging to the same peak were combined and concentrated by phenol extraction. The concentrated solutions were numbered in order of elution from the column: D2P1 (fractions 3–10); D2P2 (fractions 11–21); and D2P3 (fractions 22–25). A further subdivision of D2P3 into D2P3a and D2P3b was necessary

later, when this fraction divided into two yellow spots on paper ionophoresis and paper chromatography (Tables II and III).

Fraction D3. After a phenol extraction fraction D3 was placed on a 1 × 15-cm column of phosphocellulose and eluted successively with sodium acetate buffer of the following concentrations and *p*H: 5 mM, *p*H 4.0; 25 mM, *p*H 4.25; and 25 mM, *p*H 4.5. The flow through the column was 1.6 ml/min with a slight pressure. Fractions of 4 ml were collected and their absorbance at 260 mμ was measured. The elution pattern is shown in Figure 3. Besides other ultraviolet absorbing fractions, three corrinoid compounds emerged from the column in rather pure form. The compound present in fractions 24–30, designated D3P2, proved to be identical with compound D2P2 as judged by paper chromatography, paper ionophoresis, and the absorption spectrum. Fractions 49–60, designated D3P5, contained mainly a red cobamide coenzyme (visible absorption maximum at 520 mμ). Fractions 63–78 contained a yellow coenzyme (visible absorption maximum at 460 mμ), which was consequently numbered D3P6. As a criterion of purity of the red coenzyme, D3P5, the ratio of absorbance at 520 and 260 mμ was used. The purest fractions showed a A_{520}/A_{260} ratio of 0.19, which was not changed by further chromatography. The yellow coenzyme (D3P6), in contrast to the red coenzyme (D3P5), is inactive in the enzymatic glutamate mutase assay (Barker *et al.*, 1960a). Its absorbancy maxima are at 458, 378, 303, and 262 mμ.

Fraction D4. After phenol extraction, the dark-colored aqueous solution was run into a 1 × 7-cm column of CM-cellulose. On elution with 5 mM sodium acetate buffer, *p*H 4.0, a small amount of pink material left the column. This was not further investigated. The cobamides were eluted with 25 mM sodium acetate buffer, *p*H 4.5, whereas the dark material remained on the top. The reddish-orange fractions were combined and concentrated by phenol extraction. Ascending paper chromatography of an aliquot on Whatman No. 1 paper using solvent V revealed three red-to-orange compounds with R_F values of 0.09, 0.25, and 0.40, respectively. Further removal of ultraviolet absorbing material without separation of the pigments was achieved by chromatography on a 1 × 5.5-cm column of phosphocellulose. With 25 mM sodium acetate buffer, *p*H 4.25, only one colored peak was eluted. The three cobamide components were then separated by paper chromatography on three 15 × 40-cm strips of Whatman No. 1 paper in the system mentioned above. The zones containing the cobamides were cut out and eluted with water. According to the mobility in paper chromatography, the fast, medium, and slow compounds were designated D4F, D4M, and D4S, respectively.

The scheme for the separation of the corrinoid compounds of *M. omelianskii* is summarized in Figure 4.

Degradation of the Red Coenzyme (D3P5) by Cyanide. This was carried out according to Weissbach *et al.* (1960) and Toohey *et al.* (1961). To a solution of 0.1 μmole coenzyme in 4 ml water, 1 M KCN solution was

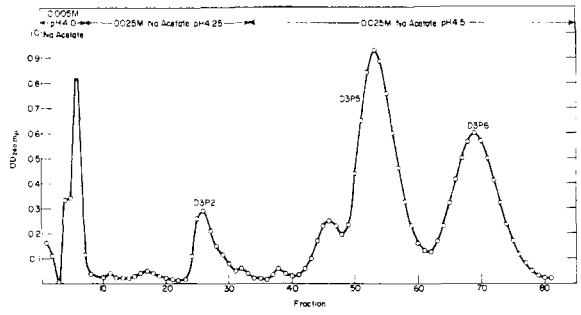

FIGURE 3: Chromatography of fraction D3 of the Dowex 50 column (Figure 1) on a phosphocellulose column.

added to give a final concentration of 25 mM and a *p*H of 10. The test tube containing the reaction mixture was placed in an ice bath and protected from light. Spectra were taken in intervals of 15–30 minutes. After 3 hours, no further increase of the absorption at 367 and 580 mμ was observed. The solution was then acidified to *p*H 3.5 with acetic acid and passed through a 0.5 × 2-cm column of Dowex 50W. The cyanocobamide passed rapidly through the column. The column was then washed with a little water until the effluent showed no absorption at 260 mμ. Finally, adenine was eluted with 25 mM sodium acetate buffer, *p*H 6.0.

Cleavage of the Nucleotide Side Chain and Isolation of the Nucleoside. This was done by the method of Friedrich and Bernhauer (1956b). The monocyano vitamin (0.1 μmole), obtained by cyanolysis of the red coenzyme D3P5, was desalted by phenol extraction. The aqueous solution was evaporated to dryness in a 5-ml tube *in vacuo* over H_2SO_4 and KOH. The following additions were made to the dried material: 0.1 ml of distilled water, 60 μl of 0.333 M $Ce(NO_3)_3$, 50 μl of 1 N NaOH, and 2 μl of a 10% solution of KCN. The tube was heated in boiling water for 1 hour with occasional stirring. Five μl of 1 M KCN was then added and the mixture was left for 1 hour at 0° for complete precipitation of cerous hydroxide. The precipitate was removed by centrifugation and washed three times with 0.3 ml water with addition of enough KCN to keep the *p*H at 8. The combined supernatants were acidified to *p*H 3.5 with a drop of 50% acetic acid and immediately passed through a 0.5 × 2-cm column of Dowex 50W. All colored material (cobinamide) was eluted with 5 ml of 10 mM KCN solution, adjusted to *p*H 6.0 with acetic acid. After the column was washed with 3 ml of distilled water, the nucleoside was eluted rapidly with 0.15 M ammonia. The nucleoside-containing fraction was detected by its absorption at 260 mμ. It was evaporated to dryness over concentrated sulfuric acid *in vacuo*. In the same way, the nucleosides from 1.4 mg of Factor III and 0.7 mg of cyanocobalamin were isolated. The yields varied between 70 and 90%.

Isolation of the Nucleotide Moiety of B_{12} Vitamins.

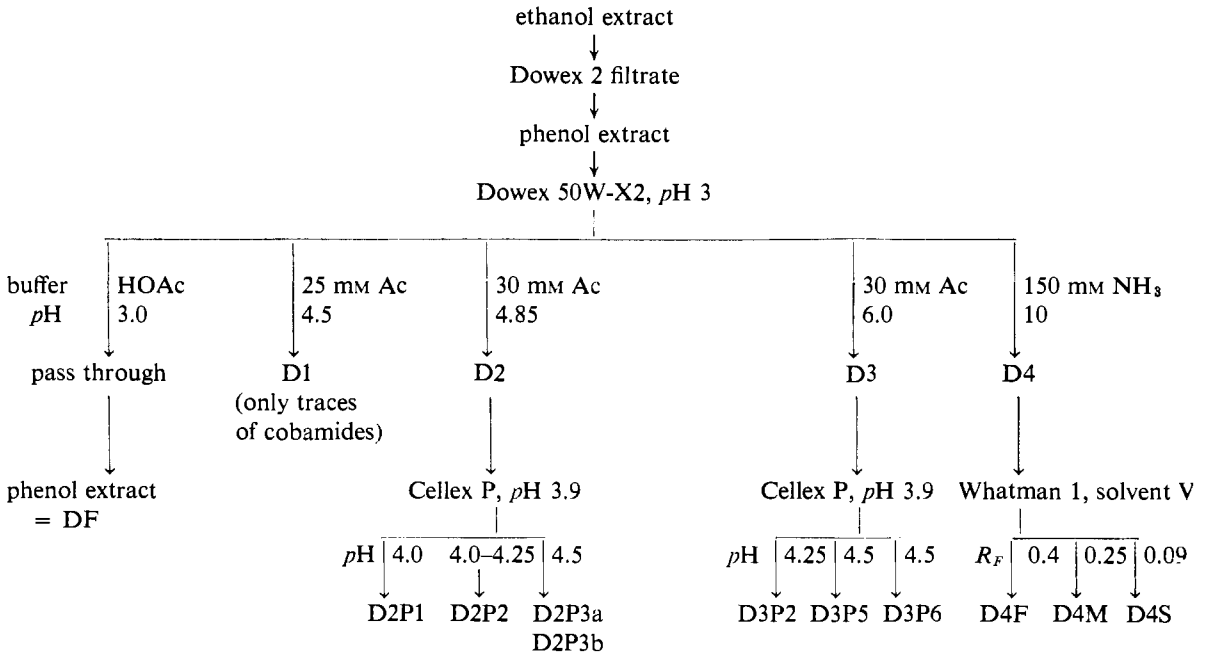

FIGURE 4: Scheme of the fractionation of the corrinoid compounds of *M. omelianskii*.

The method of Friedrich and Bernhauer (1954b, 1956a) was used. Approximately 0.1 μmole of the red coenzyme D3P5 was converted to the monocyano vitamin as described. The desalted and dried material was dissolved in 0.1 ml of 70% perchloric acid and left at room temperature for 3 hours. The solution was cooled with ice water, 0.1 ml of distilled water was added, and 2 N KOH was added dropwise to *p*H 8. After standing in the refrigerator overnight, the precipitated $KClO_4$ was centrifuged down and washed four times with 0.2-ml portions of water. After the *p*H was adjusted to 8.5 the combined supernatant solution was passed through a 0.5 × 2.5-cm column of Dowex 2 (formate). Cobinamide passed through the column without retention, and a small amount of colored material, probably derivatives of cobyric acid, was eluted with 50 mM acetic acid. Finally, the nucleotide was eluted with 0.02 N HCl. By the same method, nucleotides were isolated from 1.5 mg of cyanocobalamin and 1.6 mg of Factor III. The yields were between 80 and 90%.

Synthesis of 5-Hydroxybenzimidazolylcobamide Coenzyme. This coenzyme was synthesized according to the general method of Müller and Müller (1963). To a solution of 4.5 mg Factor III in 0.8 ml of water about 1 mg of PtO_2 was added. The solution was agitated by a stream of hydrogen gas introduced through a capillary until the color of the mixture had changed from red to brown (after 20 minutes). The catalyst was then removed by centrifugation and washed once with 0.2 ml of water. The combined supernatant solutions were transferred to a centrifuge tube of 1 cm diameter and 5 cm length, fitted with a rubber stopper bearing a long and a short hypodermic needle for inlet and outlet of reagent nitrogen. The tip of the gas-inlet needle was set just over the surface of the solution, which was agitated by a magnetic rod. Air was expelled from the reaction vessel by a stream of nitrogen for 10 minutes. Then 100 mg of NH_4Cl and about 5 mg of zinc dust were added without admitting oxygen. After 2 hours a solution of 15 mg of 2′,3′-isopropylidene-5′-tosyladenosine in 0.1 ml of absolute pyridine was injected through the gas outlet by a syringe under dim light. After 2 minutes the zinc dust was removed by centrifugation and washed twice with 0.5 ml of water. The combined supernatant solutions were passed through a phenol extraction, and hydrochloric acid was added to the resulting aqueous solution to a final concentration of 0.1 N. The solution was left at room temperature for 22 hours. After another phenol extraction, the cobamides were fractionated on a 1 × 7.5-cm column of phosphocellulose and eluted successively with 50 ml 25 mM sodium acetate buffer, *p*H 4.0; 100 ml 25 mM sodium acetate buffer, *p*H 4.25; and 100 ml of 25 mM sodium acetate buffer, *p*H 4.5. A red and a colorless absorbance peak was eluted with *p*H 4.25 buffer and a double peak showing a coenzyme spectrum emerged at *p*H 4.5. The fractions of the latter were combined, desalted by phenol extraction, and fractionated again on a 1 × 5.5-cm column of phosphocellulose. The first red peak was completely eluted with 25 mM sodium acetate buffer, *p*H 4.25, and discarded. Then the 5-hydroxybenzimidazolylcobamide coenzyme was eluted in a sharp peak with 25 mM sodium acetate buffer, *p*H 4.5. The fractions of the latter were combined

and concentrated by phenol extraction. The total recovery of 5-hydroxybenzimidazolylcobamide coenzyme was 0.28 μmole, a 9% yield.

Results

The vitamin B_{12} activity extracted from the bacteria and the distribution of the activity among the Dowex 50 column fractions DF, D1 + D2, D3, and D4, are summarized in Table I.

The characterization of the isolated corrinoid compounds by paper ionophoresis and paper chromatography is summarized in Table II and III, respectively.

Identification of the Coenzyme D3P5. The following criteria for the identification of the red coenzyme D3P5 as 5-hydroxybenzimidazolylcobamide coenzyme were fulfilled: (1) Both synthetic Factor III coenzyme and the isolated coenzyme have the same spectrum in neutral, acid, and alkaline solution. The spectrum of this coenzyme (Figure 5) differs from those of other coenzymes above *p*H 10 in exhibiting an extra peak at 315 mμ; no other coenzyme so far described has this property. The 315-mμ peak is probably caused by the ionization of the phenolic hydroxyl group, since the same absorption peak is shown by the dicyano form of Factor III as well as by the 5-hydroxybenzimidazole ribofuranoside in alkaline solution, as pointed out by Friedrich and Bernhauer (1956a, 1959). (2) The synthetic and the isolated coenzymes do not differ in electrophoretic mobility and in paper chromatography under the conditions described in Tables II and III. (3) The activities of the synthetic and natural

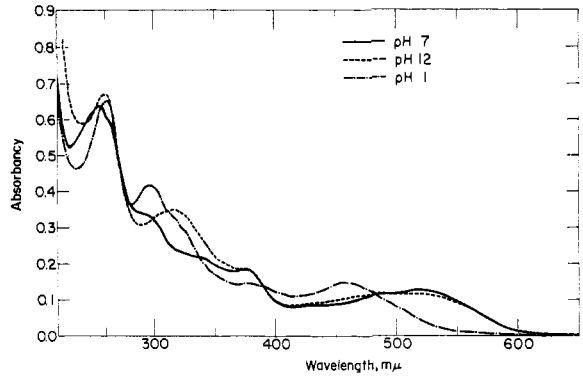

FIGURE 5: Absorption spectra of the red coenzyme of fraction D3P5 in acid (0.1 N HCl), neutral (water), or alkaline (0.01 N NaOH) solution. The coenzyme solution is approximately 2×10^{-5} M.

TABLE I: Distribution of Vitamin B_{12} Activity in Fractions Derived from *M. omelianskii*.

Fraction	B_{12} Activity[a] (μg/g wet wt)	Yield (%)
Ethanol extract	16	100
Dowex 2 filtrate	12	81
Dowex 50 pass through (DF)	5	31
Eluate *p*H 4.8–5.2 (D1 + D2)	1	6
Eluate *p*H 6.0–6.5 (D3)	5	31
Eluate *p*H 10 (D4)	2	12
Total, fractions 3–6	13	80

[a] Determined by the *E. coli* assay and expressed as cyanocobalamin.

TABLE II: Ionophoretic Mobility (cm^2 $volt^{-1}$ hr^{-1} $\times 10^3$) of Corrinoid Compounds.[a]

Buffer	Acetate				Phosphate		Borate
Concn (M)	0.1	0.05	0.05	0.05	0.05	0.05	0.1
*p*H	4.0	4.5	5.0	5.5	6.0	7.0	9.0
Compound							
D2P1	25						−175
D2P2	100						
D2P3a	75						−400
D2P3b	−100						−575
D3P5	150	100	75	75	0	0	
D3P6	225	150	88	88	0	0	
D4F	125						
D4M	200						
D4S	225						
DBC	100	75		25	0	0	
V_{1a} coenzyme	150	37					
CH_3-B_{12}	25						

[a] Paper ionophoresis on acetylated cellulose "Oxoid." Negative numbers mean migration toward the anode. The values were corrected for electroosmosis using cyanocobalamin as the uncharged reference compound.

TABLE III: Paper Chromatography of Corrinoid Compounds.[a]

Compound	R_C	R_C of Impurities
DF	0.08	0.19 (fl)
D2P1	1.12	1.44 (fl); 1.79 (fl)
D2P2	0.88	1.56 (fl)
D2P3	(a) 1.00; (b) 1.50	1.18 (fl); 1.54 (fl); 2.02 (q)
D3P5	0.55	None
D3P6	0.83	None
D4F	1.51	None
D4M	1.00	None
D4S	0.39	None
CH_3-B_{12}	1.85	None
V_{1a} coenzyme	0.70	None

[a] Ascending chromatography on Whatman No. 1 paper with 2-butanol, water, acetic acid (100:50:3) as developing solvent. The R_C values refer to the migration relative to cyanocobalamin. (fl) = fluorescent; (q) = quenching.

coenzymes were compared in the glutamate mutase assay. A single linear Lineweaver-Burk (1934) plot was obtained. The K_M values were calculated to be 1.8×10^{-6} M. The V_{max} of both samples (ΔOD_{240}/min = 0.17) were identical within the limits of error of the determination.

(4) The mono- and dicyano forms of the vitamin, obtained after cyanide degradation of D3P5, showed the spectra reported for Factor III by Friedrich and Bernhauer (1959). Adenine, formed by cyanolysis of D3P5, was recognized by its spectra in neutral, acid, and alkaline solution. (5) The cyanocobamide formed from D3P5 had the same R_F values in paper chromatography as authentic Factor III (Table IV). (6) The nucleoside

TABLE IV: Paper Chromatography of Cyanocobamides.

Compound	R_F Value in Solvent I	II	III
Factor III	0.12	0.16	0.135
D3P5	0.148	0.16	0.135
Factor III + D3P5	0.148		0.135
Cyanocobalamin	0.174	0.44	0.229

and nucleotide, obtained from degradations of the vitamin, showed the same R_F values in thin-layer chromatography as 5-hydroxybenzimidazoleribofuranoside and 5-hydroxybenzimidazole-3′-phosphoribofuranoside, respectively (Table V). Thin-layer chromatography on cellulose powder provides an excellent method for the rapid identification of nucleosides and nucleotides as recommended by Randerath and Struck (1961). The strong fluorescence of derivatives of benzimidazole in weak acid under ultraviolet light permits the detection of as little as 1 mμg of these compounds. (7) The spectra of the nucleoside in neutral, acid, and alkaline solution agreed with those spectra of 5-hydroxybenzimidazoleribofuranoside reported by Friedrich and Bernhauer (1959).

TABLE V: Thin-Layer Chromatography of the Nucleosides and Nucleotides.

Benzimidazole	R_F in Solvent VI (nucleoside)	V (nucleoside)	VII (nucleotide)
5-OH	0.45	0.68	0.45
5-CH_3O	0.70	0.79	
5,6-Dimethyl	0.75	0.79	0.62
D3P5 component	0.45	0.68	0.45

Identification of the Vitamin in Fraction DF. The absorption band at 360 mμ of fraction DF purified by paper chromatography clearly establishes the B_{12} vitamin type structure. After converting the compound to the monocyano form of the vitamin and purification by adsorption on Dowex 2 formate and chromatography on TEAE-cellulose, the spectra of the mono- and dicyano vitamin were identical with those of Factor III (Friedrich and Bernhauer, 1959). Further proof for the identity with Factor III was obtained by thin-layer chromatography in solvents I–IV, as shown in Table VI.

TABLE VI: Thin-Layer Chromatography of DF, Factor III, and Cobalamin.

Compound	I	II	III	IV
DF	0.35	0.45	2.8	3.1
Factor III	0.35	0.45	2.8	3.1
Cobalamin	0.50	0.67	3.7	4.2

Discussion

Factor III was isolated first by Friedrich and Bernhauer (1953, 1954a) from municipal sewage sludge. This material contains a large number of methane-producing bacteria, including *M. omelianskii*, as reported by Heukelekian and Heinemann (1939). Working with enrichment cultures of *M. omelianskii*, Neujahr and Callieri (1959) obtained some evidence for the presence of Factor III in this organism by

chromatographic and bioautographic methods. But as they pointed out, "8 different vitamin B_{12} factors can be attributed to such a spot." Two of those, pseudovitamin B_{12} and Factor A, they were able to exclude by "more accurate chromatographic and electrophoretic studies." But the most abundant B_{12} vitamin they found was designated Factor Met and assumed to be different from all the known nucleotide-containing B_{12} vitamins. This discrepancy with our finding that the coenzyme and vitamin forms of Factor III are the most abundant corrinoid compounds, together accounting for 54% of total B_{12} activity, may be due to differences in the strain or culture methods.

The relatively large amount of Factor III in the vitamin form (fraction DF) is of interest since the coenzyme forms have been found by Toohey *et al.* (1961) and Volcani *et al.* (1961) generally to be the predominant cobamides in bacteria. The vitamin forms (cyano-, aquo-) are usually considered to be artifacts arisen from photolysis or chemical changes during isolation. However, under the isolation conditions we have used the coenzyme form of Factor III is stable. Consequently, this vitamin is undoubtedly present in *M. omelianskii* prior to extraction. No vitamin form of any incomplete coenzyme present in fractions D2 and D3P6 could be detected.

The Michaelis constant of Factor III coenzyme (1.8×10^{-6} M) is appreciably higher than those of other 5(6)-monosubstituted benzimidazolylcobamides isolated by Toohey *et al.* (1961). The position of the substituent in those coenzymes is still an open question, whereas the position of the hydroxy group of Factor III on C-5 of the benzimidazole ring has been unequivocally established by Friedrich and Bernhauer (1957) and by Folkers *et al.* (1957).

From the spectra and paper ionophoretic properties of the other corrinoid compounds isolated, some conclusions can be drawn as to their nature. First of all, D3P6, containing about 8% of total B_{12} activity, is clearly an incomplete coenzyme because of its lack of activity in the glutamate mutase assay and because its spectrum shows an absorption peak at 303 mμ. Experiments to establish its structure are in progress.

The compounds D2P1, D2P3a, and D2P3b together represent less than 4% of total B_{12} activity. They have identical spectra, very similar to cobinamide coenzyme. Their ionophoretic mobilities at *p*H 9 (Table II) indicate one and more negative charges on the molecules, probably due to free carboxylic acid groups on the corrin nucleus. Such compounds are assumed to be precursers of cobinamide coenzyme by Bernhauer *et al.* (1962, 1963).

Fractions D2P2 (= D3P2), D4F, and D4M are neither corrinoid coenzyme nor vitamin forms. The very small fraction D4S is probably hydroxy (aquo) Factor III.

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Inter- and Intramolecular Interactions of α-Lactalbumin. III. Spectral Changes at Acid *p*H*

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ABSTRACT: The denaturation of α-lactalbumin at *p*H values below its isoelectric point is accompanied by blue shifts of the absorption spectrum in the region of 270–300 mμ characteristic of changes in the environment of tryptophan groups. The difference extinction coefficient, ΔE_{293}, is strongly *p*H dependent, but rather insensitive to large changes in ionic strength indicating the probable absence of charge perturbations. In the transition region (*p*H 3–4) ΔE_{293} is also strongly dependent on temperature. The characteristics of this thermal transition indicate that the conformational change associated with the low *p*H denaturation of α-lactalbumin is a complex one involving a series of consecutive or parallel steps. Since the transition at 25° involves the "exposure" of no additional tryptophan groups to the aqueous medium (M. J. Kronman and L. G. Holmes, 1965, *Biochemistry 4*, 526 [this issue; following paper]), it seems probable that the blue shift is the result of alterations of the environment of two or more of the three "buried" groups. It is suggested that this change might come about without unfolding through swelling of the molecule under constraint of the disulfide bridges with subsequent alteration of interactions of "buried" tryptophans with other perturbing groups.

In the first two papers in this series (Kronman and Andreotti, 1964; Kronman *et al.*, 1964), hereafter referred to, respectively, as papers I and II, we demonstrated that below *p*H 4 α-lactalbumin showed a marked propensity toward aggregation (molecular weight of aggregate 3×10^5), reflected as changes with time of the apparent component distribution in the ultracentrifuge and in solubility in ammonium sulfate at *p*H 6.6. In addition, we showed that in the same *p*H range (2–3) association to low molecular weight units could also occur even at protein concentrations where aggregate was absent. In contrast with these observations, aggregation and association were absent or were quite feeble at *p*H values close to but on the alkaline side of the isoelectric point. These observations, together with characteristics of the aggregation process, strongly suggested that the molecular states of monomeric α-lactalbumin were different in these two *p*H regions.

It was suggested (paper II) that this difference was the consequence of a conformational change which made certain groups available for intermolecular interaction, groups which heretofore had been buried within the protein molecule. While at present we cannot demonstrate that such groups are liberated during this denaturationlike process, we have sufficient information to support our hypothesis that a conformational change has occurred. For example, at *p*H 2 at protein concentrations where aggregation and association are absent, sedimentation velocity measurements reveal an increase in the frictional ratio of the monomeric α-lactalbumin molecule consistent with an increase in its hydrodynamic volume (paper II). Further evidence for such a conformational change has been obtained from rotatory dispersion measurements (M. J. Kronman and R. Blum, unpublished experiments) and changes in the ultraviolet fluorescence of tryptophan residues (M. J. Kronman, manuscript in preparation). These changes, as well as the spectral properties to be reported here, are a reflection of shifts in the balance of intramolecular interactions that stabilize the protein molecule in its native state. In this series of papers we shall attempt to describe the relationship between

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