Total Synthesis of Acetate from CO_2. I. Co-Methylcobyric Acid and Co-(Methyl)-5-methoxybenzimidazolylcobamide as Intermediates with *Clostridium thermoaceticum**

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ABSTRACT: Co-$^{14}CH_3$-cobyric acid and Co-($^{14}CH_3$)-5-methoxybenzimidazolylcobamide (Co-CH_3-Factor III_m) have been isolated from intact cells of *C. thermoaceticum* which were fermenting glucose and had been exposed to $^{14}CO_2$ for 15 sec. The Co-methyl group of these corrinoids appears to be an intermediate of the synthesis from CO_2 of the methyl group of acetate. Evidence also has been obtained for the occurrence of a Co-carboxymethylcorrinoid which may be formed by carboxylation of the Co-methylcorrinoids and thus be an intermediate in the formation of acetate. Cell-free extracts of *C. thermoaceticum* catalyze the fermentation of pyruvate and the fixation of CO_2 into both carbons of acetate. When the above ^{14}C-methyl labeled compounds were added to extracts together with pyruvate, the Co-methyl was converted to the methyl of acetate. The extracts also catalyze the conversion of Co-CH_2-$^{14}COOH$-cobalamin to carboxyl-labeled acetate. The latter reaction is stimulated by NADPH or pyruvate, which suggests that acetate is formed by reductive cleavage of the cobalt–carboxymethyl linkage. A method is described for the identification of Co-methyl ligands in B_{12} derivatives by use of iodine cleavage.

Fontaine and co-workers (1942) isolated *Clostridium thermoaceticum* and showed that it produces almost 3 moles of acetate per mole of glucose. Studies by Barker and Kamen (1945) and by Wood (1952a,b) indicate that the fermentation occurs by the following over-all reactions.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 8H + 2CO_2$$

$$8H + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$

$$\text{Sum: } C_6H_{12}O_6 \rightarrow 3CH_3COOH$$

Accordingly, two-thirds of the acetate is formed directly from glucose and one-third is formed by the reduction of carbon dioxide to acetate. Direct proof of the total synthesis of acetate from CO_2 was provided by Wood (1952a) through mass analysis of the acetate formed from $^{13}CO_2$. Thus far the only known process for synthesis of carbon to carbon bonds with the carbons derived exclusively from CO_2 is *via* the reductive pentose cycle as demonstrated in autotrophic and photosynthetic organisms. It has been shown by Ljungdahl and Wood (1965) that this cycle does not operate in *C. thermoaceticum*, nor do other mechanisms which have been proposed previously. Recently Poston *et al.* (1964) have provided a clue to the mechanism. They demonstrated that the synthesis of acetate from $^{14}CO_2$ in the presence of pyruvate by cell-free extracts of *C. thermoaceticum* is decreased by the intrinsic factor and that the methyl group of Co–$^{14}CH_3$–cobalamin is incorporated into the methyl group of acetate. This reaction as well as others which are to be reported here are shown in Figure 1.

We have confirmed the finding that Co-methylcobalamin (compound I, Figure 1) is incorporated into acetate and in addition demonstrated that extracts of *C. thermoaceticum* catalyze the cleavage of Co-carboxymethylcobalamin (compound II) to acetate. However, it has been shown that Co-methylcobalamin is not the natural intermediate of *C. thermoaceticum* metabolism; instead, Co–($^{14}CH_3$)–5-methoxybenzimidazolylcobamide (Co-methyl-Factor III_m) (compound III, Figure 1) and Co–$^{14}CH_3$–cobyric acid (compound IV) are formed by whole cells when incubated with $^{14}CO_2$. The role of these compounds as precursors of the methyl group of acetate could be demonstrated by incubating them with pyruvate and a cell-free extract of *C. thermoaceticum*. The Co-methyl group was then incorporated into the methyl group of acetate.

Materials and Methods

Preparation and Use of Cell-Free Extracts. C. thermoaceticum was grown as described by Ljungdahl and Wood (1965) under CO_2 at 55° in 1 l. of medium containing glucose. The cells after 3 or 4 days of growth were harvested by centrifugation and washed with 50–75 volumes of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.005 M cysteine. The cells were suspended

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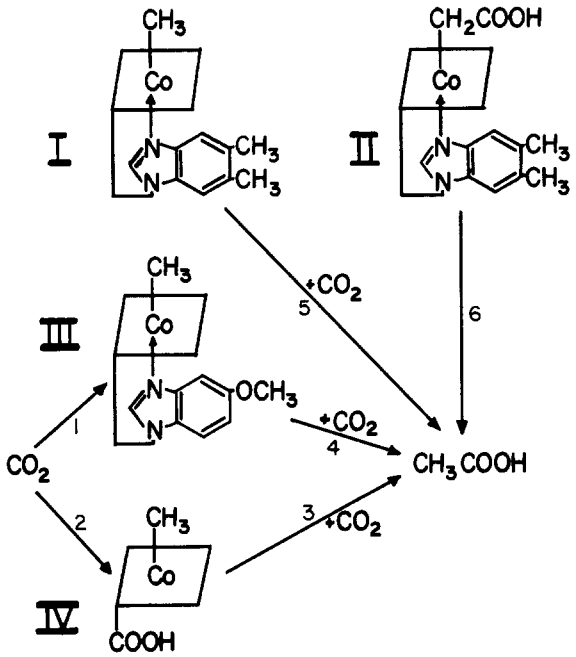

FIGURE 1: Corrinoids and the synthesis of acetate from CO_2 by *C. thermoaceticum*. The rectangle is an abbreviation for the corrin ring. I = Co-methylcobalamin, II = Co-carboxymethylcobalamin, III = Co-(methyl)-5-methoxybenzimidazolylcobamide (Co-methyl-Factor III_m), IV = Co-methylcobyric acid. Reactions 1 and 2 have been found in intact cells by use of $^{14}CO_2$. Reactions 3–6 are catalyzed by cell-free extracts. It is likely that reactions 3, 4, and 5 involve Co-carboxymethyl derivatives as intermediates.

in three volumes of the above buffer containing 0.02 M mercaptoethanol, and the suspension was passed through a French press and collected under nitrogen. The resulting mixture was centrifuged at 35,000 × *g* for 40 min, yielding a clear dark brown extract. All incubations with the cell-free extracts were made at 55° in an atmosphere of nitrogen and in darkness or dim light. In experiments with Co-$^{14}CH_3$-corrinoids the reactions were stopped by acidification to pH 2 with sulfuric acid and then carrier acetate was added. However, with Co-$CH_2{}^{14}COOH$-cobalamin it was necessary to remove the unreacted Co-carboxymethylcobalamin by filtration through charcoal (Norit A) before the sample was taken into a lighted room since Co-carboxymethylcobalamin yields acetate on photolysis (Johnson *et al.*, 1963).

Fixation of $^{14}CO_2$ by Cell Suspensions and Isolation of Co-$^{14}CH_3$-corrinoids. Cells from six bottles containing 18 l. of medium each were grown as described above and harvested in a Sharples continuous-flow centrifuge. The cell yield varied from 350 to 400 g. The cells were incubated with glucose for 10 min as described by Ljungdahl and Wood (1965) and then 0.5 mmole of potassium bicarbonate containing about 2 mcuries of ^{14}C was added. After 10–20 sec acetone was added to the cell suspension to give a final concentration of 75% in order to extract the corrinoids according to methods outlined by Bernhauer *et al.* (1963). The addition of acetone and all subsequent work was carried out in darkness or dim light because of the light sensitivity of coenzyme- and alkylcorrinoids. The acetone suspension was left at room temperature for 10–15 hr during which time it was shaken occasionally. The supernatant solution was decanted and filtered through a folded filter paper and the residue was extracted twice, each time with 2 l. of an acetone–water mixture (2:1). A small sample of the combined acetone extracts was used for isolation of an aliquot part of the free acetate formed during the incubation with the [^{14}C]bicarbonate. The remainder of the extract was evaporated in a flash evaporator to about 40 ml and this solution was absorbed on 12 g of Celite. The Celite was dried at room temperature in a stream of air and placed on a Celite column (36 × 270 mm) which had been prewashed with chloroform. The column was washed successively with 200 ml of chloroform, a chloroform–butanol mixture (2:1), ether, and finally acetone. This treatment removed material which later would interfere in the extraction with phenol. The corrinoids were then eluted from the Celite with water, from which they were extracted with a mixture of phenol and chloroform (3:2). The phenol–chloroform solution was washed with water at least ten times until the water was clear and colorless. An equal volume of chloroform was then added to the phenol–chloroform solution followed by the addition of butanol to a concentration of about 25%. The corrinoids were then extracted from the phenol–chloroform–butanol solution with water. The aqueous solution of the corrinoids was washed several times with chloroform and then with a mixture of butanol and isopropyl ether (2:1) to remove traces of phenol. The solution was concentrated to a few milliliters and streaked on several sheets of Whatman 3MM paper for descending chromatography with water-saturated 2-butanol to which 1 ml of acetic acid was added per 100 ml. Nine zones of corrinoids were separated, of which fraction VI was found to be radioactive. The further separation and identification of the unlabeled corrinoids is described by Irion and Ljungdahl (1965). Fraction VI was eluted with water and rechromatographed on 2043B paper of Schleicher and Schuell using water-saturated 2-butanol. Three zones of corrinoids separated, of which two were radioactive; of these two, one had a yellow color (compound VIc) and the other moved a little faster and was red-orange (compound VIb). They were eluted separately from the paper and rechromatographed with water-saturated 2-butanol.

In later experiments the red-orange ^{14}C-containing corrinoid (fraction VIb) was purified by ion-exchange chromatography on a carboxymethylcellulose column (36 × 160 mm). The water solution of the corrinoids obtained after the phenol extraction was applied to the column which was developed with water. A red-orange fraction containing ^{14}C, passing out of the column after

TABLE I: Incorporation of $^{14}CO_2$ into Acetate by Extracts from *C. thermoaceticum*.[a]

Additions	Incubation (min)	^{14}C in Acetate: Total (cpm)	CH_3 (cpm)	COOH (cpm)	Ratio of ^{14}C CH_3/COOH
None	20	36			
	40	63			
Formate	20	53			
	40	44			
Pyruvate	20	6,160	2,240	3,850	0.58
	40	14,950	6,125	8,750	0.70
Pyruvate, formate	20	6,090	2,070	4,490	0.46
	40	11,500	4,480	7,745	0.58
Pyruvate, formate, B_{12}	20	8,650	3,430	5,660	0.61
	40	16,700	7,090	9,650	0.73

[a] Total volume of 3.2 ml containing extract (46 mg protein); potassium phosphate (pH 7.0), 250 μmoles; mercaptoethanol, 50 μmoles; $KH^{14}CO_3$, 15 μmoles (8000 cpm/μmole); and as indicated sodium pyruvate, 54 μmoles; sodium formate, 50 μmoles; vitamin B_{12} (cyanocobalamin), 1 μmole. Incubation was under N_2 at 55°. Samples of 1.25 ml were withdrawn after 20 and 40 min, mixed with 0.4 ml of 4 N H_2SO_4 and 0.8 mmole of sodium acetate. The acetate was isolated and degraded as described in the Methods section.

700 ml of water had passed through, was chromatographed on Schleicher and Schuell 2043b paper using water-saturated 2-butanol. The main red-orange band was radioactive and the material cochromatographed and migrated in electrophoresis identically with compound VIb isolated as above using only paper chromatography.

Degradation of Co-Methylcorrinoids with Iodine. Co-Methylcobalamin when dissolved in methanol reacts in darkness with iodine, and products of this reaction are iodocobalamin and methyl iodide (Bernhauer and Irion, 1964). This reaction was used for the identification of the Co-methyl ligand of the corrinoids. One milliliter of a solution of 127 mg of I_2 in 100 ml of methanol was added to 0.05–0.4 μmole of the corrinoid. The mixture was held under nitrogen in the darkness at room temperature for about 12 hr. As carrier 0.32 mmole of methyl iodide in 0.2 ml of ethanol was added, and the test tube was connected to a trap which contained 5 ml of a 5% solution of trimethylamine in ethanol. The trap was immersed in a dry ice–Cellosolve bath. Nitrogen gas was bubbled through the tubes, and the methyl iodide was trapped in the trimethylamine solution and was crystallized as tetramethylammonium iodide (Simmonds *et al.*, 1943). The salt was recrystallized from ethanol.

Degradation of Acetate and Radioactive Measurements. The acetate from the experiments with cell-free extracts and from whole cells was purified by steam distillation followed by chromatography on Celite according to Swim and Krampitz (1954). Degradation of the acetate to obtain the individual carbons was done according to Phares (1951). The total radioactivity of the acetate was determined after combustion to CO_2 according to Van Slyke and Folch (1940) and by plating and counting the carbon dioxide as barium carbonate. The ^{14}C of tetramethylammonium iodide was likewise determined after combustion to CO_2.

The radioactivity of fractions from the chromatograms was measured after evaporating the samples on aluminum planchets using a Nuclear-Chicago low-background counter or on paper strips with a Vanguard Autoscanner 880.

Chemicals and Analytical Methods. Vitamin B_{12} was from Sigma Chemical Co. It was converted to hydroxy-(aquo-) cobalamin (B_{12b}) as described by Bernhauer and Wagner (1963). Co-$^{14}CH_3$-cobalamin, Co-CH_2-^{14}COOH-cobalamin, and Co-$^{14}CH_2$COOH-cobalamin were prepared according to Müller and Müller (1962) from the hydroxy- (aquo-) cobalamin and [^{14}C]methyl iodide (New England Nuclear Corp.) or [1-^{14}C]monochloroacetate (Nuclear-Chicago Corp.) or [2-^{14}C]monobromoacetate (Nuclear Research Chem., Orlando, Fla.). Samples of monocyanomonoaquocobyric acid (Factor V_{Ia}) and cyano-5-methoxybenzimidazolylcobamide (Factor III_m) were kindly provided by Professor K. Bernhauer, Technische Hochschule, Stuttgart, Germany. The Co-$^{14}CH_3$ derivatives of Factor V_{Ia} and Factor III_m were synthesized as above but directly from their cyano forms and were purified by paper chromatography. Barium carbonate-^{14}C was from Tracerlab, Waltham, Mass., and was converted to [^{14}C]$KHCO_3$.

Celite 535 from Johns-Manville Co. was washed with 2 N sulfuric acid, water, and ethanol and then dried at room temperature. Carboxymethylcellulose (P. Brown Co.) with an exchange capacity of 0.92 mequiv/g was washed with 0.5 N sodium hydroxide, water, 1 N hydrochloric acid, and finally water until it was at neutral pH. Paper electrophoretic, paper chromato-

graphic, and spectrometric methods used for characterization of the Co-methylcorrinoids are described by Irion and Ljungdahl (1965).

Results

Fixation of $^{14}CO_2$ with Cell-Free Extracts of C. thermoaceticum. Cell-free extracts of *C. thermoaceticum* catalyze the conversion of CO_2 to acetate during the fermentation of pyruvate, yielding acetate labeled in both carbons (Table I). The incorporation of $^{14}CO_2$ into the methyl carbon was lowered on addition of formate, resulting in a decreased ratio of ^{14}C between the methyl and the carboxyl carbons. The addition of cyanocobalamin resulted in a small increase of the incorporation of ^{14}C into acetate, and this increase was higher for the methyl carbon, as shown by the increased ratio of ^{14}C in the methyl to the carboxyl group as compared to that with pyruvate and formate without B_{12}. Lentz and Wood (1955) have previously found with cell suspensions of *C. thermoaceticum* fermenting glucose that formate is preferentially converted to the methyl group of acetate.

Conversion of Co-$^{14}CH_3$-Cobalamin to $^{14}CH_3$-COOH by Cell-Free Extracts of C. thermoaceticum. The conditions for the incorporation of the methyl group of Co-$^{14}CH_3$-cobalamin into acetate are described in Table II. In agreement with Poston *et al.* (1964) we found that pyruvate was required for this incorporation. The pyruvate could not be replaced by reduced nicotinamide–adenine dinucleotide phosphate (NADPH[1]) but some incorporation was obtained by the addition of both NADPH and adenosine triphosphate. The acetate from the incubation with pyruvate alone, having 10,016 cpm, was degraded and found to contain 10,200 cpm in the methyl group and 39 cpm in the carboxyl group.

Conversion of Co-$CH_2{}^{14}COOH$-Cobalamin to Acetate by Cell-Free Extracts of C. thermoaceticum. The formation of acetate from Co-carboxymethylcobalamin is catalyzed by cell-free extracts of *C. thermoaceticum* as shown in Table III. Some acetate is formed without the addition of enzyme, and the same amount is formed in the presence of boiled enzyme from *C. thermoaceticum*. Approximately twice the amount of acetate is formed by the enzyme preparation without any additions. The yield of acetate was increased by addition of pyruvate or of NADPH, but NADH had very little effect. An extract from *Propionibacterium shermanii* did not catalyze the reaction, which indicates that the acetate formation from Co-carboxymethylcobalamin is due to enzymatic activity present in extract of *C. thermoaceticum* and not due to a nonspecific action of protein in a bacterial extract.

Identification of the [^{14}C]Corrinoids Isolated from

TABLE II: Conversion of Co-$^{14}CH_3$-cobalamin to Acetate-^{14}C with an Extract from *C. thermoaceticum*.[a]

Additions	^{14}C in Acetate (cpm)
None	79
Pyruvate	10,016
Pyruvate, ATP	10,026
Pyruvate, NADPH	9,330
NADPH	139
ATP, NADPH	902
Pyruvate, ATP, boiled enzyme	115

[a] Total volume of 3.45 ml containing extract (65 mg protein); potassium phosphate (pH 7.0), 300 μmoles; mercaptoethanol, 60 μmoles; $NaHCO_3$, 50 μmoles; Co-$^{14}CH_3$-cobalamin, 0.385 μmole (55,000 cpm); and as indicated sodium pyruvate, 50 μmoles; ATP, 10 μmoles; NADPH, 5 μmoles. Incubation was for 35 min under N_2 at 55°. Reaction was stopped by addition of 0.25 ml of 10 N H_2SO_4 and 1 mmole of sodium acetate was added as carrier which was isolated for ^{14}C assay.

TABLE III: Conversion of Co-$CH_2{}^{14}COOH$-cobalamin to Acetate by an Extract of *C. thermoaceticum*.[a]

Addition	^{14}C in Acetate 10 min (cpm)	^{14}C in Acetate 20 min (cpm)
None	2200	3460
Pyruvate	4240	9360
NADPH	4400	9100
NADPH, ATP	4740	8000
NADH	2700	3830
Pyruvate, boiled enzyme[b]	1250	2010
Pyruvate, minus extract	1310	2170
Pyruvate, *P. shermanii* extract[c]	1230	1740

[a] Total volume of 5 ml containing extract (36 mg of protein); Co-$CH_2{}^{14}COOH$-cobalamin, 0.385 μmole (22,600 cpm); potassium phosphate (pH 7.0), 400 μmoles; mercaptoethanol, 80 μmoles; $NaHCO_3$, 200 μmoles; and as indicated sodium pyruvate, 50 μmoles; NADPH, 10 μmoles; NADH, 10 μmoles; ATP, 10 μmoles. Incubation was under N_2 at 55°. Samples of 2 ml were withdrawn after 10 and 20 min and mixed with 150 mg of charcoal, 1 ml of 1.5 N H_2SO_4, and 1 ml of 1 N acetic acid. The acetic acid was isolated after filtration. [b] The enzyme was heated in boiling water for 10 min. [c] The extract of *C. thermoaceticum* was substituted with an extract of *Propionibacterium shermanii* (29 mg of protein).

[1] Abbreviations used in this work: NADPH, reduced nicotinamide–adenine dinucleotide phosphate; NADH, reduced nicotinamide–adenine dinucleotide.

Whole Cells. Compound VIb (red-orange in color) has been identified as Co-($^{14}CH_3$)-5-methoxybenzimidazolylcobamide and compound VIc (yellow in color) as Co-$^{14}CH_3$-cobyric acid. Both compounds lost about 90% of their ^{14}C activity on exposure to light, indicating that the radioactive carbon of both compounds was bound to the cobalt atom. Addition of cyanide to the samples without exposure to light did not result in loss of ^{14}C. When treated with iodine in the dark both compounds yielded labeled methyl iodide, which was crystallized as tetramethylammonium iodide. The results are shown in Table IV and compared with results from synthetically prepared Co-$^{14}CH_3$-cobalamin and Co-$^{14}CH_3$-cobyric acid. Most of the ^{14}C was recovered as tetramethylammonium iodide, and the recoveries from the isolated compounds agree well with those of the comparable synthetic compounds. Synthetically prepared Co-$^{14}CH_2COOH$-cobalamin does not yield methyl iodide when treated with iodine.

TABLE IV: Evidence for the Formation of Co-$^{14}CH_3$-corrinoids from $^{14}CO_2$ during the Fermentation of Glucose by *C. thermoaceticum*.[a]

Corrinoid	μmole	Corrinoid (cpm)	$(CH_3)_4NI$ (cpm)	Recovery (%)
Co-$^{14}CH_3$-cobalamin, synthetic	0.385	55,500	33,100	59.7
Compound VIb	0.111	293	189	64.5
Co-$^{14}CH_3$-cobyric acid, synthetic	0.146	21,900	20,160	91.8
Compound VIc	0.044	160	133	83.0

[a] Compounds VIb and VIc were isolated from 400 g of cells which were incubated in a 50% suspension in 0.1 M phosphate buffer, pH 7, with glucose at 55° under N_2 for 10 min and then exposed for 15 sec to 0.5 mmole of potassium bicarbonate containing about 2 mcuries of ^{14}C. The Co-methylcorrinoids were allowed to react with iodine, yielding iodocorrinoids and methyl iodide. The latter after addition of carrier methyl iodide was converted to crystalline tetramethylammonium iodide and was oxidized to CO_2 for determination of ^{14}C as barium carbonate. For details see the text.

After the iodine degradation of the isolated compounds the corrinoids were converted into their cyano forms by addition of HCN. The same cyanocorrinoids were formed after photolysis and addition of HCN to compounds VIb and VIc. The cyanocorrinoids were cochromatographed on paper with authentic samples of Bernhauer's Factor III_m and cobyric acid using the three cyanide-containing solvents D, E, and F as described by Irion and Ljungdahl (1965). The cleavage products of compound VIb behaved in all solvents as Factor III_m, and those of compound VIc as cobyric acid. Also using paper electrophoresis at pH 2.7 and 7.3 the cyano derivatives of compounds VIb and VIc were found to behave as Factor III_m and cobyric acid, respectively. The spectra of the two cyano compounds were also found to be identical with those of Factor III_m and cobyric acid. A more detailed description of the identification of Factor III_m and cobyric acid, the two most abundant corrinoids in *C. thermoaceticum*, is given in the paper by Irion and Ljungdahl (1965).

The final and most conclusive evidence for the identities of compounds VIb and VIc was obtained by the direct comparison of these compounds with synthetically prepared Co-methyl-Factor III_m and Co-methylcobyric acid, respectively, using paper chromatography (Table

TABLE V: Comparison of the R_F Values of Compounds VIb and VIc from *C. thermoaceticum* with Authentic Co-Methylcorrinoids.

Corrinoid	Solvent[a] A R_F	B R_F	C R_F
Co-methylcobalamin	0.25	0.23	0.24
Compound VIb and Co-methyl-Factor III_m[b]	0.22	0.20	0.18
Compound VIc and Co-methylcobyric acid[b]	0.18	0.24	0.13

[a] Ascending chromatography in solvent systems: A, water-saturated 2-butanol; B, water-saturated 2-butanol–acetic acid (100:1); C, 2-butanol–water–25% NH_3 (100:36:14). [b] Compound VIb and Co-methyl-Factor III_m cochromatographed as did compound VIc and Co-methylcobyric acid.

TABLE VI: Comparison of the Electrophoretic Properties of Compounds VIb and VIc from *C. thermoaceticum* with Authentic Co-Methylcorrinoids.

Corrinoid	pH 2.7[a]	7.3	10.5
Co-methyl-cobalamin	+0.30		0 (neutral)
Compound VIb and Co-methyl-Factor III_m	+0.45	0 (neutral)	0 (neutral)
Compound VIc and Co-methylcobyric acid	+1.0		0 (neutral)

[a] Values at pH 2.7 are the migration relative to monocyanocobinamide = +1.

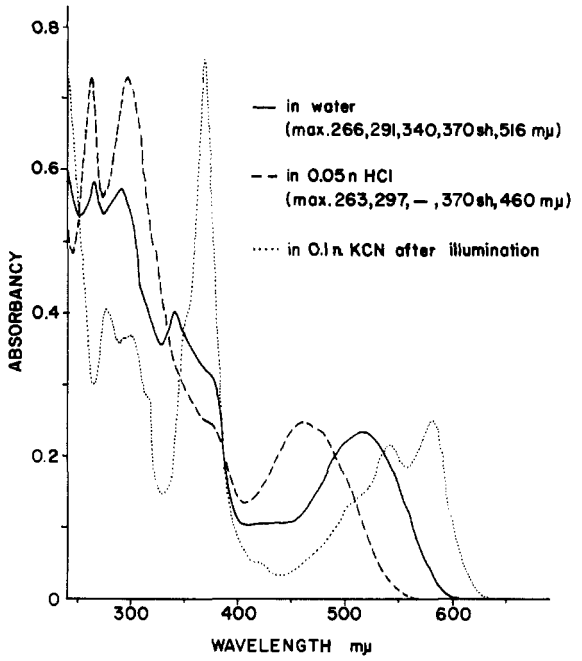

FIGURE 2: Absorption spectra of Co-(methyl)-5-methoxybenzimidazolylcobamide (compound VIb isolated from *C. thermoaceticum*). The concentration is approximately 2.4×10^{-5} M.

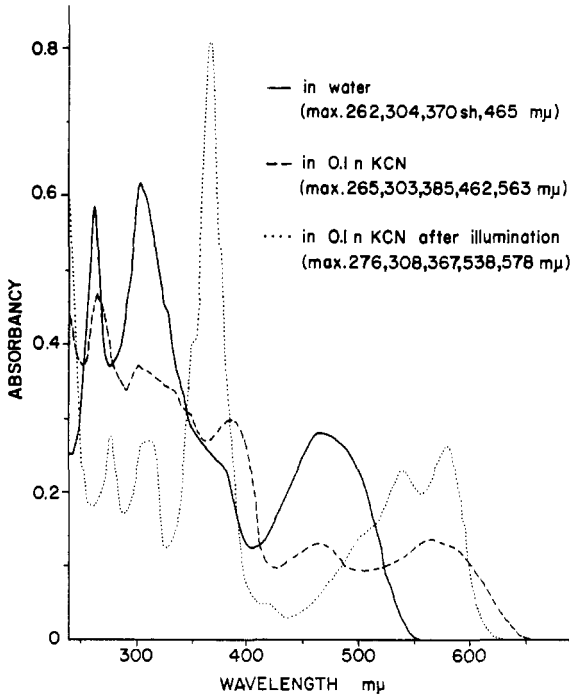

FIGURE 3: Absorption spectra of Co-methylcobyric acid (compound VIc isolated from *C. thermoaceticum*). The concentration is approximately 2.6×10^{-5} M.

V), paper electrophoresis at pH 2.7, 7.3, and 10.5 (Table VI), and spectrophotometry.

In Figure 2 the absorption spectra are shown for compound VIb (Co-methyl-Factor III_m) in water and in 0.05 N HCl (pH 1.3) and after illumination and addition of KCN. These spectra correspond exactly with those obtained using synthesized Co-methyl-Factor III_m. The shift of the visible maximum peak at 516 mμ in water to 460 mμ at pH 1.3 is characteristic of the complete coenzymes (Barker, 1962) and for complete alkylcorrinoids (Müller and Müller, 1962) and is caused by the protonation of the nitrogen in the 3 position of the imidazole ring of the nucleotide base, thus preventing the formation of a coordination bond between the nitrogen and the cobalt. The spectrum in water also indicates that compound VIb does not contain the 5′-deoxyadenosyl unit as occurs in corrinoid coenzymes. The ratio between 262 and 516 mμ is 2.47, whereas the ratio for the corresponding Co-5′-deoxyadenosyl-Factor III_m is 4.90 (Irion and Ljungdahl, 1965). On exposure to light the Co–methyl bond is cleaved and hydroxy- (aquo-) 5-methoxybenzimidazolylcobamide is formed (spectrum is given by Irion and Ljungdahl, 1965). The addition of KCN to the hydroxy (aquo) compound yields the spectrum of the corresponding dicyano compound (Figure 2, 0.1 N KCN after illumination). This spectrum is the same as that of the dicyano form of the authentic crystalline 5-methoxybenzimidazolylcobamide. Again the low absorption around 260 mμ shows the lack of an adenosine moiety which, if cleaved from the corrinoid, should have remained in the solution.

Three spectra of compound VIc which are identical with the corresponding spectra of Co-methylcobyric acid are shown in Figure 3. This compound has a yellow color in water with absorption maximum 465 mμ, indicating that the compound is an incomplete B_{12} factor. This spectrum is pH independent and is very similar in the range between 300 and 600 mμ with the spectrum of Co-methyl-Factor III_m in acid solution, but the latter has higher absorption at lower wavelength due to the absorption of the 5-methoxybenzimidazole moiety.

The spectrum of compound VIc in water also shows that it does not contain a 5′-deoxyadenosyl unit as found in corrinoid coenzyme factors. Thus the ratio between 264 and 465 mμ is 2.08, but for Co-5′-deoxyadenosylcobyric acid it is 4.72 (Irion and Ljungdahl, 1965). Addition of KCN to compound VIc (Co-methylcobyric acid) results in a change in the spectrum probably due to the formation of a Co-methylmonocyano complex. Upon acidification of this complex the spectrum of Co-methylcobyric acid is restored, showing that the methyl–cobalt bond is not cleaved by cyanide. Similar results have been obtained with Co-methylcobinamide (Müller and Müller, 1962). When a solution of compound VIc in the presence of KCN is exposed to light the cobalt–methyl bond is cleaved and the dicyano complex is formed, giving the same spectrum as found for dicyanocobyric acid

(Factor V_{Ia}) (Bernhauer *et al.* 1961). This spectrum is typical for all incomplete B_{12} factors lacking a base.

Evidences for Co-Methylcobyric Acid and Co-Methyl-Factor III_m as Intermediates in Synthesis of Acetate from $^{14}CO_2$. In Table VII the ^{14}C activities of the isolated methyl compounds are given, together with the specific activities of the acetate formed during the incubations with $^{14}CO_2$. The values for the methyl compounds were obtained by evaporating small aliquot parts on planchets and, after determination of the radioactivities, the compounds were converted to their mono and dicyano forms, respectively, for quantitative spectrophotometric estimation. The dicyanocobyric acid was determined by measuring the absorption at 367 mμ (ε 30.8 × 10^3 M^{-1} cm^{-1}) (Bernhauer *et al.*, 1961) and the monocyano Factor III_m at 361 mμ (ε 28.1 × 10^3 M^{-1} cm^{-1}) (Friedrich and Bernhauer, 1959). In every experiment the specific activity of the acetate was much lower than that of the methylcorrinoids. It is, however, probably more correct to compare the specific activities of the methylcorrinoids with those of the methyl groups of the acetate samples because, as shown by Poston *et al.* (1964) and by us, the methyl group of Co-methylcobalamin is solely incorporated into the methyl group of the acetate. When this is done the specific activities of the methylcorrinoids are 13–60 times higher than found of the methyl groups of acetate. There undoubtedly is dilution of acetate due to unlabeled acetate which was formed prior to the exposure to $^{14}CO_2$, so the comparison is not quantitative. In preparation Q it was found that the ^{14}C activities for the two methylcorrinoids were about the same. This may be so for all preparations, but unfortunately only one set of complete data is available. The amounts of Co-methyl-Factor III_m (VIb) and of Co-methylcobyric acid (VIc) obtained from different preparations have varied between 0.2 and 0.4 μmole per 400 g of wet cells.

Table VIII shows that authentic Co-methylcobyric acid and Co-methyl-Factor III_m are both incorporated into acetate and included in the table is the incorporation of Co-methylcobalamin with the same extract. The amount of Co-methylcorrinoids converted to acetate differs in each experiment; thus the incorporation of Co-$^{14}CH_3$-cobalamin into acetate is 16% in expt 1, in expt 2 74%, and in expt 3 only 3%. The extent of conversion of the other Co-$^{14}CH_3$ compounds

TABLE VII: Comparison of the ^{14}C Activities of the Acetate and Compounds VIb and VIc Formed by *C. thermoaceticum* Fermenting Glucose in the Presence of $^{14}CO_2$.[a]

Compound	Preparation			
	M	P	Q	R
	(cpm/μmole)			
Co-methyl-cobyric acid (VIc)	3640		1200	
Co-methyl Factor III_m (VIb)		2440	1230	2640
Acetate:				
total	725		246	180
methyl	280		69	42
carboxyl	450		167	110

[a] Conditions for the fermentation of glucose in the presence of $^{14}CO_2$ by *C. thermoaceticum* were the same as given in Table IV.

TABLE VIII: Conversion of Co-$^{14}CH_3$-cobalamin, Co-$^{14}CH_3$-cobyric Acid, and Co-$^{14}CH_3$-Factor III_m to ^{14}C-Acetate by Extracts of *C. thermoaceticum*.[a]

Expt	Corrinoid	(μmoles)	(cpm)	Conversion to Acetate	
				(cpm)	(%)
1	Co-CH_3-cobalamin	0.385	55,500	8,780	16
	Co-CH_3-cobyric acid	0.364	54,600	17,900	33
2	Co-CH_3-cobalamin	0.231	33,300	24,600	74
	Co-CH_3-cobyric acid	0.218	32,800	24,000	73
	Co-CH_3-Factor III_m	0.222	30,750	13,500	44
3	Co-CH_3-cobalamin	0.231	33,300	1,050	3
	Co-CH_3-cobyric acid	0.218	32,800	8,800	27
	Co-CH_3-Factor III_m	0.222	30,750	3,020	10

[a] Total volume of 4.3 ml contained potassium phosphate (pH 7.0), 360 μmoles; mercaptoethanol, 72 μmoles; sodium pyruvate, 47 μmoles; ATP, 10 μmoles; $^{14}CH_3$-corrinoids as indicated; and extract for expt 1, 134 mg; expt 2, 106 mg; and expt. 3, 73 mg protein. Incubation for 30 min at 55° in N_2.

also showed considerable variation. It appears that of the two natural precursors Co-$^{14}CH_3$-cobyric acid is the best.

Evidence for a Co-Carboxymethylcorrinoid in C. thermoaceticum. The evidence for a third compound formed from $^{14}CO_2$ in intact cells of *C. thermoaceticum* is presented in Table IX. This compound, which probably is a Co-carboxymethylcorrinoid, was isolated in a very small amount from preparation M (Table VII) and had very high radioactivity. On photolysis the following radioactive compounds were produced: CO_2, methanol, formaldehyde, and traces of formic, acetic, succinic, and glycolic acids. The latter acids were detected among the nonvolatile products. Similar products are formed on photolysis of labeled Co-carboxymethylcobalamin. Thus Co-carboxymethylcobalamin labeled in the carboxyl group gives mostly carbon dioxide, while the methyl-labeled forms ^{14}C formaldehyde, methanol, and formic acid. Succinic and glycolic acids are formed from both types of labeled Co-carboxymethylcobalamin. That most of the ^{14}C from the natural product is obtained as carbon dioxide as is the case for carboxyl-labeled Co-carboxymethylcobalamin indicates the presence of a carboxyl group. The presence of ^{14}C in methanol and formaldehyde indicates the presence of a more reduced carbon, possibly corresponding to the methyl of Co-carboxymethylcobalamin. The low recovery of acetate from the natural product may be due to the small amount (0.02 μmole) used for the photolysis. L. Ljungdahl and E. Irion (unpublished results) have observed that the composition of the cleavage products on photolysis of Co-carboxymethylcobalamin varies with the amount and is greatly influenced by the oxygen present. When the amount of Co-carboxymethylcobalamin was varied without attempting to remove air from the solution the yield of acetate decreased with the concentration of the corrinoid. Even under strictly anaerobic conditions with 0.775 μmole of Co-carboxymethylcobalamin dissolved in 1 ml of water the yield of acetate was only 50% of theoretical. Thus the low recovery of acetate does not exclude that the natural product is a Co-carboxymethylcorrinoid. Further work is underway to attempt to definitely identify the compound.

TABLE IX: Products of Aerobic Photolysis of Co-^{14}C-Carboxymethylcobalamin and a Co-^{14}C-Corrinoid Isolated from *C. thermoaceticum*, Preparation M in Table VII.[a]

Product	Co-Carboxymethylcobalamin (cpm)		Natural product from *C. thermoaceticum* (cpm)
	Carboxyl-^{14}C	Methyl-^{14}C	
Corrinoid before photolysis	45,200	30,000	4,000
CO_2	22,050	792	2,625
CH_3OH	0	730	170
HCHO	0	10,820	535
HCOOH	0	6,360	5
CH_3COOH	2,850	3,050	3
Nonvolatile products	4,020	4,615	846

[a] The corrinoids (0.775 μmole of the synthetic Co-carboxymethylcobalamin and about 0.02 μmole of the natural product) dissolved in 1 ml of water were exposed to light from a 200-w incandescent lamp at a distance of 15 cm for 1 hr. Carrier compounds were added and were isolated for determination of ^{14}C.

Discussion

The mechanism by which *C. thermoaceticum* converts two molecules of CO_2 to acetate using the glycolytic fermentation of glucose (Wood, 1952a,b; Lentz, 1956) as the reductant and as a source of energy has remained unsolved in spite of rather intensive study. The literature dealing with several other microorganisms which are able to incorporate $^{14}CO_2$ into both carbons of acetate is reviewed by Wood and Stjernholm (1962). The failure to detect the intermediate compounds of the conversion now becomes understandable because it is most probable that the conversion occurs *via* intermediate compounds linked directly to the cobalt of the corrinoids which are enzyme bound. Therefore no intermediate compound occurs free in solution which may be detected by the usual tracer techniques such as employed by Calvin and others in the study of photosynthesis. This is in accord with the finding of Ljungdahl and Wood (1965), who showed that the synthesis of acetate from CO_2 by *C. thermoaceticum* is very rapid; after only 5 sec most of the fixed $^{14}CO_2$ occurs in acetate. These studies were not done with protection from light so the intermediate cobalt complexes would have been degraded by photolysis.

The results reported here clearly demonstrate the formation of Co-$^{14}CH_3$-Factor III_m and Co-$^{14}CH_3$-cobyric acid from $^{14}CO_2$ by *C. thermoaceticum*. It is proposed that these Co-methylcorrinoids are intermediates in the synthesis of acetate from CO_2. This proposal is based on the following facts: (1) the Co-methyl of these compounds is formed from $^{14}CO_2$, (2) the methyl groups acquire a very high specific activity, and (3) the Co-methyl groups are converted to the methyl of acetate. Poston and Kuratomi (1965) have presented a preliminary report of studies showing that extracts of *C. thermoaceticum* catalyze the formation of Co-$^{14}CH_3$-cobalamin from reduced B_{12} and $^{14}CO_2$. They also reported that three protein fractions are needed for the synthesis of acetate from Co-$^{14}CH_3$-

cobalamin. However, cobalamin derivatives are not the natural intermediates because no cobalamin could be found in *C. thermoaceticum* (Irion and Ljungdahl, 1965).

Although more evidence is needed before the exact role of the corrinoids in the synthesis can be established, the over-all outline of the pathway is beginning to emerge. It is probable that a Co-carboxymethylcorrinoid is an intermediate in the formation of acetate from a Co-methylcorrinoid, as indicated by the results presented in Table IX. Further experiments are being conducted to attempt to isolate the presumed carboxymethyl derivative in large enough quantity to permit its definite identification.

The fact that *C. thermoaceticum* contains enzymes which convert carboxymethylcobalamin to acetate as shown in Table III is further evidence of the role of a carboxymethyl derivative in this fermentation. It is of interest that only NADPH was required for the formation of acetate from Co-carboxymethylcobalamin by crude extracts but pyruvate was required for the incorporation of the Co-methyl group into acetate (Table II). It seems likely that the Co-carboxymethyl is converted to acetate by reductive cleavage whereas the conversion of the Co-methyl to acetate requires a reaction involving CO_2, adenosine triphosphate, and NADPH. The possibility also exists that CO_2 first is incorporated into the carboxyl group of pyruvate *via* an exchange reaction (Ljungdahl and Wood, 1963) and then by transcarboxylation is transferred to a Co-methyl group yielding a Co-carboxymethylcorrinoid.

Co-Methylcobalamin has been reported to participate in several reactions. Blaylock and Stadtman (1963) reported that the Co-methyl group is converted to methane by extracts of *Methanosarcina barkeri*, and later they found Co-methylcobalamin to be synthesized from reduced B_{12} and $^{14}CH_3OH$ (Blaylock and Stadtman, 1964). Methane formation from Co-methylcobalamin is also catalyzed by extracts of *Methanobacillus omelianskii* (Wolin *et al.*, 1963). Guest *et al.* (1962) as well as several other investigators have found that the methyl group of Co-methylcobalamin is incorporated into methionine. In view of the many reports regarding the participation of Co-methylcorrinoids in biochemical reactions it is somewhat surprising that Co-methylcorrinoids have not been isolated previously from a natural source. However, evidence for the presence of Co-methylcobalamin in calf liver and *Escherichia coli* has been presented by Lindstrand (1964). A search for Co-methylcorrinoids in *M. omelianskii* has so far been without success (Lezius and Barker, 1965).

It seems likely that formate may be a precursor or an intermediate of the synthesis of the Co-methyl group. Lentz and Wood (1955) found that formate is preferentially incorporated into the methyl group of acetate by *C. thermoaceticum* and Ljungdahl and Wood (1963) found that this organism contains a NADP-dependent formate dehydrogenase which catalyzes a rapid exchange between $^{14}CO_2$ and formate. Further evidence that formate is a precursor of the methyl group is presented in Table I, in which it is shown that addition of ^{12}C-formate reduces the incorporation of $^{14}CO_2$ into the methyl group of acetate by extracts of *C. thermoaceticum*. Whether formyltetrahydrofolic acid has a role in this synthesis as it does in the synthesis of the methyl group of methionine is a problem for future investigation.

It is surprising that Co-methylcobyric acid and Co-methyl-Factor III_m are both present in *C. thermoaceticum*. While both compounds are active in the synthesis of the methyl group of acetate (Table VIII), it appears that Co-methylcobyric acid is a better precursor of the acetate. It is therefore possible that this compound plays the more important role in the intact cell. Another example of the possible role of incomplete B_{12} factors in enzyme reactions was reported by Elford (1964), who found that enzyme systems from hog liver and *Escherichia coli* catalyze the transfer of the methyl group of N^5-methyltetrahydrofolic acid to Factor B and in turn to homocysteine to form methionine. The occurrence of Co-methyl-Factor III_m may result from its synthesis in the cell from Co-methylcobyric acid. This suggestion is in accord with Müller and Müller (1963), who showed that incomplete Co-alkyl factors are converted by *Propionibacterium shermanii* into complete Co-alkyl factors.

In the methionine synthesis it is generally considered that the methyl group from the B_{12} derivative is transferred to the enzyme-bound B_{12} compound and then the methyl follows the normal pathway of transmethylation. Perhaps similar reactions may occur when the methyl of Co-methylcorrinoids is converted by extracts of *C. thermoaceticum* to the methyl of acetate.

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Isolation of Factor III_m Coenzyme and Cobyric Acid Coenzyme plus Other B_{12} 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 B_{12} 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 cobyric 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 B_{12} factors. Two complete factors and several incomplete factors remain to be identified.

A total synthesis of acetate from CO_2 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 chromatographic, and spectrophotometric methods. Two of these corrinoids, Co-methylcobyric 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 CO_2 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 B_{12} factors.

Methods

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

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