

# Photolytic and Reductive Cleavage of Co-carboxymethylcobalamin\*

Lars Ljungdahl and Eckart Irion

**ABSTRACT:** The photolysis of Co- $^{14}\text{CH}_2\text{COOH}$ -cobalamin and Co- $\text{CH}_2^{14}\text{COOH}$ -cobalamin in water solutions has been studied under anaerobic and aerobic conditions. Although the same products are obtained regardless of the conditions, the presence of oxygen as well as the concentration of the corrinoid greatly influences the yield of the products. Photolysis of rather concentrated solutions (0.554 mM) of Co-carboxymethylcobalamin in the presence of air yields a large amount of one-carbon compounds: carbon dioxide from the carboxyl carbon, and formaldehyde, formic acid, and a small amount of methanol from the methyl

carbon. Acetate is also formed as well as small amounts of succinic, glycolic, glyoxylic, oxalic, and diglycolic acids. The yields of carbon dioxide and acetate during aerobic photolysis are decreased using less concentrated solutions of Co-carboxymethylcobalamin. During anaerobic photolysis the yield of acetate is increased to ca. 50% of theoretical and is the major product. The cobalt-carbon bond in Co-carboxymethylcobalamin is cleaved by treatment with sodium borohydride and acetate is formed in almost a quantitative yield. This procedure can be used for the identification of Co-carboxymethyl ligands of corrinoids.

The cobalt-carbon bond in corrinoid coenzymes and Co-alkylcorrinoids is cleaved by light. This cleavage is homolytic and free radicals are formed in the primary reactions. The final product of the corrinoid moiety is a reduced product ( $\text{B}_{12\text{r}}$ ) which is rapidly oxidized in the presence of oxygen (Hogenkamp *et al.*, 1963; Pratt, 1964). The organic radical is stabilized with the formation of several products in yields which are influenced by the amount of oxygen present. The 5'-deoxyadenosyl moiety of corrinoid coenzymes yields 8,5'-cyclic adenosine under anaerobic conditions, but adenosine-5'-aldehyde (Hogenkamp, 1964) and possibly also adenosine 5'-carboxylic acid (Johnson and Shaw, 1962) are formed in the presence of air. The ethyl group of ethylcobalamin in the presence of excess oxygen gives mainly acetaldehyde, while a mixture of paraffins and olefins is produced at low oxygen tension ( $10^{-4}$  mm) (Dolphin *et al.*, 1964). Yamada *et al.* (1965) found that the photolysis of Co- $\beta,\gamma$ -dihydroxypropylcobalamin produces glycerol under anaerobic conditions, although glyceraldehyde and glyceric acid are formed in the presence of air.

Recently, Hogenkamp (1966) quantitatively determined the products formed by photolysis from the Co-methyl group of Co-methylcobalamin. In the presence of excess oxygen, formaldehyde was the major product and its yield decreased with concentration of oxygen. Methane and ethane were formed under anaerobic condition. Methanol and formic acid were also pro-

duced in small amounts but their yields were not affected much by the oxygen concentration.

The Co-carboxymethylcorrinoids, in addition to the Co-methylcorrinoids, appear to be of biochemical importance (Ljungdahl *et al.*, 1965). Their photolysis has been investigated only qualitatively. Johnson *et al.* (1963) identified acetate as a product of photolysis of Co-carboxymethylcobalamin under anaerobic condition and did not find succinic, glycolic, or oxalic acids. The present investigation of the photolysis of Co- $^{14}\text{CH}_2\text{COOH}$ -cobalamin and Co- $\text{CH}_2^{14}\text{COOH}$ -cobalamin shows that in addition to acetate the following compounds are formed: carbon dioxide, carbon monoxide, formaldehyde, methanol, as well as formic, succinic, glycolic, glyoxylic, diglycolic, and oxalic acids.

## Materials and Methods

*Synthesis of Labeled Co-carboxymethylcobalamin.* Co-carboxymethylcobalamin labeled in the carboxyl or in the methyl carbon of the carboxymethyl moiety was synthesized according to Müller and Müller (1962).

*Photolysis of Co-carboxymethylcobalamin.* The corrinoids were dissolved in 1.4 ml of unbuffered water in  $15 \times 125$  mm test tubes. When photolysis was performed in the presence of air the water was not boiled; when done in the absence of air the water was previously boiled and the atmosphere was nitrogen. For more rigorous exclusion of oxygen the solution was frozen and the tube was evacuated and then thawed and the nitrogen was replaced five times. The nitrogen used was passed through an alkaline pyrogallol solution. The tubes were stoppered with serum rubber caps through which additions were made *via* syringe needles. The tubes were exposed to light for 1 hr. The light

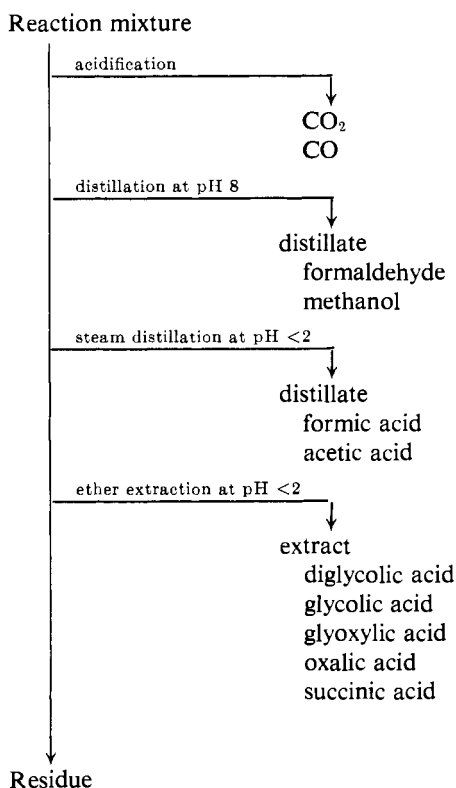
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source was a 200-w incandescent light bulb placed 15 cm from the tubes. The temperature of the tubes was maintained below 25° by immersing them from time to time into an ice bath. Before analysis of the products the tubes were stored for 15 hr in a refrigerator.

**Cleavage of Co-carboxymethylcobalamin by Sodium Borohydride.** The corrinoids were dissolved in 1.4 ml of boiled water and 40 mg of sodium borohydride was added under nitrogen. Some samples were exposed 1 hr to light as described for photolysis and then placed for 15 hr in the refrigerator. Others were placed in the refrigerator without exposure to light.

**Analysis of Products of Photolysis and Reductive Cleavage of Co-carboxymethylcobalamin.** The products of photolysis and reductive cleavage were separated as outlined in Scheme I. Before the separation, 1.5 ml of a

Scheme I: The Separation of Products Obtained by Photolysis of Co-carboxymethylcobalamin.



solution was added containing the following mmoles of compounds as carriers: sodium acetate, 0.302; sodium formate, 0.302; sodium succinate, 0.300; sodium glycolate, 0.307; sodium carbonate, 0.307; formaldehyde, 0.387; and methanol, 0.416.

The corrinoid solution at 0° was acidified with 1 ml of 3 N sulfuric acid and nitrogen gas was passed through the solution to carry the evolved CO<sub>2</sub> into a bubbler containing 2 ml of 3 N sodium hydroxide. The radioactivity of the CO<sub>2</sub> was determined after precipitation as barium carbonate. In one case the gas after passage through the sodium hydroxide solution was led into a solution of 20 mg of palladium chloride in 5 ml of 0.005

N hydrochloric acid to oxidize the carbon monoxide to CO<sub>2</sub>. This carbon dioxide was also trapped in sodium hydroxide and after addition of carrier sodium carbonate the radioactivity was determined as barium carbonate.

The corrinoid solution was next adjusted to pH 8 and diluted to 100 ml and ca. 80 ml was distilled to obtain methanol and formaldehyde. The formaldehyde was precipitated as the dimedon product by addition of 25 ml of 0.5% water solution of 5,5-dimethyl-1,3-cyclohexanedione to the distillate. The precipitate was removed by filtration and was recrystallized before part of it was oxidized to CO<sub>2</sub> for determination of <sup>14</sup>C. The filtrate containing methanol was distilled and to the distillate potassium bichromate was added to oxidize the methanol to formic acid. The latter was oxidized to CO<sub>2</sub> with mercuric chloride (Piria, 1946).

The residue of the distillation at pH 8 was acidified with sulfuric acid to pH 2 or below and formic acid and acetic acid were obtained by steam distillation. These acids were separated by chromatography on celite (Swim and Krampitz, 1954). The formic acid was oxidized to CO<sub>2</sub> with mercuric chloride and the acetate was converted to CO<sub>2</sub> by the method by Van Slyke and Folch (1940). Acetate obtained from the sodium borohydride cleavage of Co-carboxymethylcobalamin was degraded according to Phares (1951).

The acid residue from the steam distillation was extracted with ether. The ether extract was chromatographed on Celite to separate glycolic and succinic acids, which were further purified by chromatography on paper. Since these acids did not contain all the <sup>14</sup>C of the ether extract each fraction obtained in the Celite chromatogram was examined for radioactivity. Several fractions were found to contain <sup>14</sup>C including those corresponding to glyoxylic, diglycolic, and oxalic acids. These acids were added as carriers to the appropriate fractions and paper chromatography was done with a mixture (48:2) of 1-butanol (saturated with water)-formic acid and with ethyl methyl ketone-water-acetone-formic acid (40:55:2:1) as solvents. When the radioactivity cochromatographed with the carrier acid in both solvents it was assumed the radioactive material was identical with the carrier acid. The acids were oxidized to CO<sub>2</sub> which was precipitated as barium carbonate and assayed for radioactivity. The residue after the ether extraction was not further investigated, but a sample was neutralized and evaporated to dryness on a planchet for determination of <sup>14</sup>C.

**Determination of <sup>14</sup>C.** The barium carbonate on planchets was assayed for <sup>14</sup>C with a Nuclear-Chicago low-background counter. Radioactivity on paper chromatograms was detected with a Vanguard Auto-scanner 800.

## Results

**Sodium Borohydride Cleavage of Co-carboxymethylcobalamin.** Table I shows the results of the reductive cleavage of Co-carboxymethylcobalamin. The carboxymethyl group is cleaved to acetate and the yield is

TABLE I: Yield and Distribution of  $^{14}\text{C}$  in Acetate from Cleavage of Carboxymethyl- $\text{B}_{12}$  with Sodium Borohydride.<sup>a</sup>

	$^{14}\text{C}$ -Carboxyl (%)		$^{14}\text{C}$ -Methyl (%)
	Dark	Light	Dark
Total	92.8	101.8	87.1
Methyl	0.2		77.3
Carboxyl	86.7		0.1

<sup>a</sup>  $\text{Co-CH}_2^{14}\text{COOH-cobalamin}$  (0.386  $\mu\text{mole}$ , 22,600 cpm) and  $\text{Co-}^{14}\text{CH}_2\text{COOH-cobalamin}$  (0.776  $\mu\text{mole}$ , 30,000 cpm) in 1.4 ml of water, were treated with sodium borohydride (40 mg) at  $0^\circ$  under nitrogen. Carrier acetate was added and the mixture degraded to obtain the methyl and carboxyl group. Values are calculated on the basis of the specific activity of the acetate and given in per cent of  $^{14}\text{C}$  added as  $\text{Co-carboxymethylcobalamin}$ .

nearly quantitative. This cleavage is not affected by light.

*Photolysis of Co-carboxymethylcobalamin* (0.544 mM). The products formed from the carboxymethyl

moiety on photolytic cleavage of  $\text{Co-carboxymethylcobalamin}$  are listed in Table II. Several one-carbon compounds are formed showing that cleavage not only occurs between the cobalt-carbon bond but also between the methyl carbon and the carboxyl carbon. This carbon-carbon cleavage appears to be influenced by the presence of oxygen and one-carbon compounds are the major products when the photolysis occurs in air. Thus the yield of carbon dioxide is *ca.* 15% from the carboxyl group under anaerobic condition but almost 50% in the presence of air. Also some carbon monoxide is formed but the amount was not determined quantitatively. The methyl carbon of the carboxymethyl group is also converted to one-carbon compounds: formaldehyde (39% in air and 14% under  $\text{N}_2$ ), formic acid (24% in air and 8% under  $\text{N}_2$ ), and small amounts of methanol, and of  $\text{CO}_2$ .

Acetic acid is the most abundant product (39–56% yields), when the photolysis occurs under anaerobic condition, but in presence of air the yield of acetate is low. The yield of succinic acid is also increased under anaerobic conditions.

All the radioactivity in the fraction containing steam-distillable acids was accounted for as formic and acetic acids. In addition to succinic and glycolic acid, six radioactive peaks were in the eluate from the chromatography on celite of the ether extract. Three of these were identified as glyoxylic, diglycolic, and oxalic acid,

TABLE II: Yield of Products from the Carboxymethyl Group on Photolysis of Carboxymethylcobalamin in Air and in Nitrogen.<sup>a</sup>

	$\text{Co-CH}_2^{14}\text{COOH-B}_{12}$ (%)			$\text{Co-}^{14}\text{CH}_2\text{COOH-B}_{12}$ (%)		
	Expt					
	1	2	3	4	5	6
	Atm					
	$\text{N}_2^b$	$\text{N}_2^b$	Air	$\text{N}_2^b$	$\text{N}_2^b$	Air
Volatile products						
$\text{CO}_2$	13.8	14.9	49.0	0.3	0.3	2.7
CO	...	...	1.9	...	...	...
HCHO	0	0	0	12.5	14.1	39.0
$\text{CH}_3\text{OH}$	0.02	0.02	0	0.5	0.6	2.4
Acids (distilled with steam)	46.4	49.7	6.7	58.5	45.6	33.3
HCOOH	0.1	0.1	0.1	8.2	7.6	23.6
$\text{CH}_3\text{COOH}$	48.3	49.7	6.3	56.0	39.0	9.7
Acids (extracted with ether)	18.4	18.3	7.2	15.3	24.8	14.1
succinic acid	5.2	6.5	1.4	3.9	3.9	2.7
glycolic acid	2.0	2.1	3.4	3.9	2.0	3.9
Residue	6.8	4.6	2.1	4.6	3.9	1.9
Recovery	87.4	87.6	66.9	97.4	90.3	93.4

<sup>a</sup> The amount of  $\text{Co-CH}_2^{14}\text{COOH-B}_{12}$  was 0.775  $\mu\text{mole}$  with 45,200 cpm and of  $\text{Co-}^{14}\text{CH}_2\text{COOH-B}_{12}$  0.776  $\mu\text{mole}$  with 30,000 cpm. Total volume was 1.4 ml. See the text for details regarding experimental procedures. Values are calculated on the basis of the specific activities after addition of carrier and are in per cent of the total  $^{14}\text{C}$  added as  $\text{Co-carboxymethylcobalamin}$ . <sup>b</sup> Tubes evacuated and filled with nitrogen five times. In addition in expt 1 and 4 the solutions were frozen and then thawed while evacuated.

Quantitative determination of the radioactivity of these acids was not done but their total  $^{14}\text{C}$  content did not exceed 2%. The other three radioactive peaks also contained little radioactivity and have not been identified. Fumaric acid was not detected.

*Effect of the Concentration of Co-carboxymethylcobalamin on the Formation of Acetate and  $\text{CO}_2$  under Aerobic Photolysis.* The yield of both acetic acid and carbon dioxide formed from the carboxymethyl group decreased with decreasing concentration of the corrinoid (Table III). Almost no acetate was formed at the

TABLE III: The Effect of Concentration of Carboxymethyl- $\text{B}_{12}$  on the Yields of Acetate and Carbon Dioxide during Aerobic Photolysis.<sup>a</sup>

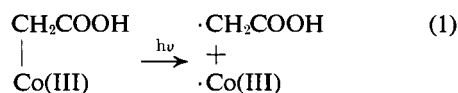
No.	Carboxymethyl- $\text{B}_{12}$ (mM)	Yield (%)	
		$\text{CO}_2$	Acetate
7	0.077	22	0.1
8	0.311	30	3.8
9	0.554	49	6.3

<sup>a</sup> Experiment 7 was with 0.192  $\mu\text{mole}$  (11,200 cpm)  $\text{Co-CH}_2^{14}\text{COOH-cobalamin}$  in 2.5 ml, expt 8 with 0.386  $\mu\text{mole}$  (22,600 cpm) in 1.2 ml, and expt 9 with 0.775  $\mu\text{mole}$  (45,200 cpm) in 1.4 ml of water. Yields are calculated on the basis of the total  $^{14}\text{C}$  added as Co-carboxymethylcobalamin. Only  $\text{CO}_2$  and acetate were determined.

lowest concentration of the corrinoid (0.077  $\mu\text{mole/ml}$ ).

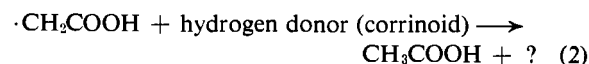
## Discussion

In analogy with other corrinoids containing cobalt-carbon bonds the initial reaction for the photolytic cleavage of Co-carboxymethylcobalamin is as follows:



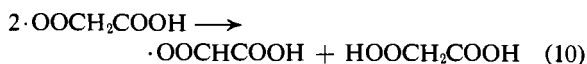
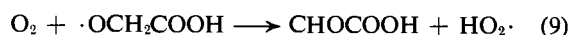
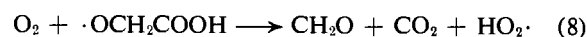
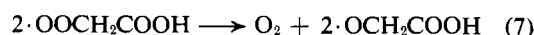
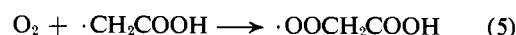
The corrinoid radical forms  $\text{B}_{12\cdot}$  [ $\text{Co(II)}$ ] or in the presence of oxygen is oxidized to hydroxy- (aquo-) cobalamin (Hogenkamp, 1964; Dolphin *et al.*, 1964; Pratt, 1964). The carboxymethyl radical undergoes several reactions and many products are obtained as is shown in Table II. The yield of these products are very dependent not only on the concentration of oxygen (Table II), but also in the presence of oxygen on the concentration of the corrinoid (Table III). Hogenkamp (1966) also found oxygen to influence the yield of products formed from the methyl radical obtained in the initial reaction of photolysis of Co-methylcobalamin.

During anaerobic conditions the main product is acetic acid, but also succinic acid, carbon dioxide, formaldehyde, and formic acid are formed in quite large yields. Acetic and succinic acids, and possibly also  $\text{CO}_2$  are obtained in the following reactions:

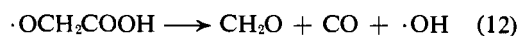


However, it is difficult to explain the formation of formaldehyde and formic acid during anaerobic photolysis. Hogenkamp (1966) also noticed the formation of oxidized products from photolysis of Co-methylcobalamin during anaerobic conditions. He suggested that oxygen remaining bound to the corrinoid in spite of repeated evacuations could account for this result. The yields of carbon dioxide, formaldehyde, and formic acid are much increased during aerobic photolysis of Co-carboxymethylcobalamin, indicating that oxygen stimulates the formation of one-carbon compounds from the carboxymethyl radical. This supports the suggestion by Hogenkamp.

Garrison *et al.* (1959) found that acetic acid in water solution saturated with oxygen when radiated was converted to glyoxylic, glycolic, and oxalic acids as well as carbon dioxide and formaldehyde. They suggested the following reactions for the formation of these compounds:



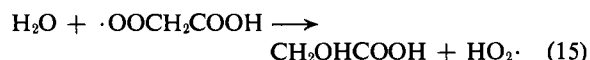
These reactions explain the formation of all products formed during aerobic photolysis of the Co-carboxymethyl group except carbon monoxide, formic, and diglycolic acids which probably can be formed as follows:





Reaction 14 has been observed in photochemical reactions in the presence of oxygen (Dever and Calvert, 1962).

The yields of carbon dioxide and acetic acid decrease during aerobic photolysis with the concentration of Co-carboxymethylcobalamin (Table III). The lower yield of carbon dioxide at lower corrinoid concentration may possibly occur because the formation of carbon dioxide is dependent on the radical concentration. When the corrinoid concentration is low the radical concentration also is low and thus the possibility for two radicals to react according to reaction 7 is decreased and subsequently the formation of  $\text{CO}_2$  in reaction 8. When the concentration of  $\cdot\text{OOCH}_2\text{COOH}$  radical is low it may be stabilized by other competing processes, for instance by the formation of organic hydroxypoxides. The following reaction suggested by Garrison *et al.* (1959) may also occur:



The higher yield of acetate obtained with increasing concentration of Co-carboxymethylcobalamin during photolysis is in accord with the following assumption. The oxygen concentration in water in equilibrium with air is *ca.* 0.25  $\mu\text{mole/ml}$  at room temperature. It can be assumed from data presented by Hogenkamp (1966) that 2 mole of oxygen reacts with the radicals formed from the photolysis of 1 mole of Co-carboxymethylcobalamin. When the concentration of Co-carboxymethylcobalamin is  $>0.25 \mu\text{mole/ml}$  there will be an excess of radicals. This in effect makes the condition for the photolysis anaerobic leading to the higher yield of acetate (reaction 2).

Co-carboxymethylcobalamin when treated with sodium borohydride is split reductively with formation of acetate and most likely  $\text{B}_{12a}$ . This cleavage is not effected by light. This is in agreement with Dolphin *et al.* (1964), who found when Co-methylcobalamin is hydrogenated in the presence of platinum that the methyl group is removed as methane. But these authors could not demonstrate a reductive cleavage of the  $\text{B}_{12}$ -coenzyme itself, and they concluded that the nucleoside substituent was too bulky to render the cobalt atom

accessible to the reducing agent. However, Yamada *et al.* (1965) performed photolysis of Co- $\beta,\gamma$ -dihydroxypropylcobalamin in the presence of sodium borohydride and found the product to be glycerol rather than propanediol. This product also was obtained during photolysis in the absence of sodium borohydride. It therefore is possible that the dihydroxypropyl group, like the 5'-deoxyadenosine moiety of coenzymes, hinders the reductive cleavage and, therefore, one obtains glycerol rather than the dihydroxy compound.

#### Acknowledgment

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