SYNTHESIS OF Co-METHYL COBALAMIN BY CELL-FREE EXTRACTS OF CLOSTRIDIUM thermoaceticum

Kazuoki Kuratomi, J. Michael Poston, and Earl R. Stadtman

Laboratory of Biochemistry, Section on Enzymes, National Heart Institute, National Institutes of Health, Bethesda, Maryland, and the Department of Microbiology, University of Maryland, College Park, Maryland.

Received April 26, 1966

Clostridium thermoaceticum catalyzes the net synthesis of acetate from CO_2 according to the following overall reaction

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

(Barker and Kamen, 1945; Wood, 1952). Our earlier observation that intrinsic factor partially inhibits the incorporation of $^{14}CO_2$ into acetate by cell-free extracts of C. thermoaceticum (Poston, et al., 1964) suggested that a vitamin B_{12} derivative might be involved (Ellenbogen, 1960). This suggestion was supported by the discovery that cell-free extracts convert the methyl moiety of Co-$^{14}CH_3$-cobalamin into the methyl group of acetate (Poston, et al., 1964) and by the recent demonstration of Ljungdahl, Irion, and Wood (1965) that Co-^{14}-CH_3-cobyric acid and Co-$^{14}CH_3$-5-methoxy-benzimidazoyl cobamide accumulate in cells of C. thermoaceticum following their exposure to $^{14}CO_2$.

The present study shows that extracts of C. thermoaceticum catalyze the de novo synthesis of Co-methyl cobalamin in the presence of pyruvate, coenzyme A, CO_2, and reduced cobalamin.

Materials and Methods---C. thermoaceticum cells were grown and harvested and extracts were prepared as described previously (Poston, et al., 1966). Co-methyl cobalamin was prepared by the method of Smith, et al. (1962). All reactions and isolations were performed in dim light and all enzymatic reactions were carried out under an atmosphere of helium.

Experimental and Results--- The system required for the fixation of $^{14}CO_2$ into acetate (Poston, et al., 1964) was supplemented with a DPNH generating system (horse liver alcohol dehydrogenase and isopropanol) and with unlabelled Co-methyl cobalamin as described in Fig. 1. After 3.5

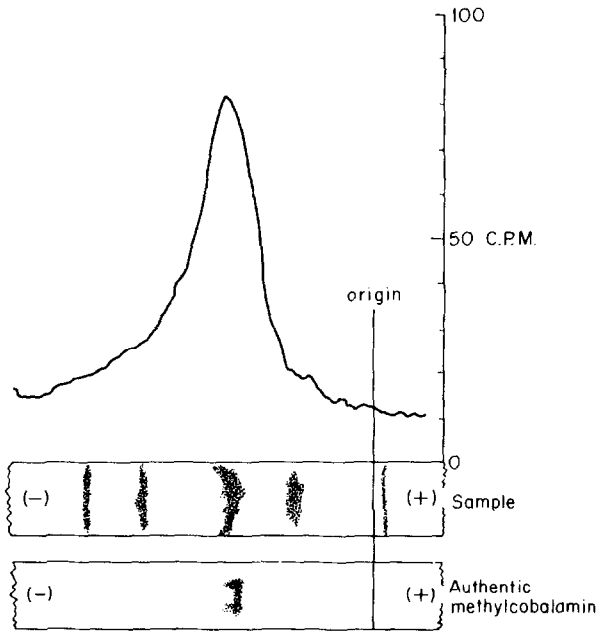

Fig. 1. The radioscan of an electrophoretic separation of cobalamin derivatives. The derivatives were isolated as described in the text from a reaction mixture containing K phosphate buffer, pH 7.4, 200 μmoles; 2-mercaptoethanol, 20 μmoles; CoASH, 2 μmoles; $MgCl_2$, 20 μmoles; ATP, 20 μmoles; Na pyruvate, 20 μmoles; cell-free French-press extract of C. thermoaceticum, 21 mg protein; Co-$^{12}CH_3$-cobalamin, 2 μmoles; $KH^{14}CO_3$, 40 μmoles, 20 μc; sodium formate, 30 μmoles, 20.4×10^5 cpm; total volume, 2.50 ml; gas phase, helium; incubation at 40° for 3.5 hours. Paper electrophoresis was carried out on Sepraphore III at 10 volts per cm in 0.5 N acetic acid.

hours, the reaction was stopped by the addition of ethanol. The B_{12} compounds were extracted by the method of Barker, et al. (1960).

In order to free the cobalamin derivatives from compounds formed from $^{14}CO_2$, the B_{12}-containing solution was then passed over a column containing 2.0 g Celite 535 mixed with 0.9 ml 0.2 N H_2SO_4 (Swim and Krampitz, 1954). Organic acids were eluted from the column with 70 ml of 20 % (v/v) n-butanol in $CHCl_3$ (saturated with 0.2 N H_2SO_4) followed by 20 ml of buffered n-butanol (50 ml n-butanol plus 1 ml 1 M potassium phosphate buffer, pH 7.4). The B_{12} compounds were eluted in a sharp band with buffered methanol (135 ml methanol plus 15 ml 0.1 M potassium phosphate buffer, pH 7.4). The pink solution was concentrated to 1.5 ml and desalted by phenol extraction as before. Finally, the B_{12} compounds were extracted into 0.5 ml water.

The ultraviolet absorption spectrum of the isolated product was essentially identical with that of Co-alkyl cobalamin (Müller and Muller, 1962; Bonnett, 1963). After exposure to light, a spectrum characteristic of hydroxycobalamin was obtained as would be expected upon photolysis of Co-alkyl cobalamin.

Electrophoresis of the B_{12}-containing mixture on Sepraphore III (Gelman Instrument Co.) in 0.5 N acetic acid separated the B_{12} compounds into several bands. Only the major band, corresponding to Co-methyl cobalamin, was radioactive, Fig. 1. Paper chromatography in two systems revealed that only the spot corresponding to authentic Co-CH_3-cobalamin was radioactive---sec-butanol: water: acetic acid, 100:50:3, R_f 0.46; water-saturated sec-butanol, R_f 0.37. Fig. 2 shows the radioscan of one such chromatogram. It is clear that exposure to light caused a loss of radioactivity. Authentic Co-$^{14}CH_3$-cobalamin also behaves in such a fashion.

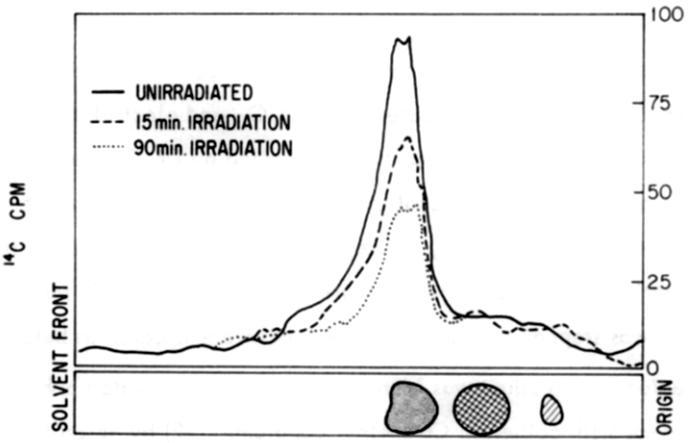

Fig. 2. The radioscan of an ascending paper chromatogram of cobalamin derivatives and the effect of irradiation on the radioactivity. The derivatives were isolated as described in the text from the reaction mixture described in Fig. 1. The chromatogram was developed in water-saturated sec-butanol. The three spots were visible because of the reddish or pink color. The paper was irradiated with a 100 wat incandescent lamp at 10 cm for the periods indicated. R_f of the radioactive Co-methyl cobalamin = 0.37.

Since formaldehyde is a major aerobic photolytic product of Co-methyl cobalamin (Dolphin, et al., 1964), carrier formaldehyde was added to the B_{12}-containing solution and the mixture was gently aerated during a 2.5-minute irradiation with a 100 watt incandescent lamp at a distance of 10 cm. The formaldehyde was isolated as the dimedon derivative and was found to be radioactive (Table I).

Table I

Degradation of the isolated radioactive cobalamin derivative by aerobic photolysis

Experiment	Before aerobic photolysis (Co-$^{14}CH_3$-cobalamin)		After aerobic photolysis ($H^{14}CHO$-dimedon)	
	cpm	mg	cpm	% yield
1	2440	177	845	34.0
2	1220	177	304	25.0

While those data present proof that a pool of extractable Co-methyl cobalamin is able to trap an isotopically labelled intermediate in the reduction of CO_2, it is possible that this does not represent de novo synthesis but, rather, an exchange of labelled methyl for unlabelled methyl groups in the Co-methyl cobalamin pool.

In order to demonstrate the de novo synthesis of Co-$^{14}CH_3$-cobalamin, extracts of C. thermoaceticum were incubated with $^{14}CO_2$ and electrolytically reduced Vitamin B_{12}. The reduced B_{12} was kindly supplied by Dr. Barbara Blaylock and consisted of an approximately equal mixture of B_{12r} and B_{12s}. The reaction mixture also contained pyruvate, coenzyme A, $MgCl_2$, $FeSO_4$, and glutathione. After incubation at the growth temperature of the organism, 57°, for 30 minutes, the reaction was stopped and the B_{12} compounds were isolated as before. The isolated radioactive product was identified (1) by co-chromatography in several solvent systems with authentic Co-methyl cobalamin, (2) by absorption spectra and (3) by its behavior upon photolysis, and (4) by its electrophoretic mobility. The formaldehyde released upon photolysis was isolated and identified as the dimedon derivative and was shown to be radioactive. By all these criteria the isolated product was indistinguishable from authentic Co-methyl cobalamin.

These data show that cell-free extracts of C. thermoaceticum catalyze the de novo synthesis of Co-methyl cobalamin from CO_2 and reduced cobalamin. These findings and the recent reports by Ljungdahl, Irion, and Wood (1965) of the existence of Co-CH_3-cobyric acid and Co-CH_3-5-methyoxy-benzimidazolyl cobamide in C. thermoaceticum point to the probable involvement of a Co-methyl corrinoid in the fixation of CO_2 into acetate by this organism.

References

Barker, H.A., and Kamen, M.D., Proc. Natl. Acad. Sci. U.S. 31, 219 (1945).
Barker, H.A., Smyth, R.D., Weissbach, H., Munch-Petersen, A., Toohey, J.I., Ladd, J.N., Volcani, B.E., Wilson, R.M., J. Biol. Chem. 235, 181 (1960).
Bonnett, R., Chem. Reviews 63, 573 (1963).
Dolphin, D., Johnson, A. W., and Rodrigo, R., Ann. N.Y. Acad. Sci., 112, 590 (1964).
Ellenbogen, J., Highley, D.R., Barker, H.A., and Smyth, R.D., Biochem. Biophys. Research Communs., 3, 178 (1960).
Ljungdahl, L., Irion, E., and Wood, H.G., Biochemistry, 4, 2771 (1965).
Muller, O., and Muller, G., Biochemische Zeit. 336, 299 (1962).
Poston, J.M., Kuratomi, K., and Stadtman, E.R., Ann. N.Y. Acad. Sci. 112, 804 (1964).
Poston, J.M., Kuratomi, K., and Stadtman, E.R., J. Biol. Chem., (1966), in press.
Smith, E.L., Mervyn, L., Johnson, A.W., and Shaw, N., Nature, 194, 1175 (1962).
Swim, H.E., and Krampitz, L.O., J. Bacteriol., 67, 419 (1954).
Wood, H.G., J. Biol. Chem., 194, 905 (1952).