COBAMIDE-DEPENDENT SYNTHESIS OF METHIONINE: LIGHT REACTIVATION OF AN INHIBITED ENZYME

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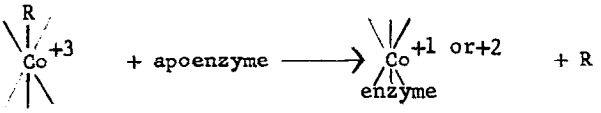
It has been shown previously that ethyl-B_{12}[1] and β-propionate-B_{12} can partially replace methyl-B_{12} in the transfer of the methyl group from methyl-folate-H_4 to homocysteine to form methionine. Other alkyl cobamides tested (propyl-B_{12}, etc.) were not only inactive in the system, but also inhibitory (Weissbach, et al., 1964). Since a reduced cobamide has been postulated as the active cobamide (Weissbach, et al., 1963; Guest, et al., 1964; Kerwar, et al., 1964) in this reaction, it appeared that those alkyl-B_{12} compounds active in the reaction combine with the apoenzyme, with a subsequent release of the alkyl moiety, leaving the active reduced cobamide species ($Co^{+1 \text{ or } +2}$) on the enzyme (Weissbach, et al., 1964).



(holoenzyme)

The present communication examines in more detail the reaction of apoenzyme with alkyl-cobamide compounds.

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[1]The following abbreviations will be used: methyl-B_{12}, 5,6-dimethylbenzimidazolylcobamide-methyl; ethyl-B_{12}, 5,6-dimethylbenzimidazolylcobamide-ethyl, etc.

Wild type Escherichia coli K_{12}, obtained from Dr. A. L. Taylor, was employed in the present studies. The organism was grown on a glucose-minimal media in the absence of any cobamide. The enzyme preparation used was a dialyzed ammonium sulfate fraction, (22 mg protein per ml) capable of forming 13 mμmoles methionine per 30 min. per mg protein from methyl-folate-H_4 using previously reported conditions (Weissbach, et al., 1963; 1964). Since the organism was grown in the absence of any cobamide the enzyme present was in the form of apoenzyme.

Preincubations were performed as follows: 0.15 - 0.25 ml of enzyme, 20 μmoles KPO_4 buffer pH 7.4, and 1 mμmole of cobamide were incubated for 5 min. at 37° in a total volume of 0.3 ml. Three tenths ml of a charcoal suspension (5 mg) was added to stop the reaction and remove the excess cobamide. After centrifugation an aliquot of the supernatant fraction (0.04 - 0.08 ml) was assayed for both activity (Weissbach, et al., 1963) and percent holoenzyme present (see below). Activity is expressed as mμmoles of methionine formed per 30 min. In some experiments (Table 3) an aliquot of the charcoal supernatant fraction was exposed (in ice) to a 200-watt bulb, at a distance of 15 cm for 5 min. before activity and percent holoenzyme were determined.

A convenient assay for holoenzyme formation was based on the cobamide requirements for the reaction with either apoenzyme or holoenzyme when the incubation was performed in air. With apoenzyme the cobamide serves two functions, to form holoenzyme as well as to lower the oxygen tension of the incubation by a nonspecific action, similar to that reported by Peel (1962) on the CO_2-pyruvate exchange reaction. Previous studies have shown that, under the conditions employed, methyl-B_{12} (and to a lesser degree ethyl-B_{12} and β-propionate-B_{12}) satisfied both cobamide requirements using apoenzyme, while other alkyl-B_{12} compounds such as propyl-B_{12}, butyl-B_{12} and deoxyadenosyl-B_{12} did not (Weissbach, et al., 1964). In fact the latter derivatives have been shown to inhibit the enzymatic synthesis of methionine from N^5-methyl-folate-H_4 in the presence of methyl-B_{12} and apoenzyme. With

holoenzyme, which contains the active cobamide on the enzyme, a cobamide is also required *in vitro* for maximum activity under the aerobic conditions used; but only to satisfy the need for anaerobiosis. Thus, all the alkyl cobamide compounds tested fulfilled this function. By comparison of activity in the presence of methyl-B_{12} and propyl-B_{12} it was possible to assay the percentage of apo- or holoenzyme present. This is shown in Table 1. With apoenzyme no

TABLE 1

ASSAY FOR HOLOENZYME FORMATION

Cobamide added to reaction mixture[1]	Apoenzyme[2] Activity	$\frac{\text{Propyl-}B_{12} \times 100}{\text{Methyl-}B_{12}}$	Holoenzyme[2] Activity	$\frac{\text{Propyl-}B_{12} \times 100}{\text{Methyl-}B_{12}}$
Methyl-B_{12}	1.9	<5	2.2	>90
Propyl-B_{12}	<0.1		2.0	
None	<0.1		0.2[3]	

[1]The reaction mixture contained in a total volume of 0.2 ml: homocysteine, 50 mμmoles; S-adenosylmethionine, 10 mμmoles; ATP, 100 mμmoles; TPNH 100 mμmoles, 2-mercaptoethanol, 40 μmoles, KPO_4 buffer, pH 7.4 10 μmoles; cobamide, 10 mμmoles; DL-^{14}C-methyl-folate-H_4, 30 mμmoles and enzyme. The methionine formed was assayed as described previously (Weissbach, *et al.*, 1963). Activity is expressed in terms of mμmoles methionine formed per 30 minutes.

[2]Apoenzyme was prepared from cells grown without vitamin B_{12}, while holoenzyme was prepared from cells grown with the vitamin (0.01 μmoles/ml) present in the medium.

[3]Under the conditions used holoenzyme did not show an absolute cobamide requirement. Generally 10-30% of maximum activity observed in the presence of a cobamide was seen if no cobamide was present. This is very likely due to some degree of anaerobiosis in the incubations, since high levels of mercaptoethanol are employed. Under strictly anaerobic conditions no cobamide is needed with holoenzyme.

significant activity (<5%) is seen if propyl-B_{12} is used in place of methyl-B_{12} in the assay; while with holoenzyme propyl-B_{12} can completely replace methyl-B_{12}. In the present study the results are expressed in percent holoenzyme, i.e., the percent of enzyme protein present as holoenzyme, as determined by the $\frac{\text{propyl-}B_{12}}{\text{methyl-}B_{12}}$ activity ratio.

The short-chain alkyl-B_{12} compounds were synthesized by the method of Smith, *et al* (1962). Deoxyadenosyl-B_{12} was a gift of Dr. D. Perlman, Squibb Institute. Large amounts of *E. coli* K_{12} cells were kindly supplied by

Mr. D. Rogerson.

Preincubation of the enzyme with methyl-B_{12} yielded excellent formation of holoenzyme. Ethyl-B_{12} and β-propionate-B_{12} were as active as methyl-B_{12} in forming holoenzyme, agreeing with earlier results (Weissbach, et al., 1964) with these compounds. Other alkyl cobamides tested such as propyl-B_{12}, butyl-B_{12} and deoxyadenosyl-B_{12} did not yield significant formation of holoenzyme. It was also observed that preincubation with propyl-B_{12}, but not deoxyadenosyl-B_{12}, resulted in a marked inhibition (60-80%) of the enzyme. A typical experiment is shown in Table 2. No holoenzyme was formed with methyl-B_{12} (Experiment 4, Table 2) when the preincubation was carried out in ice.

TABLE 2

ACTIVITY AND HOLOENZYME FORMATION AFTER PREINCUBATIONS OF APOENZYME WITH ALKYL-COBAMIDE COMPOUNDS

	Preincubation	Activity	% Holoenzyme
1.	Enzyme + methyl-B_{12}	4.0	53
2.	Enzyme + propyl-B_{12}	1.4	<3
3.	Enzyme + deoxyadenosyl-B_{12}	4.6	<3
4.	Enzyme + methyl-B_{12} (0°)	3.8	<3

Preincubations were done for 5 min. at 37° as described in text except for #4. Activity is expressed as mμmoles methionine formed per 30 min. using equal aliquots from the preincubated tubes.

Enzyme, inactivated by preincubation with propyl-B_{12}, could be readily activated by exposing it to light. This treatment not only completely restored the enzymatic activity, but yielded holoenzyme, as shown in Table 3. No significant effect of light was observed on enzyme extracts preincubated with either methyl-B_{12} or deoxyadenosyl-B_{12}. The latter alkyl derivative therefore neither forms holoenzyme nor an inhibited enzyme.

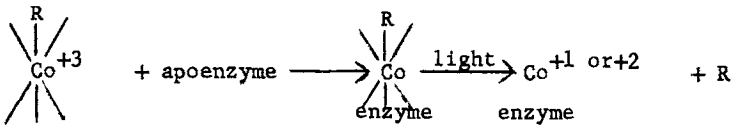
TABLE 3

EFFECT OF LIGHT ON ENZYME AFTER PREINCUBATION WITH ALKYL-COBAMIDE DERIVATIVES

Preincubation	Treatment	Activity	% Holoenzyme
Enzyme + methyl-B_{12}	dark	5.3	62
	light	5.5	57
Enzyme + propyl-B_{12}	dark	2.1	<3
	light	5.2	60
Enzyme + deoxyadenosyl-B_{12}	dark	4.5	<3
	light	4.5	<3

Preincubation conditions and light treatment are described in the text. Activity is expressed as mμmoles methionine formed per 30'.

The above experiments support the view that cleavage of the carbon-cobalt bond is an essential step in the biological activity of alkyl cobamides in this reaction. The data suggest that the enzyme can break the carbon-cobalt bond in methyl-B_{12}, but cannot cleave the carbon-cobalt bond in propyl-B_{12}. Thus, reaction of apoenzyme with propyl-B_{12} yields an inactive enzyme.



(inhibited enzyme) (holoenzyme)

R = propyl

In this case, light accomplishes what the enzyme is unable to do, i.e., cleave the carbon-cobalt bond which is known to be light sensitive (Weissbach, et al., 1960; Dolphin, et al., 1962). The light cleavage of the carbon-cobalt bond has been shown to proceed through B_{12r} (Brady and Barker, 1961) which is further support for a role of a reduced cobamide species in this reaction.

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