*MAMMALIAN METHYLMALONYL ISOMERASE AND VITAMIN B_{12} COENZYMES**

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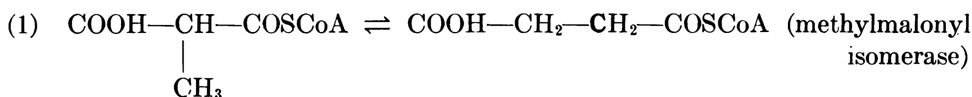
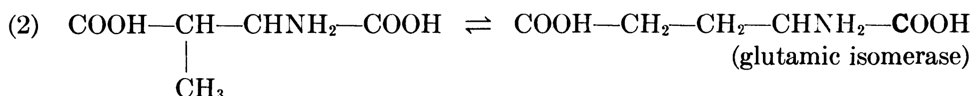
In 1955 Flavin *et al.*[1-4] found that the main pathway of propionic acid oxidation in animal tissues proceeds via the reaction sequence propionate $\xrightarrow{ATP,CoA}$ propionyl CoA[5] $\xrightarrow{ATP,CO_2}$ methylmalonyl CoA → succinyl CoA → succinate. Isomerization of methylmalonyl CoA to succinyl CoA (reaction 1) is also a key step in propionate formation by propionic acid bacteria.[6] In analogy to Lynen's β-methylcrotonyl carboxylase,[7] propionyl carboxylase (the enzyme catalyzing the carboxylation of propionyl CoA to methylmalonyl CoA) has been found to be a biotin enzyme.[8] Although reaction 1 resembled Barker's glutamate-β-methylaspartate isomerization[9] (reaction 2),[10] which requires cobamide coenzymes (vitamin B_{12} or pseudovitamin



(1) COOH—CH(CH_3)—COSCoA ⇌ COOH—CH_2—CH_2—COSCoA (methylmalonyl isomerase)



(2) COOH—CH(CH_3)—$CHNH_2$—COOH ⇌ COOH—CH_2—CH_2—$CHNH_2$—COOH (glutamic isomerase)

B_{12} derivatives),[11] it was not until recently realized that the Barker coenzymes are also required by methylmalonyl isomerase. This we owe to the work of Smith and Monty,[12] Gurnani *et al.*,[13] and Stadtman *et al.*[14] The first two groups of investigators used liver preparations of vitamin B_{12}-deficient rats; the latter prepared the apoenzyme from enzyme fractions of propionic acid bacteria by treatment with charcoal. Inactivation of glutamic isomerase by treatment of *Clostridium tetanomorphum* extracts with charcoal had in fact led Barker and collaborators to the discovery and isolation of the cobamide coenzymes.

In our experience, charcoal treatment of methylmalonyl isomerase preparations from sheep kidney did not affect their activity. The same was true of exposure to light or cyanide which, as shown by Barker *et al.*,[11] leads to rapid inactivation of the cobamide coenzymes. Incubation with intrinsic factor, which binds vitamin B_{12} was also ineffective. However, precipitation of the enzyme with acid from ammonium sulfate solutions, a device first used by Warburg and Christian[15] for the resolution of kidney D-amino acid oxidase into apoenzyme and its coenzyme (flavin adenine dinucleotide), led to almost complete resolution. The resolved enzyme was reactivated by dimethylbenzimidazolyl- or benzimidazolylcobamide coenzyme but adenylcobamide coenzyme, the pseudo vitamin B_{12} derivative, had little or no effect. This paper deals with these and other experiments bearing on the

strength of the apoenzyme-coenzyme binding in the native and reconstituted holoenzyme.

Materials and Methods.—The methylmalonyl isomerase preparation used was the dialyzed ammonium sulfate fraction obtained from sheep kidney cortex as described by Beck *et al.*[3] Its specific activity (0.8–1.0 μmole/hr/mg protein) was a little higher than previously reported. It will be referred to as the enzyme. The apoenzyme was prepared by a modification of the method of Warburg and Christian[15] described by Cori and Illingworth[16] for the removal of bound pyridoxal phosphate from crystalline muscle phosphorylase. In a typical resolution, carried out at 0°, 1.2 ml of enzyme (31.5 mg protein/ml) was diluted to 6.3 mg protein/ml with 1.2 ml of 0.02 *M* Tris buffer, pH 7.4, and 3.6 ml of distilled water. 6.6 ml of saturated (at 0°) ammonium sulfate were then added followed by 1.7 ml of 0.004 *N* HCl. Both additions were made dropwise with vigorous stirring. The resulting pH was 3.45–3.6 (glass electrode). The turbid solution was centrifuged for 7 min at 12,000 rpm in the high speed head of the International refrigerated centrifuge. The precipitate was suspended in 8 ml of 75 per cent saturated ammonium sulfate, previously adjusted to pH 8.7 with NN_4OH, and recentrifuged. This operation was repeated once and the precipitate was then taken up in 1.2 ml of 0.02 *M* Tris buffer, pH 7.4; any insoluble material was removed by centrifugation. This resolution procedure was successfully repeated many times. While the degree of resolution increased with increasing acidity, the yield of undenatured apoenzyme decreased; in our experiments it varied between 35 and 50 per cent.

A purified preparation of methylmalonyl isomerase from *Propionibacterium shermanii* was kindly provided by Dr. H. G. Wood, Western Reserve University School of Medicine. This enzyme was extensively resolved and had but little activity in the absence of added cobamide coenzymes. With saturating concentrations of DBC coenzyme and under our assay conditions,[3] its specific activity was 0.5 μmoles/min/mg protein, 30 to 40 times higher than that of the kidney preparation.

Methylmalonyl isomerase activity was measured in two ways. In most cases (direct assay) the succinyl CoA formed from methylmalonyl CoA as substrate was determined enzymatically as succinate, following alkaline hydrolysis of the thiolester, through reduction of cytochrome *c* in the presence of succinoxidase.[3] In some experiments ($C^{14}O_2$ fixation assay) methylmalonyl isomerase was incubated with propionyl CoA and $C^{14}O_2$, in the presence of an excess of propionyl carboxylase, and the radioactivity incorporated into dicarboxylic acids determined before and after oxidation with $KMnO_4$.[2] The former value gives the amount of methylmalonate plus succinate formed whereas the latter gives that of succinate only as succinate is resistant to permanganate treatment while methylmalonate is destroyed by oxidation to volatile products. Propionyl carboxylase from pig heart[4] (specific activity about 12, free of methylmalonyl isomerase) was provided by Dr. Y. Kaziro of this department. Synthetic propionyl CoA[2] and methylmalonyl CoA[3] were prepared as previously described. Since synthetic methylmalonyl CoA is a racemic mixture of two stereoisomers, only one of which is attacked by the isomerase,[17] the amounts given in the tables are one-half of the total thiolester present. Cytochrome *c*, type III, from horse heart was obtained from the Sigma Chemical Company, St. Louis, Missouri. We are greatly indebted to Dr. Karl Folkers,

Merck Institute for Therapeutic Research, Rahway, New Jersey, for generous supplies of the DBC coenzyme, Dr. H. A. Barker, University of California, Berkeley, California, for gifts of BC and AC coenzymes, and Dr. Leon Ellenbogen, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for gifts of intrinsic factor. The vitamin B_{12}-binding capacity of the highly purified intrinsic factor preparation was 0.5 μg per mg.[18]

Protein was determined spectrophotometrically.[19] Experiments involving the use of cobamide coenzymes were performed in the dark room with a dim red light.

Results.—Effect of DBC coenzyme on methylmalonyl isomerase activity: As shown in Table 1 (experiments 1*a* and 1*b*), DBC coenzyme produced a slight stimulation

TABLE 1

EFFECT OF DIMETHYLBENZIMIDAZOLYLCOBAMIDE COENZYME (DBC) ON ACTIVITY OF SHEEP KIDNEY METHYLMALONYL ISOMERASE (DIRECT ASSAY)

Experiment No.	Enzyme	Additions	Succinate formed (μmoles)
1a	Unresolved	None	0.132
1b	"	DBC (1.5×10^{-5} *M*)	0.214
2a	Resolved	None	0.015
2b	"	DBC (1.6×10^{-6} *M*)	0.127
2c	"	Vitamin B_{12} (1.2×10^{-4} *M*)	0.018
2d	"	DBC + vitamin B_{12}	0.123
2e	Boiled	None	0.007
2f	None	None	0.006
3a	Resolved	None	0.005
3b	"	DBC (4.2×10^{-7} *M*)	0.138
4a	"	None	0.013
4b	"	DBC (10^{-7} *M*)	0.108
4c	"	DBC (10^{-6} *M*) previously illuminated*	0.013
4d	"	DBC (10^{-7} *M*) pretreated with KCN†	0.016
4e	"	DBC (10^{-7} *M*) preincubated with intrinsic factor (2 mg)‡	0.016

Samples contained potassium phosphate buffer, pH 7.4, 50 μmoles; methylmalonyl CoA, 0.35 μmoles and, when present, enzyme with 0.32 to 0.38 mg of protein (except in experiment 3, 0.55 mg). Other additions as indicated. Final volume made up with water to 0.9 ml. Incubation 20 min at 30°. Coenzyme (or vitamin B_{12}) preincubated with enzyme for 12–13 min prior to the addition of methylmalonyl CoA.
* DBC coenzyme (1.8×10^{-5}*M*) illuminated before use in a Beckman cell (d = 1 cm) with a 100-watt tungsten lamp at a distance of 12 cm for 150 min at 0°.
† DBC coenzyme (5×10^{-6} *M*) made 0.1 *N* with KCN and kept for 30 min at room temperature before use.
‡ DBC coenzyme and intrinsic factor were preincubated for 30 min at 30°. Enzyme was then added and the mixture incubated for a further 13 min prior to the addition of methylmalonyl CoA.

of the isomerase activity of unresolved enzyme. This effect, previously noted by Stern and Friedman[20] with ox liver fractions, varied from preparation to preparation and was mostly small. On the other hand, the resolved enzyme had negligible activity in the absence of coenzyme (experiments 2, 3, and 4). Vitamin B_{12} at much higher concentrations was inactive (experiment 2*c*). As expected, prior exposure of the coenzyme to light, cyanide, or intrinsic factor (experiment 4) led to loss of activity. Table 2 shows the effect of the coenzyme on methylmalonyl isomerase activity as determined by the $C^{14}O_2$ fixation assay.

Coenzyme specificity and affinity constants: The rate of methylmalonyl CoA isomerization as a function of the concentration of added DBC or BC coenzyme is shown in Figure 1. Coenzyme concentrations were determined spectrophoto-

metrically at wavelength 367 mμ in 0.1 *N* KCN according to Barker *et al.*[21] Maximal rates with either coenzyme were obtained at concentrations around 3×10^{-6} *M*. On the other hand, 10^{-5} *M* AC coenzyme gave only 15 per cent of the maximal rate, a value given by about 10^{-8} *M* DBC coenzyme. Thus, the effect of the former could have been due to a very slight contamination (0.1 per cent) with the latter.

TABLE 2

EFFECT OF DIMETHYLBENZIMIDAZOLYLCOBAMIDE COENZYME ON ACTIVITY OF SHEEP KIDNEY METHYLMALONYL ISOMERASE ($C^{14}O_2$ FIXATION ASSAY)

Methylmalonyl CoA isomerase	Coenzyme addition	$C^{14}O_2$ fixed (cpm) In methylmalonate	In succinate
Unresolved	None	2820	3400
"	1.5×10^{-5} *M*	1930	3540
Resolved	None	4615*	855*
"	1.5×10^{-5} *M*	2500*	3090*
None	"	5310	90

Samples contained, in μmoles, Tris buffer, pH 7.4, 100; $MgCl_2$, 6; ATP, 3; $KHC^{14}O_3$ (14000 cpm/μmole), 10; propionyl CoA, 0.5; glutathione, 2; propionyl carboxylase, 0.1 unit; unresolved or resolved methylmalonyl isomerase (1 mg protein); and coenzyme (preincubated with isomerase as in Table 1) as indicated. Final volume made up with water to 1.0 ml. Incubation 15 min at 30°.
* Average of duplicate runs.

It is apparent that the AC coenzyme has little if any effect with the kidney methylmalonyl isomerase. A less likely possibility is that this coenzyme is inactivated by the relatively crude enzyme preparation used.

The apparent affinity constants (K_m values), calculated from Lineweaver–Burk[22] plots of the data in Figure 1, are given in Table 3 (column 2). Column 3 gives the similarly obtained values for *P. shermanii* methylmalonyl isomerase.[23] The table

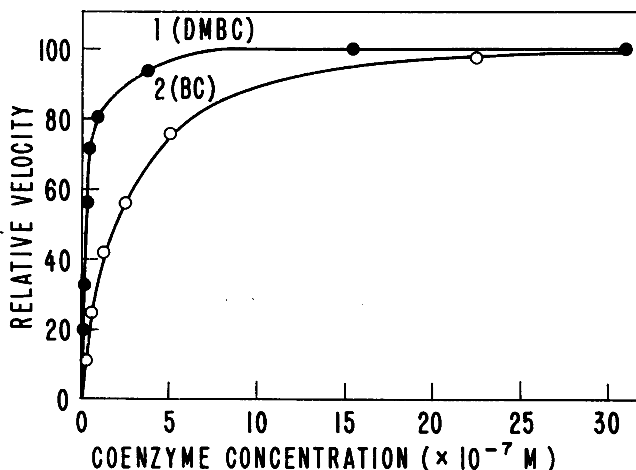


FIG. 1.—Rate of isomerization of methylmalonyl CoA as a function of cobamide coenzyme concentration. Conditions of Table 1 with resolved enzyme. Curve 1, enzyme with 0.9 mg protein; curve 2, enzyme with 0.6 mg protein. Relative velocity given as per cent of V_{max}. DMBC = dimethylbenzimidazolylcobamide coenzyme; BC = benzimidazolylcobamide coenzyme.

also lists for comparison (column 4) the corresponding values of Barker *et al.*[21] for *C. tetanomorphum* glutamic isomerase. It may be seen that both kidney and bacterial methylmalonyl isomerases have highest affinity for the DBC coenzyme while the reverse is true for glutamic isomerase. The affinity for BC coenzyme is about the same in all cases. Curiously enough both bacterial enzymes have high affinity for the AC coenzyme whereas, as noted above, this coenzyme has little or no affinity for the kidney methylmalonyl isomerase.

TABLE 3

COENZYME AFFINITIES OF COBAMIDE ENZYMES

	K_m (*M*)		
Coenzyme	Kidney methylmalonyl isomerase	*P. shermanii* methylmalonyl isomerase	Glutamic isomerase*
DBC	2.1×10^{-8}	2.4×10^{-8}	1.8×10^{-5}
BC	2.0×10^{-7}	1.3×10^{-7}	2.4×10^{-7}
AC		1.0×10^{-7}	1.4×10^{-6}

Conditions for the experiments with *P. shermanii* methylmalonyl isomerase were those of Table 1 except that only 17 to 20 μg of enzyme protein were used and the incubation was for 15 to 19 min.
* Values from Barker *et al.*[21]

Coenzyme binding strength of native and reconstituted holenzyme: As previously mentioned, such procedures as exposure to light, treatment with charcoal, or incubation with intrinsic factor, which might be expected to bring about resolution of methylmalonyl isomerase, do not significantly affect the activity of the native enzyme (Table 4, experiments 1*a* and *b*, 2*a* and *b*, and 3). However, when applied

TABLE 4

EFFECT OF LIGHT, CHARCOAL TREATMENT, AND INTRINSIC FACTOR ON SHEEP KIDNEY METHYLMALONYL ISOMERASE (DIRECT ASSAY)

Experiment No.	Enzyme	Coenzyme (DBC) addition	Treatment	Succinate formed (μmoles)
1a	Unresolved	None	None	0.126
1b	"	"	Illuminated	0.119
1c	Resolved	"	None	0.032
1d	"	10^{-7} *M*	"	0.150
1e	"	"	Illuminated	0.070
2a	Unresolved	None	None	0.115
2b	"	"	Charcoal	0.125
2c	Resolved	"	None	0.025
2d	"	10^{-7} *M*	"	0.167
2e	"	"	Charcoal	0.094
2f	"	"	Charcoal before DBC coenzyme	0.181
3a	Unresolved	None	None	0.110
3b	"	"	Intrinsic factor (1 mg)	0.104
3c	"	"	" " (5 mg)	0.111
3d	"	"	" " (10 mg)	0.098
4a	Resolved	"	None	0.013
4b	"	10^{-7} *M*	"	0.108
4c	"	"	Intrinsic factor (2 mg)	0.037

Conditions as in Table 1 with the following exceptions. Isomerase (in mg protein) experiment 1, 0.5; experiment 2, unresolved, 0.5, resolved, 0.8; experiment 3, 0.33; experiment 4, 0.39. Preincubation of coenzyme with isomerase, experiment 4, 30 minutes. Illumination, 60 min at 0° in a Beckman cell (d = 1 cm) with two 100 Watt lamps at 12 cm. Charcoal treatment, 1 mg Nuchar C per mg protein added to enzyme solution (2 mg protein/ml), stirred 15 min at 0° and filtered. Protein was redetermined in the supernatant as some is removed by the charcoal. When used, intrinsic factor was incubated with the reaction mixture for 15 min at 30° prior to adding methylmalonyl CoA.

to the reconstituted holoenzyme, i.e., to the resolved enzyme after preincubation with coenzyme, the same procedures lead to partial inactivation (Table 4, experiments 1*c* to *e*, 2*c* to *f*, and 4). This suggests either that the natural coenzyme is not identical with DBC coenzyme, or that holoenzyme binds the coenzyme less tightly in the reconstituted than in the native form. Considering that the enzyme is exposed to a low pH for resolution, the latter is not an unlikely possibility.

Discussion.—The fact that the AC coenzyme has very low, if any activity with kidney methylmalonyl isomerase is not without interest, since this coenzyme is active not only with glutamic isomerase of *C. tetanomorphum*, an organism that contains mainly AC coenzyme, but also with methylmalonyl isomerase of *P. shermanii*, an organism that contains predominantly DBC coenzyme. It may be noted that the behavior of the cobamide coenzymes with the mammalian enzyme parallels that of the corresponding vitamin forms in the animal organism, since both vitamin B_{12} and its benzimidazole analogue are active as growth factors and against pernicious anemia while pseudovitamin B_{12} (the adenine analogue) is inactive.[24]

Eggerer *et al.*[25] have recently established that methylmalonyl CoA isomerization (cf. reaction 1) involves migration of the thiolester rather than the carboxyl[17] group. This is in line with the fact[26] that glutamate isomerization (cf. reaction 2) occurs by migration of a two-carbon fragment, involving carbons 1 and 2 of glutamate, rather than by rearrangement of the C_5-carboxyl group. Rearrangement of a group along a propionic acid residue is thus a common feature of the two reactions; in both cases a methyl group is produced when the straight-chain compound is converted to the branched-chain isomer.

In view of the reported presence of methylmalonic acid in human urine,[27] a study of the levels of this compound in the urine of pernicious anemia patients, and of the effect of vitamin B_{12} thereon, might be of some interest.

Summary.—Methylmalonyl isomerase apoenzyme was prepared from sheep kidney cortex enzyme fractions by acidification in the presence of ammonium sulfate. Activity was restored by dimethylbenzimidazolyl- and benzimidazolylcobamide coenzymes but adenylcobamide coenzyme had little or no effect. In contrast, the three coenzymes were active with methylmalonyl isomerase preparations from *P. shermanii*. A study of the effects of illumination, charcoal treatment and incubation with intrinsic factor on the native and the cobamide coenzyme-reactivated kidney enzyme, suggested that the strength of the apoenzyme-coenzyme binding in this enzyme is greater before than after resolution.

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[5] Abbreviations: ATP, adenosine triphosphate; CoA, coenzyme A; DBC, dimethylbenzimidazolylcobamide; BC, benzimidazolylcobamide; AC, adenylcobamide; Tris, tris(hydroxymethyl)aminomethane.

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