*CONGENITAL METHYLMALONIC ACIDEMIA: ENZYMATIC EVIDENCE FOR TWO FORMS OF THE DISEASE**

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Abstract.—Methylmalonic acidemia is an inherited metabolic disorder thus far found in children and characterized by the excessive excretion of methylmalonate in the urine. Typically these children exhibit vomiting, lethargy, ketoacidosis, and failure to grow. Many of the patients are mentally retarded and die early in life. Two variants of this disease are known. In one, the administration of vitamin B_{12} will reverse or prevent these clinical findings, whereas in a second variant vitamin B_{12} therapy is of no value.

This paper presents the first enzymatic evidence (obtained with cell-free liver extracts) that bears on two important aspects of the disease. It has been found that methylmalonylCoA carbonylmutase activity is essentially absent in the livers of patients suffering from one variant (vitamin B_{12}-unresponsive) of the disease. Secondly, it has been found that the livers of patients with the second variant (vitamin B_{12}-responsive) of the disease show normal enzymatic behavior in the presence of the coenzyme form of vitamin B_{12}, but are identical to the vitamin B_{12}-unresponsive variant in the absence of the added coenzyme. Thus the enzyme studies fully support the clinical observations that two types of this disease exist.

Introduction.—Two variants of inherited methylmalonic acidemia have been described since the original report of this inborn error of metabolism by Oberholzer *et al.*[1] The metabolic pathway involved is the conversion of propionate to succinate, one step of which requires the cobamide coenzyme form of vitamin B_{12} (DBCC),‡ and in which methylmalonylCoA is an intermediate. The pathway of propionate metabolism in mammals is depicted in Figure 1.

Although none of the patients with methylmalonic acidemia are vitamin B_{12} deficient, they all excrete massive quantities of urinary methylmalonate. One variant of the disease is refractory to the administration of vitamin B_{12} and usually results in early death.[2] A second variant is responsive to massive doses of vitamin B_{12} and is compatible with normal growth and development.[3]

Although the nature of the defect has not been defined, it appears reasonable to believe that methylmalonylCoA carbonylmutase (MM-mutase) activity is the site of the lesion, since urinary methylmalonic acid is excreted in large amounts, whereas the next metabolic product of D,L-methylmalonylCoA metabolism, succinate, is absent. In the vitamin B_{12}-responsive variant of this disorder there is a decrease in the excretion of methylmalonic acid after vitamin B_{12} is administered, which indirectly indicates that MM-mutase activity is involved.

The present communication definitely implicates MM-mutase activity in both

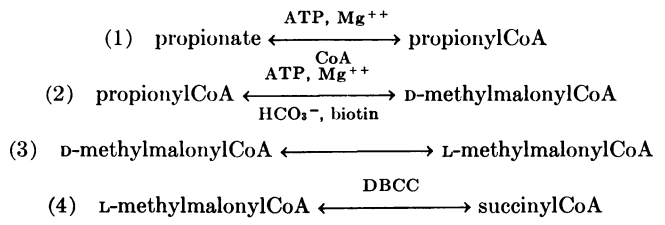

FIG. 1.—The enzymatic pathway of propionate metabolism in mammals. The enzymes catalyzing the respective reactions are: (1) propionate activating enzyme, (2) propionylCoA carboxylase, (3) methylmalonylCoA racemase, and (4) methylmalonylCoA carbonylmutase.

variants of methylmalonic acidemia. In the variant unresponsive to DBCC the defect appears to reside in the MM-mutase structural protein. In the second variant the defect involves either the metabolism of DBCC or its binding to the MM-mutase apoenzyme, the ultimate result of which is a diminished MM-mutase activity.

Materials and Methods.—Coenzyme-A was purchased from P-L Biochemicals as the lithium salt. ^{3}H-methylmalonic acid was obtained from the New England Nuclear Corp.

Unlabeled D,L-methylmalonylCoA was prepared by the method of Overath *et al.*[4] All such preparations used in this paper are racemic mixtures. D,L-MethylmalonylCoA labeled with tritium in the methyl group was prepared from ^{3}H-methylmalonic acid by the procedure of Trams and Brady[5] and purified by chromatography on DEAE-cellulose.[6] The material was shown to have a radiochemical purity greater than 90% when its hydroxamic acid derivative was chromatographed in pyridine:2-butanol:water (1:1:1).[7] The concentration of D,L-methylmalonylCoA was determined by the hydroxamate procedure of Lipmann and Tuttle.[8]

Liver extract preparation: The livers of four patients with methylmalonic acidemia and of three control subjects were obtained and frozen within 15 hr after death, except for control 1, whose liver was obtained 30 hr after death (Table 1). 750 mg of wet liver plus 1 ml of 0.25 *M* sucrose were homogenized by hand in a glass homogenizer at 0°. All procedures were carried out in the dark, as were the subsequent incubations. Only an ordinary flashlight was used when visualization was necessary. After centrifugation at 4300 × *g* for 1 hr the supernatant fluid was frozen until the assays were performed. Protein was determined according to the method of Lowry *et al.*[9]

Conversion of propionate-1-^{14}C to ^{14}C-methylmalonic acid and ^{14}C-succinate: The assay components are given in the legend to Table 2. Incubations were carried out in the dark in 10 × 75 mm test tubes at 37°. The reaction was terminated after 30-min incubation

TABLE 1. *Source of liver samples and DBCC content.*

Patient	Age at death	Cause of death	Time frozen before assay	Liver content of DBCC*
L. A.	22 mo	Methylmalonic acidemia	1½ mo	0.10
D. W.	4 mo	Methylmalonic acidemia	10 mo	0.09
C. D.	36 mo	Methylmalonic acidemia	13 mo	0.14
R. H.	21 mo	Methylmalonic acidemia	40 mo	<0.01
Control 1	70 yr	Lymphoma	1 day	5.8
Control 2	5 mo	Meningococcemia	1 mo	0.15
Control 3	48 mo	Acute lymphocytic leukemia	13 mo	0.29
Control range				0.08–0.25†

* DBCC values are expressed in μg/gm wet liver.

† The range consists of seven additional controls in whom only DBCC was assayed.

TABLE 2. *Metabolism of propionate-1-^{14}C by liver homogenates.*

Patient	^{14}C-methylmalonate cpm/3 mg Liver Protein		^{14}C-Succinate cpm/3 mg Liver Protein	
	No DBCC	DBCC added	No DBCC	DBCC added
L. A.	3,820	3,690	72	89
D. W.	2,140	3,420	32	142
C. D.	2,820	3,400	26	40
R. H.	2,600	689	190	6,200
Control 1	617	300	3,480	5,140
Control 2	1,400	570	2,740	5,300
Control 3	612	166	2,910	5,200

The incubation mixtures contained (in μmoles): propionate-1-^{14}C, 0.43 (2.18 μc); Mg^{++}, 2.5; ATP, 1.2; CoA, 0.52; glutathione (reduced form), 2.5; $KHCO_3$, 5.0; and tris-HCl, 25.6 (pH 7.3). 12 μg. of DBCC were added as indicated. 2.2 to 4.6 mg liver protein were added to initiate the reaction in a total volume of 375 μl.

by the addition of 300 μl water, and the vessels were immersed in a boiling water bath for 15 min. 100 mg NaCl, 120 μg each of methylmalonic acid and succinate, and 2 drops of 12 *N* H_2SO_4 were added. The entire mixture was then extracted five times with 1.5-ml aliquots of anhydrous ether, the ether phase being retained. Counting of the aqueous phase indicated that all the extractable radioactivity was removed after three extractions. The ether phases were combined and concentrated by evaporation. The ether concentrate was spotted on Whatman #1 paper and chromatographed in a two-way system using ethanol:ammonia:water (200:9:40) as the first solvent and isopentyl formate:formic acid-water (220:40:10) as the second solvent. After being dried the chromatograms were stained with bromcresol green (0.02%); the methylmalonic acid and succinate spots were located on the paper and then cut out and counted in a liquid scintillation counter.

Conversion of ^3H-methylmalonylCoA to ^3H-succinate: The assay components are given in the legend in Table 3. The incubations were carried out as discussed above, except that the reactions were terminated by boiling for 4 min. Ether extractions and the concentration of the extracts were performed as indicated above. The concentrate was spotted on Whatman 3-MM paper. One-way ascending chromatography was performed overnight with isopentyl formate:formic acid:water (220:40:10). Nonradioactive standards of methylmalonic acid and succinate (120 μg each) were spotted on either side of the samples. After the standards were stained with bromcresol green, the positions corresponding to succinate and methylmalonic acid were cut out and counted in a liquid scintillation counter.

DBCC determinations: Assays were performed as described elsewhere,[10] by the enzymatic conversion of 1,2-propanediol to propionaldehyde by the DBCC dependent enzyme, dioldehydrase.

Results.—Patient material: Complete clinical and biochemical studies concerning the four patients have been published elsewhere,[2, 11] and are summarized

TABLE 3. *Metabolism of ^3H-methylmalonylCoA by liver homogenates.*

Patient	^3H-Succinate cpm/mg Liver Protein	
	No DBCC	DBCC added
L. A.	6	17
D. W.	44	189
C. D.	16	41
R. H.	452	7,770
Control 1	4,920	6,010
Control 2	3,040	4,540
Control 3	3,710	5,000

The incubation mixtures contained: ^3H-methylmalonylCoA, 0.11 μmole (62,500 cpm); tris-sulfate pH 7.3, 20 μmoles; and DBCC, 12 μg. 1.3 to 2.8 mg liver protein were added to initiate the incubations in a total volume of 200 μl.

in part in Table 1. Two of the three control subjects were randomly selected children dying of various causes. To determine the effect of long-term storage of liver samples, the controls were selected so that the delay prior to assay was similar to the delay after death of the patients. One of the interesting findings from these studies is the remarkable stability of the enzyme activities for the entire metabolic pathway to prolonged storage in the frozen state.

DBCC content of liver: Normal DBCC concentrations in seven control subjects ranged from 0.08 to 0.25 μg/gm wet liver (Table 1). The level of DBCC was normal in patients L. A., D. W., and C. D. In patient R. H. the level of DBCC present was close to the sensitivity of the method. Whether this is the result of prolonged storage (40 months) is not known, although human livers stored for more than two years have normal concentrations.[12] The high level of DBCC in the livers of control patients 1 and 3 is to be noted. Deranged vitamin B_{12} metabolism with elevated levels of vitamin B_{12} has been described in patients in the lymphoma-leukemia group.[13]

Propionate-1-^{14}C metabolism: When labeled propionate was used as the substrate in incubations with liver extracts (Table 2), the controls readily converted propionate-1-^{14}C to ^{14}C-succinate. Addition of DBCC increased ^{14}C-succinate production by an average of 71 per cent. In contrast, the extracts from patients L. A., D. W., and C. D. converted propionate to succinate only in trace amounts with or without added DBCC. R. H., on the other hand, converted normal quantities of propionate-1-^{14}C to ^{14}C-succinate but was similar to the other patients without added DBCC. The average accumulation of methylmalonic acid in the patients was 3.2-fold greater than the controls, and in the case of three of the patients (L. A., D. W., and C. D.) this accumulation is not reduced by the addition of DBCC. Thus the propionate activation and the carboxylation steps are not sites of decreased enzymatic activity.

D,L-*MethylmalonylCoA conversion to succinate:* Control liver homogenates readily convert ^{3}H-methylmalonylCoA to ^{3}H-succinate (Table 3). With the addition of DBCC, there was an average increase of 33 per cent in the extent of conversion. It would appear, therefore, that under the conditions of the assay used here, the enzyme system from control subjects is not saturated with respect to DBCC. Of the patients, only R. H. had a dramatic increase in ^{3}H-methylmalonylCoA conversion, i.e., a 17-fold rise. Without added DBCC, R. H. was able to convert ten times as much D,L-methylmalonylCoA as the other patients but only 12 per cent of mean control values. Most important, however, is the failure of liver extracts from one variant of the disease (patients L. A., D. W., and C. D.) to show any significant conversion under saturating levels of DBCC. In the experiments reported here racemic D,L-methylmalonylCoA was used as the substrate, and the failure to find significant succinate formation implicates the MM-mutase activity as being at least one site of the defect.

Discussion.—The enzymatic evidence presented can be used to define the defect in methylmalonic acidemia. It may be summarized as follows: (1) Liver extracts from patients with the disease convert propionate to D,L-methylmalonyl-CoA without further significant conversion to succinylCoA, whereas with extracts of normal livers the reverse is true, i.e., little D,L-methylmalonylCoA

accumulates, but there is extensive succinylCoA accumulation. (2) With racemic D,L-methylmalonylCoA as substrate, the extracts from the patients catalyze little if any conversion to succinylCoA.

The evidence from the propionate experiments suggests that neither the activation of propionate to propionylCoA nor the carboxylation of the latter to D-methylmalonylCoA is the site of the block in methylmalonic acidemia. This leaves two enzymatic steps as possible sites: the racemase that converts D- to L-methylmalonylCoA and the mutase reaction by which the latter substrate is converted to succinylCoA. The fact that racemic D,L-methylmalonylCoA is not significantly converted to succinylCoA by extracts from the patients points to the mutase reaction as the deficient enzyme activity.

What has been summarized above pertains to liver extracts from all patients with methylmalonic acidemia that have been examined to date, and in each case there is an apparent block at the mutase step. Beyond that it is possible to differentiate the disease further by the same enzymatic procedures. The mutase reaction is the only step in the pathway that is dependent upon the presence of DBCC, the coenzyme form of vitamin B_{12}. When it is present in excess in the same enzymatic assay procedures, the liver extracts of methylmalonic acidemia patients can be divided into two groups. One group, represented in this study by patients L. A., D. W., and C. D., shows no difference in response in the presence or absence of excess DBCC. It is concluded that in this group the MM-mutase apoenzyme is defective and is the primary lesion. The most likely possibilities involve an amino acid alteration in the structural protein of the apoenzyme or chain-terminating and deletion mutations, any of which could lead to a loss or defect at the catalytic site.

The second group, represented in this study by patient R. H., can give a differential enzymatic response dependent upon whether DBCC is present in excess or not. When it is limiting, or absent, the enzymatic response is almost identical to that of the first group mentioned above, viz., a failure to convert D,L-methylmalonylCoA to succinylCoA and an accumulation of D,L-methylmalonylCoA with propionate as substrate. However, when DBCC is present in excess these extracts are indistinguishable from extracts of normal liver. Several possibilities exist to explain this: the primary effect could still be in the apoenzyme by virtue of an amino acid substitution, but in this case only the binding of DBCC is affected, not the catalytic site. Other possibilities would place the defect at the mutase reaction as secondary to a primary lesion involving vitamin B_{12} metabolism. Two such possibilities are: defects in the conversion of vitamin B_{12} to DBCC, and excessive destruction of DBCC. The significance of the low level of DBCC in the liver of patient R. H. is difficult to assess at this time, but it could possibly be evidence for a defect in the formation of DBCC or evidence for its excessive destruction.

The enzymatic picture described here for the first time is in complete accord with the clinical picture of this disease. Clinically, two forms of methylmalonic acidemia are recognized by their response to vitamin B_{12} therapy, one form of which is responsive and the other unresponsive. Patient L. A. was given massive vitamin B_{12} therapy, but his clinical picture was not altered. Similarly, the

enzymatic picture of liver extract from this patient is not altered by the presence of excess DBCC. Unfortunately, we have no clinical observations in regard to vitamin B_{12} therapy with the other three patients, since they expired prior to the recognition of this metabolic disorder. However, another patient has been described who is responsive to vitamin B_{12} therapy.[3] Presumably patient R. H. would have been responsive to this therapy.

The failure of liver extract from patients L. A., D. W., and C. D. to convert either propionate of D,L-methylmalonylCoA to succinate could theoretically result from the presence of excess D,L-methylmalonylCoA deacylase activity rather than from a defect in MM-mutase activity. This possibility is unlikely for two reasons: in one variant of methylmalonic acidemia as represented by patient R. H. the addition of DBCC permits normal conversion of both propionate and D,L-methylmalonylCoA to succinate, and the carbonylmutase is the only DBCC-dependent enzyme in the pathway. Secondly, in quantitating the utilization of unlabeled D,L-methylmalonylCoA[14] in the liver extracts from L. A., D. W., and C. D. there was no increased disappearance of D,L-methylmalonylCoA compared to appropriate controls for nonenzymatic hydrolysis. There is also no effect of DBCC. These findings are incompatible with the presence of significantly increased activity of D,L-methylmalonylCoA deacylase.

In all the results reported here the experimental conditions were such that the various enzymatic entities assayed were not limiting, with the exception of those cases where there are missing activities. Both the time of incubation and the amounts of tissue extract used were in excess of assay conditions where the rates of the reactions are proportional to these factors. Experiments are now in progress to characterize the individual enzymatic activities with conditions chosen such that the assay for each is linearly related to the activity. This should provide a more definitive answer to the question of the DBCC-responsive form of the disease where an altered binding of DBCC to the carbonylmutase is one of the possible explanations. Secondly, with more accurate enzymatic assays and with the availability of tissue extracts from the parents and other siblings of several of these patients, it will be possible to examine some questions of the genetic basis of this disease. One would like to know whether enzymatic evidence can be obtained for an autosomal recessive trait in methylmalonic acidemia. Thirdly, there is the question of whether more than one enzymatic activity is involved, thus indicating a possible defect in metabolic control. Nothing can be said about the racemase activity from the results reported in this communication.

There are some indications that the above questions can be successfully approached. Preliminary experiments with extracts of skin fibroblasts from one of the patients have given results almost identical to those reported here. Since the fibroblast lines can be maintained in tissue culture, it will be possible to examine entire families without the necessity of resorting to liver biopsies for tissue samples. Secondly, as reported here, the entire enzyme system appears to be extremely stable to prolonged storage in frozen tissue specimens, and the DBCC-MM-mutase complex is more dissociable than has previously been reported for this enzyme from other mammalian sources.[15] This latter fact should

make possible an exact comparison of the DBCC binding constants in normal and vitamin B_{12}-responsive forms of this disease.

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‡ Abbreviations used: MM-mutase, methylmalonylCoA carbonylmutase (EC 5.4.99.2); DBCC, vitamin B_{12} coenzyme (5,6-dimethylbenzimidazolylcobamide 5′-deoxyadenosine); CoA, coenzyme A.

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