VITAMIN B_{12} DEPENDENT METHYLMALONICACIDURIA: DEFECTIVE B_{12} METABOLISM IN CULTURED FIBROBLASTS

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SUMMARY

Intact fibroblasts from children with vitamin B_{12} dependent methylmalonicaciduria failed to isomerize methylmalonate to succinate, and contained less than 10 percent of the normal amount of 5'-deoxyadenosylcobalamin (B_{12} coenzyme) when grown in a standard culture medium. Both metabolic abnormalities disappeared, however, when a B_{12}-supplemented growth medium was employed. These findings, plus the demonstration that methylmalonyl-CoA mutase apoenzyme activity in whole cell homogenates from these mutant lines was not impaired, indicate that B_{12} dependent methylmalonicaciduria is caused by a defect in the biosynthesis or metabolism of 5'-deoxyadenosylcobalamin. To our knowledge, such a defect in human vitamin metabolism has not been demonstrated previously in tissue culture.

Methylmalonyl Coenzyme A Carbonylmutase (MMA-CoA mutase), the enzyme which catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA, requires a B_{12} coenzyme, 5'-deoxyadenosylcobalamin (1,2). In patients with acquired B_{12} deficiency, this isomerization reaction is blocked, leading to excessive urinary excretion of methylmalonic acid (MMA) which is rapidly reversed by B_{12} replacement (3,4). Since 1967, thirteen young children have been described who excrete massive amounts of MMA, but who are not B_{12} deficient (5-11). Five of these children, however, have responded to administration of pharmacologic doses of vitamin B_{12} or 5'-deoxyadenosylcobalamin with a marked reduction in MMA excretion (7,10-13). The present studies were undertaken to define the bio-chemical mechanism of this unique, and presumably inherited, vitamin dependency.

EXPERIMENTAL

Patients. Skin biopsies were obtained from two boys with B_{12} dependent methylmalonicaciduria (R.P., age 2 yr.; and C.K., age 2 yr.) and from several controls: S.P. and L.Y. (healthy adult females); R.C. (an adult male with gout); J.P. and L.J. (adults with cystinuria); R.R. (an adult male with Wilson's disease); S.R. (a 4 yr. old healthy boy); M.R. (a 5 yr. old girl with glucose-galactose malabsorption); S.J. (a 7 yr. old girl with Type I glycogen storage disease); and A.G. (a 6 yr. old girl with propionyl-CoA carboxylase deficiency).

Tissue Culture. Skin biopsy explants and their subcultures were grown in Diploid Growth Medium (Grand Island Biologicals) containing 10 percent fetal calf serum and neomycin (100 μg/ml). The vitamin B_{12} concentration of this medium was 25-30 picograms (pg) per ml. Fibroblast monolayers, grown to confluence in 32 ounce glass bottles or in large Bellco Roller bottles (1410 cm^2), were harvested with 0.25 percent trypsin, washed twice with buffered 0.9 percent sodium chloride and centrifuged at 600 G for 5 minutes at 20°C. Cell counts and packed weight determinations were obtained immediately prior to or following the last centrifugation.

Methods. Oxidation of C^{14} labelled propionate, methylmalonate and succinate to $C^{14}O_2$ was measured by suspending intact fibroblasts in Krebs-phosphate buffer (pH 7.4) for 3 hr. at 37°C, as described previously (12). The concentration of 5'-deoxyadenosylcobalamin in fibroblasts disrupted by sonication was determined with bacterial dioldehydrase by the method of Abeles et al. (14). Conversion of DL-methyl-H^3-malonyl-CoA to succinyl-CoA by whole cell homogenages, prepared by alternate freezing and thawing, was determined as described by Cardinale et al. (15). All cell preparation was carried out in subdued light. Assays of 5'-deoxyadenosylcobalamin and methyl-H^3-methyl-CoA isomerization were conducted in a dark room illuminated only by a 3 volt flashlight battery.

Materials. Propionate-3-C^{14}, succinate-1-4,-C^{14}, methyl-C^{14}-malonate and methyl-H^3-malonate were purchased from New England Nuclear. DL-methyl-H^3-

malonyl-CoA was synthesized from methyl-H^3-malonate as described by Trams and Brady (16). Dioldehydrase, purified from A. aerogenes, was a gift from Dr. R.W. Abeles.

RESULTS AND DISCUSSION

$C^{14}O_2$ Production. The data in Figure 1 demonstrate that intact fibroblasts from R.P. and C.K., grown in a medium containing 25 pg/ml of vitamin B_{12}, oxidized far less labelled propionate or methylmalonate to $C^{14}O_2$ than control cells did. Succinate oxidation was normal in both mutant cell lines, however, documenting the specificity of the biochemical defect and localizing the block to the B_{12} catalyzed reaction in which L-methylmalonyl-CoA is converted to succinyl-CoA. Morrow et al. (17) have also demonstrated defective propionate oxidation by fibroblasts from a patient with methylmalonicaciduria.

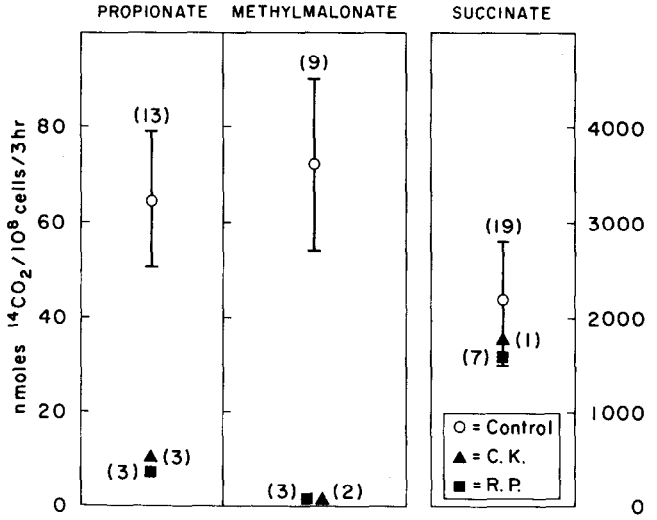

Figure 1 Oxidation of labelled propionate, methylmalonate and succinate to $C^{14}O_2$ by intact fibroblasts from controls and two patients with B_{12} dependent methylmalonicaciduria (R.P. and C.K.). Control data is presented as mean of number of observations in parentheses ± one standard deviation.

5'-deoxyadenosylcobalamin Content. Cell sonicates from six control cultures, grown in medium containing 25 pg/ml of vitamin B_{12}, had 5.9 ± 2.0 nanograms (ng) of 5'-deoxyadenosylcobalamin per gm wet weight (Table 1). Under

Table 1

Concentration of 5'-deoxyadenosylcobalamin in Cultured Fibroblasts*

Subjects Studied		5'-deoxyadenosylcobalamin (ng/gm. wet wgt.)
Controls:	M.R.	3.0
	S.R.	6.4;7.0+
	S.J.	8.0;10.2
	L.Y.	6.0
	S.P.	6.3
	R.C.	4.3
		$\bar{x} \pm 1$ S.D. = 5.9 ± 2.0
Patient:	R.P.	0.4;0.6

* All cells were grown in Diploid Growth Medium containing 10% fetal calf serum. Vitamin B_{12} concentration of this medium was 25-30 pg/ml.

\+ Each value represents a single determination. Where two values are given, they represent assays performed on successive subcultures.

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these same conditions, cells from R.P. contained only 0.4-0.6 ng/gm wet weight ($p < 0.01$). When the vitamin B_{12} concentration of the growth medium was increased to 25,000 pg/ml, however, three significant findings were observed (Figure 2): the concentration of 5'-deoxyadenosylcobalamin in control cells did not change; R.P.'s cells contained normal quantities of the B_{12} coenzyme; and his cells oxidized propionate nearly as well as control cells did. Thus the B_{12} dependency, previously demonstrated *in vivo*, was also observed in cultured fibroblasts.

Methylmalonyl-CoA Isomerization. The findings described above are consistent with a defect in 5'-deoxyadenosylcobalamin biosynthesis or metabolism, but could also reflect impaired binding of B_{12} coenzyme by a mutant mutase apoenzyme. Therefore, mutase activity was assayed directly by incubating whole cell homogenates with DL-methyl-H^3-malonyl-CoA and isolating the succinyl-H^3-CoA formed. As shown in Table 2, no significant defect in succinate formation was observed in cells from R.P. or C.K., either in the absence of added 5'-deoxyadenosylcobalamin or when enzymatic activity was enhanced by saturating amounts

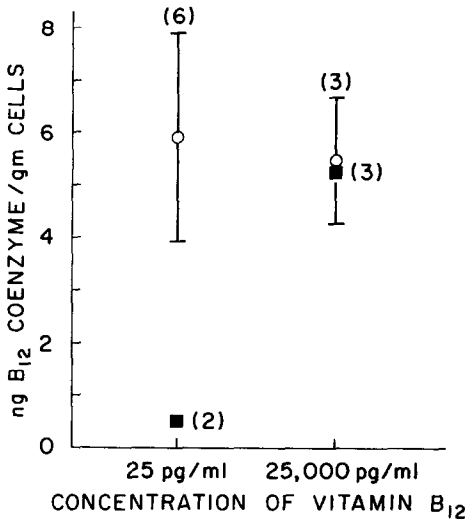

Figure 2 Effect of increasing concentration of vitamin B_{12} in growth medium on content of 5'-deoxyadenosylcobalamin in fibroblasts from controls (open circles) and R.P. (closed squares). Control data is presented as mean ± one standard deviation.

Table 2

Conversion of DL-Methyl-H^3-Malonyl-CoA to Succinyl-CoA by Fibroblast Homogenates

Subjects Studied		Enzymatic Activity*	
		Without B_{12} Coenzyme	With B_{12} Coenzyme ($2x10^{-5}$ M.)
Controls:	A.G.	0.07;0.06+	0.63
	R.R.	0.9	1.03
	J.P.	0.12;0.16	2.88
	L.J.	0.12	3.25
	E.B.	0.09	1.68
		$\bar{x}$ ± 1 S.D. = 0.10 ± 0.03	$\bar{x}$ ± 1 S.D. = 1.89 ± 1.1
Patients:	R.P.	0.06;0.09;0.04	1.40;0.97;1.07
	C.K.	0.15	1.61;1.71

* Activity expressed as μmoles succinate formed/gm wet weight/30 minutes.

\+ Each value represents a single determination. Where more than one value is given, they represent assays on serial subcultures. No significant differences were noted in comparing control values with these of R.P. and C.K.

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of the B_{12} coenzyme ($2x10^{-5}$ M). Furthermore, when 5'-deoxyadenosylcobalamin was added to the incubation medium in concentrations ranging from 10^{-11} M. to

10^{-4} M., enzymatic activity in R.P.'s cells mirrored that observed in two control lines, indicating that his mutase apoenzyme did not have reduced affinity for coenzyme (Table 3).

Table 3

Effect of Addition of 5'-Deoxyadenosylcobalamin on Conversion of Methylmalonyl-CoA to Succinyl-CoA

Concentration of 5'-deoxyadenosylcobalamin (moles/L)	Enzymatic Activity* A.G.	E.B.	R.P.
None	0.06	0.09	0.06
10^{-11}	0.06		0.05
10^{-10}	0.07		0.06
10^{-9}	0.07	0.14	0.07
10^{-8}	0.26	0.45	0.24
10^{-7}	0.52	1.16	0.75
10^{-6}	0.56		
10^{-5}	0.63	1.68	1.20
10^{-4}	0.66	1.75	1.24

* Activity expressed as μmoles succinate formed/gm wet wgt/30 minutes. The apparent K_m for coenzyme appears to be between 10^{-8} and 10^{-7} M. in all cell lines.

Recently, Morrow et al. (18) have published very similar results using liver homogenates from four patients with congenital methylmalonicaciduria. Three of their patients had normal hepatic B_{12} coenzyme content but demonstrated a profound defect in methylmalonyl-CoA isomerization which was not enhanced by the in vitro addition of B_{12} coenzyme. The fourth patient, however, had a much reduced hepatic B_{12} coenzyme content and showed a striking increase in mutase activity when 5'-deoxyadenosylcobalamin was added in vitro.

Their results and ours, are consistent with the hypothesis that patients with the B_{12} responsive form of congenital methylmalonicaciduria have a defect in the biosynthesis or metabolism of 5'-deoxyadenosylcobalamin while the B_{12} unresponsive patients suffer from a mutation of the mutase apoenzyme.

One additional report warrants comment. Mudd et al. (19) described a 7 wk. old boy who had methylmalonicaciduria, a low serum methionine concentration, and elevated plasma and urinary homocystine and cystathionine concentrations.

The liver of this patient had a very much reduced content of 5'-deoxyadenosylcobalamin and also showed a defect in the methyltransferase enzyme which converts homocysteine to methionine. These workers proposed that the child had a defect in B_{12} metabolism which affected both B_{12} dependent reactions defined in mammalian systems: methylmalonyl-CoA isomerization; and homocysteine methylation (20). It is possible that R.P. and C.K. have the same defect as that reported by Mudd et al., but we have failed to find any abnormalities in plasma or urinary methionine, homocystine or cystathionine in R.P., despite repeated searches for such findings. At least three distinct enzymes are required for the step-wise biosynthesis of 5'-deoxyadenosylcobalamin from its vitamin precursor in Clostridium tetanomorphum (21,22). It seems likely that a similar pathway exists in man and that several inherited defects in vitamin B_{12} metabolism may ultimately be defined, some affecting only methylmalonate metabolism and others affecting sulfur amino acid metabolism as well.

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