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3-Hydroxypropionate: Significance of β-Oxidation of Propionate in Patients with Propionic Acidemia and Methylmalonic Acidemia

(inborn errors/metabolic blocks/valine oxidation)

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ABSTRACT **[1-^{14}C]Propionate administered intravenously was metabolized to methylmalonate, to 3-hydroxypropionate, and to methylcitrate in the urine of a patient with methylmalonic acidemia. L-[*U*-^{14}C]Isoleucine and L-[*U*-^{14}C]valine were also converted to urinary methylmalonate and to 3-hydroxypropionate in the patient. Two patients with propionic acidemia due to a defect in propionyl-CoA carboxylase metabolized [*1*-^{14}C]propionate to urinary methylcitrate and 3-hydroxypropionate. The appearance of radioactive 3-hydroxypropionate in the urine after the administration of these compounds indicates that β-oxidation of propionyl-CoA through acryloyl-CoA was functioning in these patients. The conversion of valine to 3-hydroxypropionate suggests that valine is oxidized by way of propionate and propionyl-CoA in man.**

In animals the oxidation of propionate proceeds through a preliminary carboxylation of propionyl-CoA to methylmalonoyl-CoA through conversion to succinoyl-CoA to oxidation by way of the Krebs cycle (1). Two inborn errors of propionate metabolism occur in humans that are seen as metabolic blocks in the pathway from propionyl-CoA to to succinoyl-CoA. In one condition there is a defect in propionyl-CoA carboxylase (2–5), and in the other a defect in methylmalonoyl-CoA mutase (6–8).

Patients with these disorders oxidize [*1*-^{14}C]propionate inefficiently to respiratory $^{14}CO_2$ (9). Normal individuals oxidize the tracer rapidly to $^{14}CO_2$. The decrease in oxidation in propionate in the patients was of such an order of magnitude that it appeared that the major pathway of propionate oxidation in man was blocked in these patients. This hypothesis is consistent with the site of the block. However, there was always some oxidation of propionate to CO_2, even in patients in whom no activity of propionyl-CoA carboxylase could be demonstrated. This observation suggested that alternative pathways for propionate oxidation might be present. During a systematic study of the organic acids of urine after injection of [*1*-^{14}C]propionate (10), 3-hydroxypropionate was found as a significant product of propionate in all patients with propionic acidemia studied. Furthermore, [*U*-^{14}C]valine and [*U*-^{14}C]isoleucine were converted to 3-hydroxypropionate in a patient with methylmalonic acidemia.

MATERIALS AND METHODS

Isotopic tracers were obtained from New England Nuclear Corp., Boston, Mass. Before injection, the solution containing the sodium salt of the tracer was sterilized by passing it through a Millipore filter, and it was made isotonic with sodium chloride. The specific activities of three batches of [*1*-^{14}C]sodium propionate were 9.78 Ci/mol, used for V. B., D. G., and L. G.; 14.4 Ci/mol for C. E.; and 3.0 Ci/mol for A. M. L-[*U*-^{14}C]Valine and L-[*U*-^{14}C]isoleucine had specific activities of 200 Ci/mol and 263 Ci/mol, respectively. Urine was collected for 24 hr after injection, stored on ice during collection, and kept frozen at −20° until analyzed.

Subjects. Two patients with propionic acidemia (V. B. and C. E.) (9, 10) and one with methylmalonic acidemia (L. G.) (10, 11) were studied with labeled propionate. Two control subjects were D. G., a patient with cerebral gigantism (12), and A. M., a patient with Cornelia de Lange syndrome (13). Labeled valine and isoleucine were given to L. G. In each instance the dose of isotopic material was 2 μCi/kg. Each patient (V. B., C. E., and L. G.) had elevated concentrations of propionate in his blood (14). Defective oxidation of propionate was demonstrated *in vivo*, in V. B. and L. G., or *in vitro*, with fibroblasts in cell culture, in all of these patients (9). Methylmalonic acid was excreted only by L. G.

Silicic Acid Chromatography. Organic acids were separated by chromatography on the silicic acid column of an Organic Acid Analyzer constructed as described by Kesner and Muntwyler (15). The detailed procedures for sample preparation, fraction collection, and assay of radioactivity in the fractions have been described (10).

Isolation of Radioactive Compounds. Silicic acid column chromatography was performed without the addition of indicator solution. Fractions were collected from the bottom of the column. 1 ml of each 12-ml fraction was used for liquid scintillation counting. Another 1-ml aliquot of each fraction was used to locate peaks of acid by titration with indicator solution. Radioactive fractions were evaporated to dryness in an oven at 60°.

Identification of Radioactive Compounds Isolated. Paper chromatography was done by the method of Nordman and Nordman (16), and radioactive spots were located by autoradiography. Combined gas–liquid chromatography and mass spectrometry of the trimethylsilyl derivatives of isolated compounds were done as described (10). Authentic 3-hydroxypropionate was obtained from the Aldrich Chemical Co., Inc., Milwaukee, Wis.

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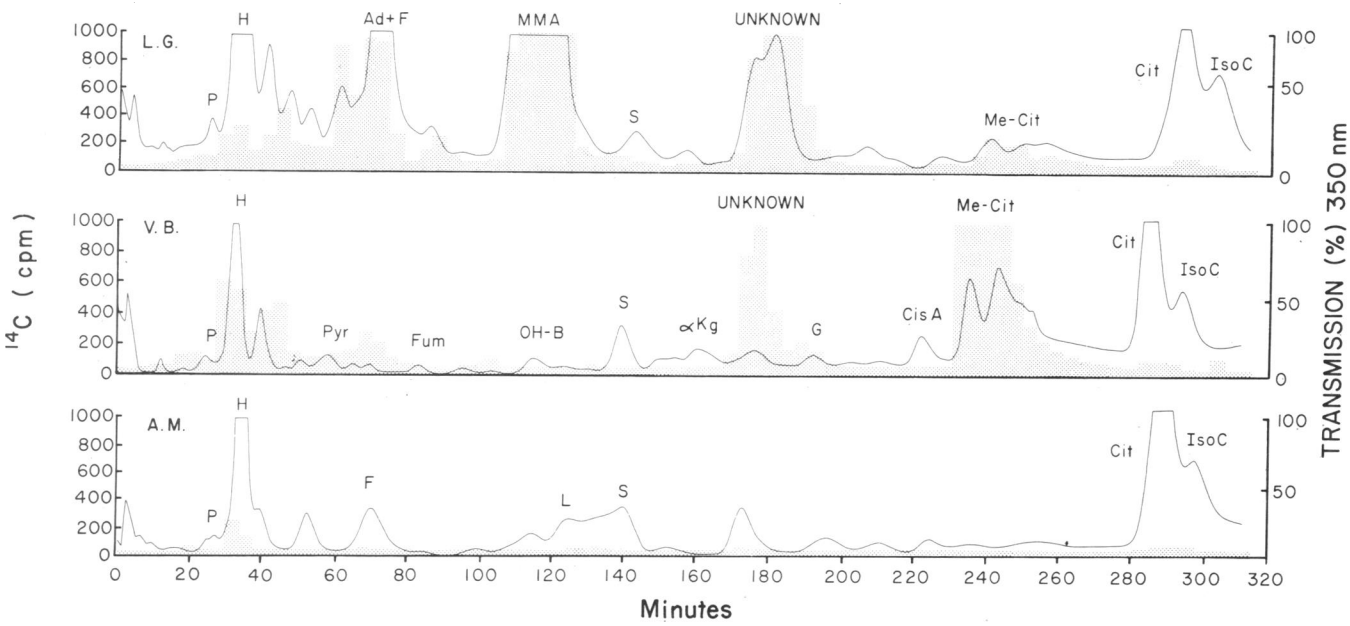

FIG. 1. Pattern of excretion of organic acids in urine after injection of labeled propionate. Each patient received an identical dose of 2 μCi/kg of [*1*-^{14}C]propionate. The chromatograms were obtained on silicic acid columns in an organic-acid analyzer. The effluent was collected in 15.6-ml fractions, each representing 4-min periods. The *continuous line* indicates the presence of acid and the *shaded area*, radioactivity. P, propionate; N, hippurate; Pyr, pyruvate; Fum, fumarate; OH-B, hydroxybutrate; S, succinate; α-Kg, α-ketoglutarate; G, glycolate; CisA, *cis*-aconitate; Cit, citrate; IsoC, isocitrate; F, formate; L, lactate; Ad, adipate; MMA, methylmalonate; and MeCit, methylcitrate.

Determination of Radioactivity in Whole Urine. Aliquots of 0.5–1.0 ml of urine were mixed with 15 ml of Bray's solution containing 0.4 g of thixotropic gel. The samples were kept at room temperature at least 24 hr until chemical luminescence disappeared, and then counted in a liquid scintillation spectrometer.

RESULTS

Isolation and identification of 3-hydroxypropionate

The patterns obtained by chromatography on silicic acid columns of the metabolic products excreted in urine after administration of labeled propionate are shown in Fig. 1. In control individuals, such as A. M., virtually no isotope is found in the organic acids of the urine. There was a small amount of isotope excreted as propionate. In patients with propionic acidemia, such as C. E. and V. B., the largest radioactive peak in urine has been identified as methylcitrate (10). In L. G., the patient with methylmalonic acidemia, methylmalonate was the largest product of propionate found in the urine. The second largest radioactive peak appeared at about 180 min, in the area of pyrrolidone carboxylate on the chromatogram. The chromatograms of V. B. and C. E. also showed large, radioactive peaks in the same area.

The unknown peak compound was identified as 3-hydroxypropionate in the following manner. Two dimensional paper chromatography of the radioactive compound from L. G. revealed spots of aconitate, of pyrrolidone carboxylate, and of an unknown compound. The spots were cut out of the paper and assayed for radioactivity. Only the unknown spot was radioactive. For V. B., a similar radioactive spot was found in the same area of the chromatogram, but no acid was detected there by spraying with bromocresol green.

Gas–liquid chromatography mass spectrometry was done on the trimethylsilyl [$(CH_3)_3Si$] derivative of the pooled peak fractions (Fig. 2). In addition to the unknown, the unlabeled pyrrolidone carboxylate and aconitate were characterized in this way. The mass spectrum of the unknown revealed a compound with a molecular ion at 234. This was consistent with a molecular weight for the parent compound of 90 and a derivative with two $(CH_3)_3Si$ groups. We suspected that the unknown compound might be 3-hydroxypropionate. The $(CH_3)_3Si$ derivative of authentic 3-hydroxypropionate gave a mass spectrum identical to that of the unknown compound. Paper chromatography of authentic 3-hydroxypropionate showed a location relative to standard glycolate and succinate that was the same as the unknown. Finally, authentic 3-hydroxypropionate chromatographed in the same position as the unknown on chromatography on the original silicic acid column.

Evidence that 3-hydroxypropionate is not an artifact of chemical conversion from propionate during assay

It is possible for 3-hydroxypropionate to be formed nonenzymatically from propionate. Such a possibility might be particularly likely under conditions of derivatization and gas–liquid chromatography in the presence of some water. It appears much less likely that 3-hydroxypropionate could result artifactually under the conditions used because it was found directly on silicic acid chromatography, before it was subjected to derivatization or high temperature. Actually, very little labeled propionate was found in the urines of patients or control subjects. Furthermore, C. E.'s urine was analyzed within 3 days after collection, and it contained labeled 3-hydroxypropionate. In order to exclude the possi-

TABLE 1. *Radioactivity in methylmalonate, 3-hydroxypropionate, and methylcitrate after injection of labeled precursors*

Patient	Isotope	Date	Isotope given (μCi)	Radioactivity (μCi/day) Whole urine	Methyl-malonate	3-OH-propionate	Methyl-citrate
L. G.	[*U*-^{14}C]Isoleucine	8/13/68	16.4	0.390	0.203	0.007	0
		8/14/68		0.108	0.062	0.002	0
	[*1*-^{14}C]Propionate	8/16/68	16.2	2.630	2.111	0.207	0.017
		8/17/68		0.075	0.046	0.002	0.000
		9/30/68		0.010	0.006	0	0
	[*U*-^{14}C]Valine	10/17/68	18.4	0.601	0.435	0.018	0.004
V. B.	[*1*-^{14}C]Propionate	4/22/69	13.95	0.755	0	0.059	0.185
C. E.	[*1*-^{14}C]Propionate	7/22/71	16.3	1.056*	0	0.192	0.122
D. G.	[*1*-^{14}C]Propionate	8/23/68	30.2	0.248	0	0	0
A. M.	[*1*-^{14}C]Propionate	11/1/68	20.6	0.408	0	0	0

The values shown represent the results of analysis of collections of urine on the day designated for 24 hr after injection of the tracer.
* This urine sample also contained 0.221 μCi/day of propionylglycine.

bility of chemical conversion of propionate to 3-hydroxypropionate in the analytical procedure, we added 4 μmol of sodium propionate and 1,612,400 dpm of [*1*-^{14}C]sodium propionate to 5 ml of urine from a dehydrated baby, and the solution was analyzed by a procedure identical to that used for the patients. Radioactive propionate was recovered in a yield of 95.5%. There was no detectable radioactivity in the area of 3-hydroxypropionate, nor was an acid peak detected in this area. When the ^{14}C-labeled propionate was added directly to the column of the organic-acid analyzer it was found to be at least 99% pure. Again, no radioactivity was found in hydroxypropionate. In these procedures hydroxypropionate impurity in the propionate precursor of as little as 0.005% would have been detected. Furthermore, substrate quantities of unlabeled hydroxypropionate have now been found in the urine of two patients with propionic acidemia.

Radioactivity in 3-hydroxypropionate and other products of propionate, of valine, and of isoleucine

The isotope recovered in the urine in products of propionate, valine, and isoleucine metabolism is listed in Table 1.

In L. G., most of the total radioactivity recovered in the urine in 24 hr was found in methylmalonate and in 3-hydroxypropionate. In V. B. and C. E., the organic acids accounted for a much smaller part of the total radioactivity in whole urine. Isotope was also found in propionylglycine in C.E. L.G. was studied with more than one tracer; therefore, it was important to establish that isotope in a compound did not represent simply persistence of label from a previous precursor in a product that is poorly metabolized because of a metabolic block. The data in Table 1 make this point clearly. Isotope in 3-hydroxypropionate and in methylcitrate was negligible even 24–48 hr after injection. There was, on the other hand, persistence of isotope in methylmalonate a month-and-a-half after injection. The data establish valine and isoleucine, as well as propionate, as precursors of methylmalonate in man.

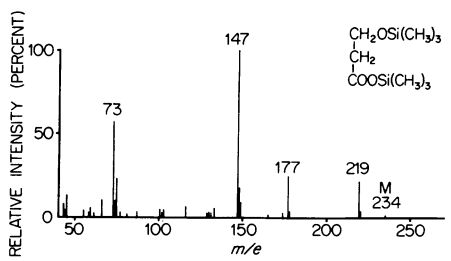

FIG. 2. Mass spectrum of the abnormal metabolite of propionate. The $(CH_3)Si$ derivative of the pooled, dried peak fractions was injected into the LKB 9000 mass spectrometer with a gas–liquid chromatographic inlet system equipped with a 1% OV-1 column.

Fig. 3 illustrates the percent incorporation of the effective carbons§ of the tracer into urinary products. In L. G., 13% of the isotope of [*1*-^{14}C]propionate was metabolized to urinary methylmalonate in 24 hr and 1.3% was incorporated into 3-hydroxypropionate. In contrast, V. B. and C. E. metabolized 1.3% and 0.7%, respectively, of isotope of propionate into methylcitrate, and 0.4% and 1.2%, respectively, to 3-hydroxypropionate. No methylmalonate was found in V. B. or C. E. In control subjects, D. G. and A. M., there was virtually no radioactivity in urinary organic acids. [*U*-^{14}C]Valine and [*U*-^{14}C]isoleucine were metabolized to urinary methylmalonate. The conversions were 2.95 and 2.47%, respectively, and to 3-hydroxypropionate, 0.15 and 0.08%, respectively.

DISCUSSION

Hydroxypropionate would be expected to arise from propionate through a β-oxidation or an ω-oxidation of propionyl-CoA. These pathways are illustrated in Fig. 4, which also shows the major metabolic interrelations relevant to this study and the sites of the metabolic defects in propionic acidemia and methylmalonic acidemia. The β-oxidation of propionate has generally been thought to constitute a minor pathway in animal tissues (1).

The intermediate expected in this conversion is acryloyl-CoA, which would be formed in a reaction analogous to the formation of 2,3-unsaturated fatty acid acyl-CoA as catalyzed by Acyl-CoA dehydrogenases.

§ In this calculation we assumed that the labeling of each carbon was uniform and that three of the five or six carbons of valine and isoleucine, respectively, were available for propionate formation.

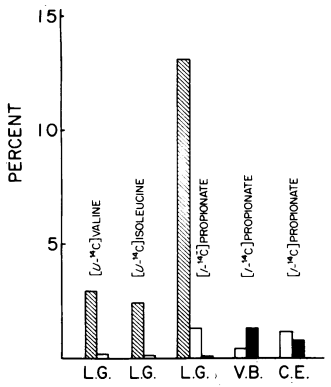

FIG. 3. Percent conversion of administered precursor to urinary metabolites. ▨ Methylmalonate; ▭ 3-Hydroxypropionate; ▬ Methylcitrate.

$$\text{Propionyl-CoA} \rightleftarrows \text{Acryloyl-CoA} + 2H \quad [1]$$

$$\text{Acryloyl-CoA} + H_2O \rightleftarrows \text{3-Hydroxypropionyl-CoA} \quad [2]$$

$$\text{3-Hydroxypropionyl-CoA} + H_2O \rightleftarrows \text{3-Hydroxypropionate} + \text{CoA} \quad [3]$$

$$\text{3-Hydroxypropionate} + \text{NAD} \rightleftarrows \text{Malonic semialdehyde} + \text{NADH} + H^+ \quad [4]$$

$$\text{Malonic semialdehyde} + \text{CoA} \rightleftarrows\rightleftarrows \text{Acetyl-CoA} + CO_2 \quad [5]$$

Mammalian crystalline enoyl-CoA hydratase (crotonase, EC 4.2.1.17) catalyzes reaction [2] (17). 3-Hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4) catalyzes reaction [3], releasing 3-hydroxypropionate. Conversion to malonic semialdehyde in reaction [4] is catalyzed by a specific enzyme, 3-hydroxypropionate dehydrogenase (EC 1.1.1.59) (18). Evidence is sufficient for the existence of the pathway in animal systems. However, its significance has not been studied nor has its existence in man. In the presence of an accumulation of propionic acid or propionyl-CoA, it would also be possible for hydroxypropionate to arise by direct ω-oxidation and hydroxylation. ω-Oxidation of short-chain fatty acids has been reported (19).

The patients in this study all had a metabolic block in the major pathway of propionate oxidation. In previous studies on these patients, [*1*-^{14}C]propionate was found to be quite inefficiently oxidized to respiratory CO_2 (9). Assay for propionyl-CoA carboxylase indicated a complete defect in the activity of the enzyme in fibroblasts cultured from skin (9). On the other hand, the amounts of propionate accumulated in body fluids (14) were not so high, suggesting that there might be alternative metabolic pathways. It has been speculated that odd-numbered fatty acids might derive from propionyl-CoA (5, 20). Our investigation of the metabolic fate of propionate in these patients led to the discovery of methylcitrate (10) and propionylglycine (21) as products of propionate metabolism.

The conversion to 3-hydroxypropionate provides an additional answer to the question of the fate of propionate (Fig. 4) in these patients. L. G., the patient with methylmalonic acidemia, excreted larger amounts of labeled 3-hydroxypropionate after administration of [*1*-^{14}C]propionate than did V. B., in whom the defect in propionic acid metabolism was more proximal. However, the conversion of propionate to urinary hydroxypropionate in C. E., the other patient with propionic acidemia, was virtually the same as in L. G. The concentrations of propionate in the plasma of all three patients were quite similar. We have concluded that the degree of hydroxypropionaturia is proportional to the propionic acidemia in both conditions and that the orders of magnitude of propionic acidemia and hydroxypropionaturia were not significantly different in these three patients. The β-oxidation of propionate could be an important pathway for the metabolism of excess propionyl-CoA in these patients. In the patients studied, hydroxypropionate and methylcitrate were the major products of propionate found. The amounts of either compound excreted in the urine represent minimal estimates of the amounts formed or the quantitative importance of the pathways, since these compounds may be further metabolized. Aconitase can catalyze the conversion of methylcitrate to methylisocitrate (22). On the other hand, the formation of propionylglycine seems to be a quantitatively less significant pathway (21). It is possible that propionyl-CoA may be used for the formation of even-numbered fatty acids through its β-oxidation pathway, as well as for the synthesis of odd-numbered fatty acids through direct use of propionyl-CoA (23). The quantitative significance of conversions to hydroxypropionate and methylcitrate can be appreciated by consideration of the formation of CO_2 from these patients during the study (9). In control individuals, as much as 50% of the isotope administered may be recovered in $^{14}CO_2$ within 2 hr after administration of tracer. In contrast, the conversion to $^{14}CO_2$ in patients with propionic acidemia was about 6%, while conversions to hydroxypropionate were about 1%.

These studies of the metabolism of propionate serve to define the abnormal chemical environment in which the cells of these patients must develop, and could thus contribute to an understanding of the pathogenesis of the clinical manifestations observed. The use of alternate pathways and secondary metabolic disturbances in these patients is complex. We have previously reported the excretion of tiglic acid in C. E. and in another patient and its absence in V. B. (24). Propionylglycine was also found in C. E. but not in V. B. These findings could reflect genetic heterogeneity in

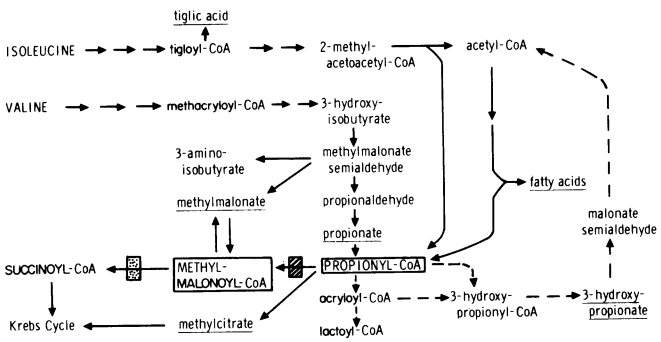

FIG. 4. Metabolic pathways relevant to propionic acidemia and methylmalonic acidemia. The *vertical, cross-hatched* bars indicate the metabolic defects in propionyl-CoA carboxylase in patients with propionic acidemia, and the *vertical stippled bar*, the defect in methylmalonyl-CoA mutase in methylmalonic acidemia. The *underlined compounds* have been found in body fluids of patients with these conditions.

these conditions. For instance, V. B. has been reported to be biotin-responsive (25). However, after biotin treatment, the plasma concentrations of propionate in V. B. were reduced to a level similar to those found in C. E. (25). Biotin deficiency could also be a secondary phenomenon in V. B. It is alternatively possible that the excretion of tiglic acid and of 3-hydroxypropionate are related. Certainly considerably more labeled hydroxypropionate was found in C. E. We have speculated that acryloyl-CoA might compete with tiglyoyl-CoA for enoyl hydratase (24). In this way a primary accumulation of propionyl-CoA and its β-oxidation could lead to accumulation of tigloyl-CoA and consequent accumulation and excretion of tiglic acid. The absence of tiglic acid in the urine of V. B. could reflect a smaller pool of hydroxypropionyl-CoA and acryloyl-CoA or possibly a greater avidity of his enzyme for tiglyoyl-CoA.

The formation and excretion of 3-hydroxypropionate after administration of [*U*-^{14}C]valine and [*U*-^{14}C]isoleucine is of some interest in the intermediary metabolism of these compounds in man. Isoleucine is generally recognized to be a precursor of propionyl-CoA. However, the conversion of valine to 3-hydroxypropionate provides the first evidence that valine is metabolized to propionyl-CoA in man. Methylmalonic semialdehyde, which is formed from β-hydroxyisobutyrate during valine catabolism, may be converted to propionyl-CoA in microoragnisms, but it has been generally thought to be converted directly to methylmalonic acid and methylmalonoyl-CoA in animals (26). Our data suggest that the pathway is from methylmalonic semialdehyde to propionyl-CoA to methylmalonoyl-CoA. This is consistent with the labeling pattern of liver glycogen after administration of labeled valine to rats, which indicated the participation of a 3-carbon intermediate (27); and the isolation of labeled propionic acid after incubation of rat-liver homogenate with labeled valine (28).

It is proposed that this is the major pathway for the degradation of valine. It is also possible that the data could reflect reversibility of the reaction from propionyl-CoA to methylmalonoyl-CoA. However, the fact that valine was toxic in a patient with propionic acidemia, presumably due to deficiency of propionyl-CoA carboxylase (2, 29), supports these considerations. If methylmalonic semialdehyde were metabolized directly to methylmalonate, the degradation of valine should proceed normally in such a patient and, therefore, should not be toxic. Furthermore, the ratio of urinary [^{14}C]-3-hydroxypropionate to methylmalonate was 3.4% after [*U*-^{14}C]valine. These values also indicate that valine was converted to methylmalonate by way of propionyl-CoA as in the case of isoleucine.

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