

Metabolism of [1-(14)C] and [2-(14)C] leucine in cultured skin fibroblasts from patients with isovaleric acidemia. Characterization of metabolic defects.

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Research Article

Leucine metabolism in cultured skin fibroblasts from patients with isovaleric acidemia was compared with that in normal fibroblasts and in cells from patients with maple syrup urine disease using [1-(14)C] and [2-(14)C] leucine as substrates. Inhibitory effects of methylenecyclopropylacetic acid on leucine metabolism in normal cells were also investigated. Production of $^{14}\text{CO}_2$ from [2-(14)C] leucine was very reduced (96-99%) in both types of mutant cells. Radioactive isovaleric acid accumulated in assay media with isovaleric acidemia cells but not in those with maple syrup urine disease cells. Unexpectedly, $^{14}\text{CO}_2$ production from [1-(14)C] leucine was partially depressed (80%) in isovaleric acidemia cells whereas in maple syrup urine disease cells it was strongly depressed (99%) as expected. These two mutant cells were clearly distinguished by detection of ^{14}C -isovaleric acid accumulation after incubation with [2-(14)C] leucine. A pattern of inhibition of leucine oxidation similar to that seen in isovaleric acidemia cells was induced in normal cells by the addition of 0.7 mM methylenecyclopropylacetic acid to the assay medium. The partial inhibition of [1-(14)C] leucine oxidation seen in isovaleric acidemia cells and also in normal cells in the presence of the inhibitor appears to be, at least in part, due to an accumulation of isovalerate in the cells. Isovaleric acid (5-10 mM) inhibited [1-(14)C] leucine oxidation 32-68% when added to the assay medium with normal [...]

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Metabolism of [1-¹⁴C] and [2-¹⁴C]Leucine in Cultured Skin Fibroblasts from Patients with Isovaleric Acidemia

CHARACTERIZATION OF METABOLIC DEFECTS

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ABSTRACT Leucine metabolism in cultured skin fibroblasts from patients with isovaleric acidemia was compared with that in normal fibroblasts and in cells from patients with maple syrup urine disease using [1-¹⁴C] and [2-¹⁴C]leucine as substrates. Inhibitory effects of methylenecyclopropylacetic acid on leucine metabolism in normal cells were also investigated.

Production of ¹⁴CO₂ from [2-¹⁴C]leucine was very reduced (96–99%) in both types of mutant cells. Radioactive isovaleric acid accumulated in assay media with isovaleric acidemia cells but not in those with maple syrup urine disease cells.

Unexpectedly, ¹⁴CO₂ production from [1-¹⁴C]leucine was partially depressed (80%) in isovaleric acidemia cells whereas in maple syrup urine disease cells it was strongly depressed (99%) as expected. These two mutant cells were clearly distinguished by detection of ¹⁴C-isovaleric acid accumulation after incubation with [2-¹⁴C]leucine.

A pattern of inhibition of leucine oxidation similar to that seen in isovaleric acidemia cells was induced in normal cells by the addition of 0.7 mM methylenecyclopropylacetic acid to the assay medium. The partial inhibition of [1-¹⁴C]leucine oxidation seen in isovaleric acidemia cells and also in normal cells in the presence of the inhibitor appears to be, at least in part, due to an accumulation of isovalerate in the cells. Isovaleric acid (5–10 mM) inhibited [1-¹⁴C]leucine oxidation 32–68% when added to the assay medium with normal cells.

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Addition of flavin adenine dinucleoside to culture medium or assay medium or both did not restore oxidation of either leucine substrate in isovaleric acidemia cells.

INTRODUCTION

Isovaleric acidemia, an inborn error of leucine metabolism, is characterized clinically by bouts of recurrent ketoacidosis and coma which are accompanied by a peculiar offensive odor described like "sweaty feet" (1, 2). The severe acidotic attacks are frequently observed during the newborn period (2, 3) and often result in death (3, 4). At least 19 cases have been reported (5). The major biochemical features of this disease are continuous excretion of large amounts of isovaleryl glycine in urine (6) and episodic accumulation of serum isovaleric acid (1) and urinary β-hydroxyisovaleric acid (7) during the periods of acute ketoacidosis. From the identification of these abnormal metabolites and also from the inability of patient's leukocytes to oxidize [1-¹⁴C]isovaleric acid, it was assumed that isovaleric acidemia is due to a block in leucine metabolism at the step of isovaleryl coenzyme A (CoA)¹ dehydrogenation (1, 6). The altered metabolism of leucine in patients with this disease is illustrated in Fig. 1. Further biochemical studies on the nature of the enzyme defect have been hampered by a difficulty in the direct assay of isovaleryl CoA dehydrogenase activity in crude tissue preparations such as homogenates of liver and leukocytes. These difficulties were due to strong nonspecific reduction of

¹Abbreviations used in this paper: CoA, coenzyme A; FAD, flavin adenine dinucleoside; MCPA, methylenecyclopropyl acetic acid; MSUD, maple syrup urine disease.

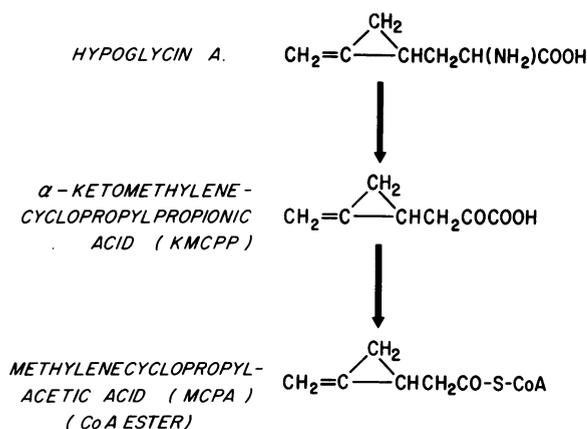


FIGURE 2 Structure and metabolism of hypoglycin A.

METHODS

Skin biopsies were obtained from three patients with isovaleric acidemia, two patients with classical MSUD, and six control subjects. Biopsies were also obtained from the mother of the isovaleric acidemic siblings and from the parents of a MSUD patient. Cells were also cultivated from amniotic fluids obtained from six normal pregnant women between the 14th and 16th wk of pregnancy. The two isovaleric acidemia patients (S. A. and B. A.), now 12 and 10 yr old, are the two siblings originally reported by Tanaka et al. (1, 7). The third patient (K. Fa.) is 5 yr old.

Fibroblasts were grown in Eagle's medium supplemented with 15% fetal calf serum and "non-essential" amino acids. When confluent, monolayers of fibroblasts were washed with phosphate buffered saline and trypsinized. Cells were suspended in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 3 mM glucose and radioactive substrate. Assays were done in two different sizes. Preliminary experiments and experiments on the effects of isovaleric acid and flavin adenine dinucleoside (FAD) were done in small scale using 5×10^5 cells in micro reaction vessels (Bolab, Inc., Derry, N. H.). The final volume of the assay medium was 50 μ l, and the amount of the radioactive substrate was 0.075 μ mol (sp act: 0.4 μ Ci/ μ mol). The major experiments were done using a 25-ml Erlenmeyer flask with a center well. In this system 2×10^7 cells were incubated in 2 ml of the medium which contained 1.5 μ mol (sp act: 0.2 μ Ci/ μ mol) of radioactive substrate. All of the incubations were done at 37° under 95% O₂-5% CO₂ atmosphere for 3 h unless otherwise mentioned. At the end of the incubation, the reaction was stopped by addition of 2 N H₂SO₄. Radioactive carbon dioxide was collected into 1 M hyamine hydroxide (0.3 ml) and counted by a liquid scintillation spectrometer. In the large volume experiments where [2-¹⁴C]leucine was used as substrate, the assay medium was analyzed for radioactive isovaleric acid or isovalerylglycine. In these experiments, unlabeled isovaleric acid (1 mg) or isovalerylglycine (1 mg) was injected as 0.2% solutions into the assay medium before the addition of H₂SO₄.

For the analysis of radioactive isovaleric acid, the assay medium was directly steam distilled according to the method previously described (1). $\frac{1}{2}$ ml of 0.1 N NaOH was added to the distillate and evaporated to dryness. The residue was redissolved in 20 μ l formic acid and then injected into a gas-liquid chromatographic column (6 foot \times 4 mm) which

was packed with 20% neopentylglycol adipate-2% phosphoric acid on silanized Anakrom (Analabs, North Haven, Conn.). The gas-liquid chromatographic equipment utilized was a Barber-Coleman Model 5000 (Barber-Colman Company, Rockford, Ill.) equipped with a hydrogen flame detector and a radioactivity monitoring system. The split ratio of gas-liquid chromatographic effluent was 5% to the hydrogen flame detector and 95% to the radioactivity monitoring system.

The analysis of radioactive isovalerylglycine was also done by radio-gas-liquid chromatographic. The preparation of samples for the analyses was done by the method previously described (8).

D,L-[1-¹⁴C] and D,L-[2-¹⁴C]leucine were obtained from New England Nuclear (Boston, Mass.) and ICN (ICN Chemical & Radioisotope Division, Waltham, Mass.), respectively. Methylene cyclopropylacetic acid⁸ was prepared from hypoglycin A by oxidative deamination with snake venom L-amino acid oxidase (13). Hypoglycin A was the gift of Dr. E. C. DeRenzo, Lederle Laboratories, Pearl River, N. Y.

RESULTS

Oxidation of D,L-[1-¹⁴C] and D,L-[2-¹⁴C]leucine to carbon dioxide. Production of ¹⁴CO₂ from D,L-[1-¹⁴C]leucine by normal fibroblasts was linear for 3 h as shown in Fig. 3. The ability of fibroblasts to oxidize [1-¹⁴C]leucine was saturated at 1.5 mM of the substrate as shown in Fig. 4.

Production of ¹⁴CO₂ by normal fibroblasts ranged from 1,245 to 3,887 pmol/10⁶ cells/h with the mean value of 2,143 pmol/10⁶ cells/h (Table I). When [2-¹⁴C]leucine was the substrate, ¹⁴CO₂ produced was from 234 to 868 pmol/10⁶ cells/h with the mean value of 453 pmol/10⁶ cells/h. The amount and the specific activity of [2-¹⁴C]leucine used as substrate was identical to those for [1-¹⁴C]leucine.

Radioactive CO₂ evolves from the second step of [1-¹⁴C]leucine degradation whereas breakdown of [2-¹⁴C]leucine requires twelve steps before ¹⁴CO₂ is produced. This probably accounts for the difference in the amounts of ¹⁴CO₂ produced from the two differently labeled leucines.

The oxidation of [2-¹⁴C]leucine by isovaleric acidemia cells was strongly reduced measuring 0.3-1.5% of the control values as shown in Table I. These data were compatible with the previous hypothesis that this disease is due to a deficiency of isovaleryl CoA dehydrogenase activity. Unexpectedly, however, oxidation of [1-¹⁴C]leucine by isovaleric acidemia cells was 17-24% of the mean control value. Oxidation of both substrates

⁸ The purity of methylenecyclopropylacetic acid was 77%. Isovaleric acid was the sole contaminant. Isovaleric acid is produced from leucine which was present in the hypoglycin A sample. The chromatographic behavior of hypoglycin A is very similar to that of leucine in many chromatographic systems, and its further purification was not possible. However, the amount of isovaleric acid added in the assay with 0.7 mM MCPA was 0.16 mM, and did not have a significant effect on leucine oxidation as shown in Table IV.

by MSUD cells was extremely low as expected, being less than 1.3% and 3.7% of mean control values for [1-¹⁴C] and [2-¹⁴C]leucine, respectively. Therefore, isovaleric acidemia cells can be clearly distinguished from MSUD cells by the difference in ability to oxidize [1-¹⁴C]leucine.

Due to the unexpected observation of a low oxidation of [1-¹⁴C]leucine in isovaleric acidemia cells, the radioactivity of [1-¹⁴C]leucine used was checked in our laboratory. More than 98% of the radioactivity migrated with leucine on high voltage paper electrophoresis using 6% formic acid as a buffer. 99% of the radioactivity was lost after ninhydrin treatment, indicating that the ¹⁴C is, in fact, located at carbon 1 of leucine (14). Thus, we confirmed that the [1-¹⁴C]leucine was radiochemically pure.

The ability to oxidize both substrates by cells from an obligate heterozygote for isovaleric acidemia was lower than the lowest control and was about 40% of the mean control value (Table I). The ability to oxidize [1-¹⁴C]leucine by cells from the parents of a MSUD patient was about 20% of the mean control value. Oxidation of [2-¹⁴C]leucine by these cells was not measured.

Six cell lines cultured from normal amniotic fluids actively oxidized both substrates. They produced ¹⁴CO₂ in the amounts of 1074±91 SD and 227±77 SD pmol/10⁶ cells/h from [1-¹⁴C] and [2-¹⁴C]leucine, respectively. These were about a half of the mean value for normal skin fibroblasts.

Inhibition by MCPA of [1-¹⁴C] and [2-¹⁴C]leucine oxidation in normal fibroblasts. MCPA, at the concentration of 0.7 mM, inhibited [2-¹⁴C]leucine oxidation in

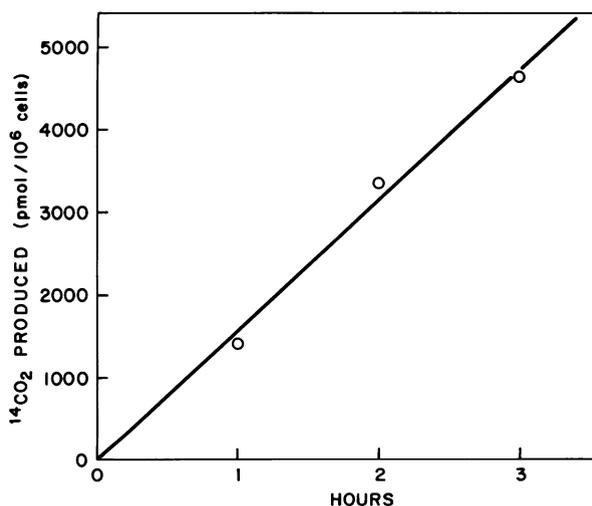


FIGURE 3 Time course of [1-¹⁴C]leucine oxidation in normal fibroblasts. Experiments were done in duplicate and the assays were done in microvessels. The conditions are described in the text.

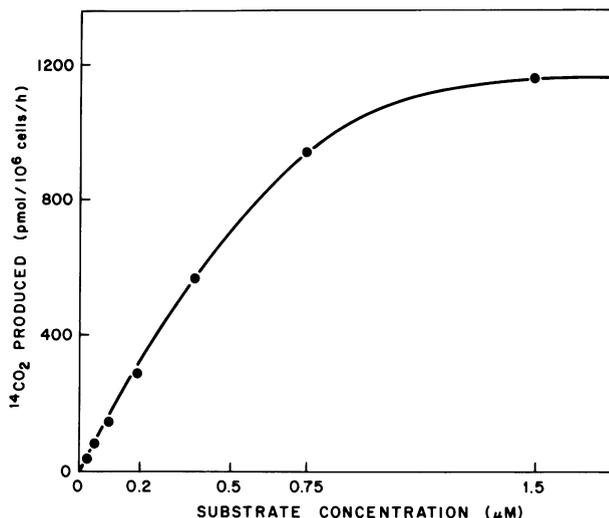


FIGURE 4 ¹⁴CO₂ production from [1-¹⁴C]leucine in normal fibroblasts at different substrate concentrations. Assays were done in microvessels. The specific activity of [1-¹⁴C]leucine was 0.4 μCi/μmol. Other conditions for the assay are described in the text.

normal cells by 94–97%. It also inhibited ¹⁴CO₂ production from [1-¹⁴C]leucine, but the inhibition was about 55–65% as shown in Table II. The pattern of inhibition of leucine oxidation in normal cells in the presence of MCPA is similar to the situation observed with isovaleric acidemia cells (Table I).

Analyses of radioactive isovaleric acid and isovaleryl-glycine in assay media with [2-¹⁴C]leucine as substrate. The inability of MSUD cells to oxidize either [1-¹⁴C] or [2-¹⁴C]leucine is consistent with the enzyme defect of MSUD being located at the oxidative decarboxylation step (Fig. 1). However, the pattern of decreased ¹⁴CO₂ production from [2-¹⁴C]leucine seen in isovaleric acidemia cells may be explained by a metabolic block at any step after the oxidative decarboxylation. Therefore, after incubating cells with [2-¹⁴C]leucine, the assay medium was analyzed for accumulated radioactive intermediates to locate the metabolic block in isovaleric acidemia cells more specifically.

First, the assay medium was analyzed for radioactive organic acids including isovaleryl-glycine and β-methylcrotonyl-glycine. Glycine (3 mM) was added to the assay medium in this experiment since the addition of glycine greatly enhanced the isovaleryl-glycine formation from leucine by rat liver slices in the presence of α-ketomethylenecyclopropylpropionic acid (8, 10). The addition of glycine did not alter ¹⁴CO₂ production from [2-¹⁴C]leucine by isovaleric acidemia cells nor by normal cells in the presence or absence of MCPA. Neither radioactive isovaleryl-glycine nor β-methylcrotonyl-glycine was detected in significant amounts in the assay media

TABLE I
Radioactive Carbon Dioxide Production from [1-¹⁴C] and [2-¹⁴C]leucine by Cultured Fibroblasts of Patients with Isovaleric Acidemia, MSUD, and Control

	Substrates	
	[1- ¹⁴ C]leucine	[2- ¹⁴ C]leucine
	<i>pmol/10⁶ cells/h</i>	
Control (6)	2,143±870	453±199
Isovaleric acidemia		
Homozygotes		
B. A.	362	5
S. A.	412	7
K. Fa.	516	1
Heterozygote		
Mrs. A.	879	177
MSUD		
Homozygotes		
C. C.	3	17
S. Fo.	27	12
Heterozygotes		
Mr. Fo.	408	—*
Mrs. Fo.	439	—*

A part of the data on [2-¹⁴C]leucine oxidation has been published in a preliminary communication (12).

Values represent mean±SD. Number of cell lines tested is presented in parenthesis. All experiments were done in duplicate.

Assays were done in 25-ml center well flasks. For experimental details, see text.

* Not measured.

with isovaleric acidemia cells or in those with normal cells with or without MCPA. Therefore, we analyzed the assay media for radioactive short chain fatty acids.

In the assay media with normal cells there was a small, ambiguous radioactive peak which appeared in the region of isovaleric acid (Fig. 5, right). In contrast, a high radioactive isovaleric acid peak was detected in the assay media with isovaleric acidemia cells (Fig. 5, left) and also in that with normal cells in the presence of MCPA (Fig. 6). The amount of radioactivity was calculated as a ratio of the area of the radioactive peak over that of the peak by mass detection since the same amount of nonlabeled isovaleric acid was added as a carrier to each medium after incubation. The amount of radioactive isovaleric acid was at least significantly higher in the assay media with isovaleric acidemia cells than in the control cells. The amount of radioactive isovaleric acid was also high in the assay medium when normal cells were incubated with MCPA (Table III). It was very low in an assay medium with MSUD cells. No radioactive peak was detected in the region of β -methylcrotonic acid in the assay media with isovaleric

acidemia cells nor with normal cells in the presence of MCPA.

Inhibition of ¹⁴CO₂ production from [1-¹⁴C]leucine by isovaleric acid. One of the possible reasons for the partial depression of [1-¹⁴C]leucine oxidation in isovaleric acidemia cells is that oxidative decarboxylation of α -ketoisocaproic acid is secondarily inhibited by the accumulated product, isovalerate. Therefore, the effect of isovaleric acid on ¹⁴CO₂ production from [1-¹⁴C]leucine by normal fibroblasts was tested. Isovaleric acid inhibited the activity to 67% at 5 mM and to 32% at 10 mM (Table IV).

Effect of FAD on leucine oxidation by cultured fibroblasts. The data presented thus far are compatible with the concept that isovaleric acidemia cells have a deficiency of isovaleryl CoA dehydrogenase activity. Although this enzyme has not been characterized, its function is similar to that of green acyl CoA dehydrogenase (15). Substrates for these two enzymes, namely isovaleryl CoA and *n*-butyryl CoA, respectively, differ only in a methyl substitution. It may be assumed, therefore, that the prosthetic group of isovaleryl CoA dehydrogenase is similar to that of green acyl CoA dehydrogenase, i.e., flavin. Therefore, the effect of FAD addition to the medium was tested. FAD was added either in the culture medium for five days before harvesting, or in the assay medium, or both. The concentration of riboflavin in normal serum-supplemented tissue culture medium is 0.3 μ M. Normal cells cultivated with 0.12 mM FAD had an activity of [1-¹⁴C]leucine oxidation twice as high as those cultured in the conventional culture medium although cell growth was inhibited by this concentration of FAD. The addition of the same amount of FAD in the assay medium did not increase leucine oxidation nor did the addition of FAD to both the culture and assay media in an amount of 0.012 mM.

The ¹⁴CO₂ production from [1-¹⁴C] and [2-¹⁴C]leucine

TABLE II
Inhibitory Effect of MCPA on ¹⁴CO₂ Production from [1-¹⁴C] and [2-¹⁴C]Leucine by Normal Fibroblasts and Normal Amniotic Fluid Cells

Cell lines	MCPA added	Substrates	
		[1- ¹⁴ C]-leucine	[2- ¹⁴ C]-leucine
		<i>pmol/10⁶ cells/h</i>	
Skin fibroblasts	0	1,232	275
	0.7	426	17
Amniotic fluid cells	0	956	167
	0.7	423	5

Incubations were done in 25-ml center well flasks. For experimental details, see text.

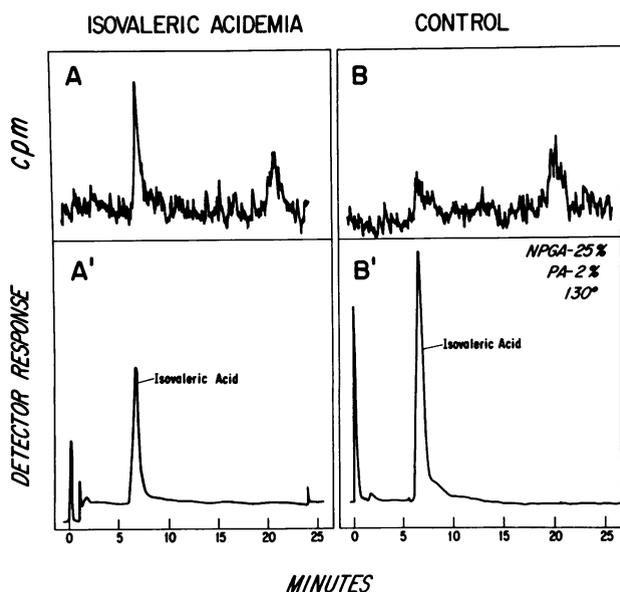


FIGURE 5 Radio-gaschromatogram of short chain fatty acids from assay media with $[2-^{14}\text{C}]$ leucine. The upper curves (A and B) are by radioactivity detection. The lower curves (A' and B') are by mass detection. 1 mg of carrier isovaleric acid was added to each sample. The curves on the left hand side (A and A') are from a patient with isovaleric acidemia and those on the right hand side (B and B') are from a control.

by isovaleric acidemia cells was not augmented to any degree by the addition of FAD in either fashion (Table V).

Oxidation of $[1,4-^{14}\text{C}]$ succinic acid and $[1-^{14}\text{C}]$ valine to $^{14}\text{CO}_2$. Succinate oxidation by an isovaleric acidemia cell line (B.A.) was essentially the same as that of the two control cells lines. Therefore, it may be concluded that the decreased $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ and $[2-^{14}\text{C}]$ leucine is not due to a decreased viability of the

TABLE III
Amount of Radioactive Isovaleric Acid Accumulated in Assay Media after Incubation with $[2-^{14}\text{C}]$ Leucine

Cell lines	Radioactive isovaleric acid produced*
Control (skin fibroblasts) (2)	0.15 (0.17, 0.13)
Control (amniotic fluid cells) (3)	0.12 (0, 0.06, 0.30)
Control (amniotic fluid cells) + MCPA‡ (1)	0.98
Isovaleric acidemia (3)	1.17 (0.76, 0.96, 1.80)
MSUD (1)	<0.1

Numbers of cell lines tested are presented in parentheses in cell line column. * The amount of radioactive isovaleric acid is expressed as the ratio of area of radioactive peak per peak area by mass detection. The radioactivity was detected at a sensitivity of 300 cpm full scale. Mass detection was done with a hydrogen flame detector at a sensitivity of 10^{-9} ampere full scale. 1 mg of carrier isovaleric acid was added to all samples.

‡ 0.7 mM.

cells. Production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ valine by the isovaleric acidemia cells was about 45% that of a control (Table VI).

DISCUSSION

The very low $^{14}\text{CO}_2$ production from $[2-^{14}\text{C}]$ leucine by isovaleric acidemia cells and the accumulation of radioactive isovaleric acid in the assay media together support our previous concept that isovaleric acidemia is due to a deficiency of isovaleryl CoA dehydrogenase activity (1, 6). The partial depression of $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ leucine by isovaleric acidemia cells was unexpected. However, the $[1-^{14}\text{C}]$ leucine oxidation was about 20% of the mean control value and was still much higher than that by classical MSUD cells. Radioactive isovaleric acid does not accumulate in the assay medium when MSUD cells are incubated with $[2-^{14}\text{C}]$ leucine. Isovaleric acidemia cells may be readily distinguished by these measurements. We did not have an opportunity to apply the present methods to cell lines from patients with β -methylcrotonyl CoA carboxylase deficiency (16), another inborn error of leucine metabolism at the step

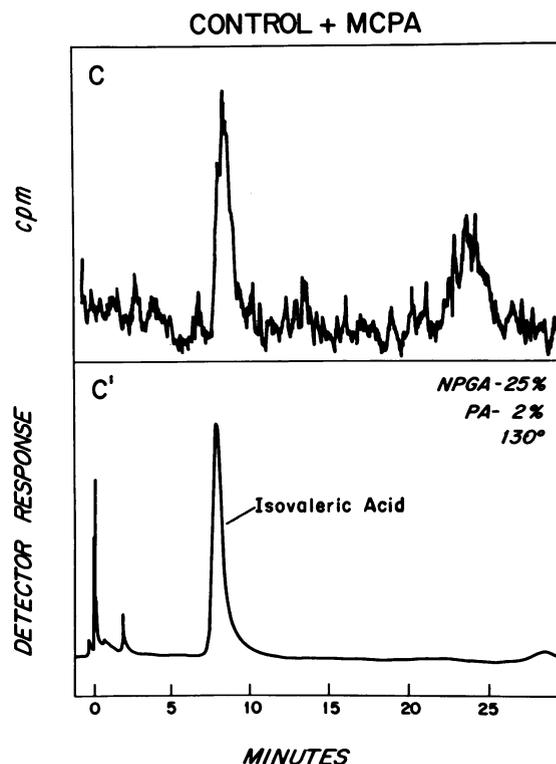


FIGURE 6 Radio-gaschromatogram of short chain fatty acids from assay medium of normal fibroblasts incubated with $[2-^{14}\text{C}]$ leucine in the presence of MCPA. The upper curve (C) is by radioactivity detection and the lower curve (C') by mass detection. The concentration of MCPA was 0.7 mM. 1 mg of carrier isovaleric acid was added.

TABLE IV
Effect of Isovaleric Acid on $^{14}\text{CO}_2$ Production from
[1- ^{14}C]Leucine by Normal Fibroblasts

Isovaleric acid added		$^{14}\text{CO}_2$ produced	
mM	$\mu\text{mol}/10^6$ cells/h	% Activity	
0	1,530	100	
0.1	1,440	94	
0.5	1,260	82	
1.0	1,140	75	
5.0	1,030	67	
10.0	490	32	

Data are mean of duplicates. Differences between duplicates were less than 5%. Assays were done in microvessels.

immediately distal to isovaleryl CoA dehydrogenase. The patterns of $^{14}\text{CO}_2$ production by these mutant cells from [1- ^{14}C] and [2- ^{14}C]leucine may be similar to those observed with isovaleric acidemia cells. Theoretically, however, radioactive β -hydroxyisovaleric or β -methylcrotonic acids would accumulate predominantly in the assay medium when β -methylcrotonyl CoA carboxylase deficient cells are incubated with [2- ^{14}C]leucine. The usefulness of these methods to distinguish isovaleric acidemia cells from β -methylcrotonyl CoA carboxylase deficient cells must be tested in the future.

An abnormality in leucine metabolism similar to that seen in isovaleric acidemia cells was induced in normal fibroblasts by the addition of MCPA, a toxic metabolite of hypoglycin A. The findings indicate that hypoglycin A and its metabolites inhibit isovaleryl CoA dehydrogenase as has been shown in rats (8, 10), and that acquired isovaleric acidemia may be observed in patients with the Jamaican vomiting sickness (9, 10).

TABLE V
Effect of Flavin Adenine Dinucleoside on $^{14}\text{CO}_2$ Production
from Radioactive Leucine by Isovaleric Acidemia Cells

FAD added		$^{14}\text{CO}_2$ production	
Culture medium*	Assay medium	[1- ^{14}C]-leucine	[2- ^{14}C]-leucine
mM		$\mu\text{mol}/10^6$ cells/h	
0	0	552	17
0	0.12	443	12
0.12	0	624	5
0.012	0.012	624	5
0.12	0.12	751	5

The experiments were done in duplicate using microvessels.
* Cells were grown in the incubation medium with FAD for 5 days before harvesting.

TABLE VI
Oxidation of [1,4- ^{14}C]Succinate and [1- ^{14}C]Valine to $^{14}\text{CO}_2$ by
Isovaleric Acidemia Fibroblasts and Normal Fibroblasts

Cell lines	Substrates	
	[1,4- ^{14}C]-succinate*	[1- ^{14}C]-valine†
	$\mu\text{mol}/10^6$ cells/h	
Normal fibroblasts A	2,000	—§
Normal fibroblasts B	2,000	1,070
Isovaleric acidemia fibroblasts (B. A.)	1,630	494

These experiments were done in duplicate using microvessels.

* Final concentration, 1.4 mM.

† Final concentration, 3.4 mM.

§ Not measured.

The inhibition of $^{14}\text{CO}_2$ production from [1- ^{14}C]leucine observed in experiments with isovaleric acidemia cells is of special interest in relation to the primary enzyme deficiency and its effects on metabolic regulation in patients with this disease. The inhibition of $^{14}\text{CO}_2$ production from [1- ^{14}C]leucine does not appear to be due to dual deficiency of two successive enzymes as in the case of orotic acidemia (17) for the following reasons. First, the degree of depression of [1- ^{14}C]leucine oxidation is not nearly so severe as that of [2- ^{14}C]leucine oxidation in the variant cells. Secondly, the pattern and degrees of inhibition of the oxidation of these two substrates induced by MCPA were remarkably similar to those observed with isovaleric acidemia cells. Thus it is more likely that the inhibition of [1- ^{14}C]leucine oxidation is secondarily caused by a metabolic effect such as accumulated intermediate(s) which increase because of the primary enzyme defect. This hypothesis is supported by the findings of inhibited $^{14}\text{CO}_2$ production from [1- ^{14}C]leucine by isovaleric acid. These data indicate that the intracellular accumulation of isovalerate may be responsible, at least in part, for the partial inhibition of [1- ^{14}C]leucine oxidation in isovaleric acidemia cells. It must be pointed out that whole cells were used in these experiments and the effective intracellular concentrations of isovalerate are not known. However, serum isovaleric acid accumulates to a level as high as 3 mM during ketoacidotic episodes in patients with isovaleric acidemia (1, 2), a concentration in the same order of magnitude that as that used in our experiments. It is also possible that isovalerate exerts an inhibitory effect in the in vitro assay only after it is converted to isovaleryl CoA. Therefore, the effect of isovalerate on oxidative decarboxylation must be tested using [1- ^{14}C]- α -ketoisocaproic acid as a substrate and isovaleric acid or isovaleryl CoA as inhibitor in a cell free system to draw a definitive conclusion.

Oxidation of other substrates, [1,4-¹⁴C]succinate, and [1-¹⁴C]valine, were studied in an isovaleric acidemia cell line as well as in two controls. The rate of succinate oxidation by isovaleric acidemia cells was in the same range as that of the controls. Therefore, general metabolic activity of the isovaleric acidemia cell line appears to be normal. In contrast, ¹⁴CO₂ production from [1-¹⁴C]-valine by isovaleric acidemia cells was about 50% of a control. Whether this is due to a cross inhibition of α-ketoisovalerate decarboxylation by accumulated metabolites remains to be determined.

Radioactive carbon dioxide from [2-¹⁴C]leucine by cells from an obligate heterozygote for isovaleric acidemia was about 40% of the mean control value. Intermediate values of ¹⁴CO₂ production from [1-¹⁴C]leucine were likewise observed in two cell lines heterozygous for MSUD as has been previously reported (18, 19). Langenbeck et al. commented that due to a large variation of data, diagnosis of the heterozygous state of MSUD was not possible when cultured fibroblasts were incubated using branched-chain α-keto acids as substrates (20). The possibility of using these methods with [¹⁴C]-leucine as substrate for the detection of heterozygotes of these inborn metabolic diseases must be determined by much larger numbers of assays on heterozygous cells and controls. Even with much more data, it is likely that there will be considerable overlap of heterozygous and control values using an assay that measures CO₂ production.

Vitamin responsiveness has never been tested clinically in cases of isovaleric acidemia because the biochemical characteristics of isovaleryl CoA dehydrogenase are currently unknown. However, the type of substrate and the nature of the reaction are analogous to those of green acyl CoA dehydrogenase, a flavin enzyme (15, 21). Therefore, we examined the effect of FAD on leucine oxidation by isovaleric acidemia cells. Oxidation of neither [1-¹⁴C] nor [2-¹⁴C]leucine was restored by the addition of FAD either in culture medium or assay medium.

Investigation on the accumulated abnormal metabolites in the assay medium resulted in the identification of free isovaleric acid rather than isovalerylglycine as the product from [2-¹⁴C]leucine in isovaleric acidemia fibroblasts, and also in normal fibroblasts in the presence of MCPA. This was in contrast to the previous findings that the major abnormal metabolite from leucine in vivo in patients with isovaleric acidemia (6) and also in hypoglycin A treated rats (13) was isovalerylglycine, and that the product from [2-¹⁴C]leucine by rat liver slices in vitro in the presence of α-ketomethylenecyclopropylpropionic acid was also isovalerylglycine (8). These observations would be explained if cultured fibroblasts lack glycine-N-acylase. This enzyme is known to be

present in liver and kidney (22) but its activity has not been tested in fibroblasts.

The procedures described in this paper may be utilized clinically for prenatal diagnosis of isovaleric acidemia. A preliminary report on this subject, has been published (12). The ability of normal amniotic fluid cells to oxidize both substrates were about 50% of normal skin fibroblasts. One of the cell lines that was used for the study of MCPA inhibition was derived from amniotic fluid. Therefore, it is quite likely that amniotic fluid cells from an affected fetus would show the same defect of leucine metabolism as that detected in skin fibroblasts from isovaleric acidemia patients.

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