Proc. Natl. Acad. Sci. USA
Vol. 77, No. 1, pp. 580–583, January 1980
Medical Sciences

Demonstration of a specific mitochondrial isovaleryl-CoA dehydrogenase deficiency in fibroblasts from patients with isovaleric acidemia

(isovaleric acid/butyryl-CoA dehydrogenase/leucine/tritium release assay/mitochondria)

WILLIAM J. RHEAD* AND KAY TANAKA†

Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

Communicated by Kurt J. Isselbacher, October 4, 1979

ABSTRACT **To study the enzymatic basis of isovaleric acidemia, we have developed assay methods for isovaleryl-CoA and butyryl-CoA dehydrogenases that measure the amount of tritium released from the respective [2,3-^{3}H]acyl CoAs. Because assay of these enzymes in human fibroblast homogenates was subject to interference by nonspecific reactions, we have isolated mitochondria from cultured skin fibroblasts by protease treatment, homogenization, and differential centrifugation. By using this assay method with these isolated mitochondria, we have demonstrated a specific deficiency of isovaleryl-CoA dehydrogenase [isovaleryl-CoA: (acceptor) oxidoreductase, EC 1.3.99.10] activity in cultured skin fibroblasts from five patients with isovaleric acidemia. In contrast, mitochondrial butyryl-CoA dehydrogenase [butyryl-CoA: (acceptor) oxidoreductase, EC 1.3.99.2] activity in these cells was preserved at normal levels. These results have been reproduced by using the conventional dye reduction assays. These observations give further support to the hypothesis that isovaleryl CoA is dehydrogenated by a specific enzyme and that isovaleric acidemia is due to a deficiency of this enzyme.**

Isovaleric acidemia, first described in 1966 (1), is an inborn error of leucine catabolism that results in the accumulation of isovaleric acid and its derivatives in the serum and urine of affected patients, leading to episodic vomiting, lethargy, coma, and ketoacidosis (2). Aside from isovaleric acid and its derivatives, no other metabolites of the branched chain amino acids or short chain fatty acids such as *n*-butyrate, 2-methylbutyrate, or isobutyrate accumulate in the body fluids of these patients (1–3). These *in vivo* observations were subsequently verified *in vitro* when both leukocytes and fibroblasts from individuals with isovaleric acidemia were unable to oxidize [1-^{14}C]isovaleric acid and [2-^{14}C]leucine, respectively (1, 4). Thus, it appeared that isovaleric acidemia was due to a specific deficiency of the dehydrogenation of isovaleryl CoA and was unaccompanied by the accumulation of other metabolites that were dehydrogenated in an analogous manner. Butyryl-CoA dehydrogenase [butyryl-CoA: (acceptor) oxidoreductase, EC 1.3.99.2], also called the green or short chain acyl-CoA dehydrogenase, had previously been viewed as the enzyme responsible for the oxidation of the branched chain fatty acids derived from leucine, valine, and isoleucine, as well as the straight chain fatty acids with four to six carbons, although the evidence for isovaleryl CoA dehydrogenation by this enzyme was based on scanty experimental data (5). The identification of isovaleric acidemia and its characterization cast doubt on the validity of this interpretation and suggested that a substrate-specific isovaleryl-CoA dehydrogenase [isovaleryl-CoA: (acceptor) oxidoreductase, EC 1.3.99.10] did exist (1, 3). However, several attempts in this laboratory to demonstrate a specific deficiency of isovaleryl-CoA dehydrogenase activity in tissues and fibroblasts from patients with isovaleric acidemia met with difficulty, as did those by others to separate isovaleryl-CoA dehydrogenase physically from butyryl-CoA dehydrogenase (6). The failure of these efforts was due, in large part, to the lack of sensitivity and specificity inherent in the dye reduction methods used for the assay of these activities (7).

We have recently developed a more sensitive assay method for isovaleryl-CoA dehydrogenase and butyryl-CoA dehydrogenase that utilizes [2,3-^{3}H]acyl CoAs as substrates to assay these activities in cultured skin fibroblasts from patients with isovaleric acidemia. Activities of these enzymes are determined by measuring the amount of tritium released into the assay media. We report in this communication the demonstration of a specific deficiency of isovaleryl-CoA dehydrogenase in mitochondria isolated from fibroblasts derived from patients with isovaleric acidemia by using these methods.

MATERIALS AND METHODS

Materials. [2,3-^{3}H]Isovaleric acid (10 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and [2,3-^{3}H]butyric acid (10 mCi/mmol) were synthesized by New England Nuclear via catalytic tritiation of 3-methylcrotonic and crotonic acids, respectively, and their purity was confirmed by paper chromatography. CoA esters of the free acids were synthesized by the mixed anhydride method (8) in our laboratory. The tritiated acyl CoAs were found to be 89–98% pure by using paper chromatography. Pure pig liver electron transferring flavoprotein was a gift from Carole L. Hall, Georgia Institute of Technology, Atlanta, GA.

Fibroblasts were cultured from skin biopsies of five patients with isovaleric acidemia and four normal individuals and grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, glutamine, and nonessential amino acids in 750-cm^2 glass roller bottles. They were subcultured (1:4) every 2–3 weeks.

Preparation of Human Fibroblast Mitochondria. The methods of Millis and Pious (9) and Danner *et al.* (10) were modified by increasing the length of exposure to protease and vigor of homogenization, as well as the centrifugal forces used in differential centrifugation. For each cell line, two to three roller bottles of confluent fibroblasts were washed with phosphate-buffered saline and harvested with 0.25% trypsin (5 min, 37°C). The cells from each roller bottle were collected in 40 ml of MTE buffer (270 mM mannitol/10 mM Tris·HCl, pH

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Division of Medical Genetics, Department of Pediatrics, University of Iowa Hospital and Clinics, Iowa City, IA 52241.

† To whom reprint requests should be addressed.

7.4/0.1 mM EDTA) and centrifuged at 1100 × *g* for 5 min. The cell pellets of each cell line were then combined, recentrifuged, and thoroughly resuspended in buffer (400 mg of wet weight per 10 ml of MTE). Protease VI (Sigma) was added at 2.5 μg per 400 mg of wet weight and the suspension was mixed thoroughly by inversion and incubated at 0°C for 9 min. The cell suspension was then homogenized with 15 passes of a 40-ml glass/Teflon grinder and the volume was doubled with MTE buffer. The homogenate was then centrifuged at 700 × *g* for 10 min. The 700 × *g* supernatant was centrifuged at 15,000 × *g* for 10 min. The 15,000 × *g* pellet (mitochondria plus lysosomes) was resuspended in 0.5 ml of MTE containing 0.4 mg of digitonin (final concentration of digitonin was approximately 0.5 mg/ml) and incubated at 0°C for 20 min. The volume was then doubled with MTE and the mitochondria were again pelleted by centrifugation at 15,000 × *g* for 10 min. The white fluffy layer overlying the brown mitochondrial pellet was removed with a pasteur pipette, the mitochondria were sonicated in 200 μl of NaP_i buffer (12.5 mM, pH 7.5) by using a Branson sonifier with a small tip (setting no. 3) at 0°C for 30 sec, and the sonicate was centrifuged at 40,000 × *g* for 30 min. This mitochondrial sonic supernatant was used in the assays described below.

Marker enzymes for subcellular compartments (glutamate dehydrogenase, *N*-acetyl-β-glucosaminidase, glucose-6-phosphate dehydrogenase) were assayed by using existing methods (11–13). Protein was determined by the biuret reaction (14).

Tritium Release Assay for Isovaleryl- and Butyryl-CoA Dehydrogenases. The demonstration of isovaleryl-CoA dehydrogenase deficiency in crude homogenates of leukocytes and fibroblasts obtained from patients with isovaleric acidemia by the dye reduction method has been unsuccessful due to the strong nonspecific reduction of the electron acceptor by endogenous reducing agents. Furthermore, when assayed by the tritium release method, crude homogenates of isovaleric acidemia fibroblasts showed only a 30–50% decrease in isovaleryl-CoA dehydrogenase activity compared to normal controls. To lower the nonspecific reducing activity, isolation of mitochondria from cultured skin fibroblasts and subsequent assay for dehydrogenase activities were attempted, because preliminary experiments with rat liver have shown that both isovaleryl-CoA dehydrogenase and butyryl-CoA dehydrogenase are localized in the mitochondrial matrix (15). Dehydrogenation of the tritiated CoA ester by mitochondrial sonic supernatants (25–50 μg of mitochondrial protein per assay) was performed in a total volume of 100 μl. The final concentrations of all reagents were as follows: 10 m NaP_i, pH 7.5; 18.8 mM phenazine methosulfate; 100 μM [2,3-^{3}H]acyl CoA. After incubation at 37°C for 15 min, the reaction was stopped by addition of 5 μl of 0.5% iodine in 0.5 M HCl/75% ethanol. A 90-μl aliquot of the resulting mixture was passed over a 0.5-ml column of AG-1 anion exchange resin, acetate form (Bio-Rad), packed in a pasteur pipette, and the column was washed with 1 ml of deionized water. The tritium in the eluate was then quantitated by liquid scintillation. More than 99% of the tritiated water in the reaction mixture was recovered by this procedure, whereas less than 0.2% of unreacted substrate escaped trapping by the ion-exchange resin. A comprehensive description of the tritium release assay will be published elsewhere.

Dye Reduction Assay for Isovaleryl- and Butyryl-CoA Dehydrogenase. The enzyme-catalyzed reduction of dichloroindophenol by nonradioactive acyl CoAs was performed as described by Hall (7) with the following modifications: total volume of assay mix, 0.47 ml; pig liver electron transferring flavoprotein, 2.6 μg per assay; acyl CoA, 50 μM. Rates of dye reduction were calculated by using the absorbance change in the interval from 2 to 4 min after addition of substrate.

Table 1. Distribution of marker enzymes among subcellular fractions isolated from fibroblasts after protease VI treatment and homogenization

Fraction	% total activity*		
	Glutamate dehydrogenase	Glucose-6-phosphate dehydrogenase	*N*-acetyl β-glucosaminidase
700 × *g* pellet	23	13	37
15,000 × *g* pellet	74	1	20
15,000 × *g* supernatant	3	86	43

* Total activity was defined as the sum of activities of all three fractions; this total accounts for all cell material, because none was discarded during the experiment.

RESULTS

Characterization of Mitochondria Prepared from Cultured Skin Fibroblasts. We utilized protease VI treatment to facilitate homogenization of fibroblasts (9, 10). Our modification of this procedure gave a good yield and purification of mitochondria, as the results of a typical isolation experiment demonstrate (Tables 1 and 2). Approximately three-quarters of the total cellular mitochondria, as measured by the marker enzyme for the matrix, glutamate dehydrogenase, was recovered in the final 15,000 × *g* precipitate (Table 1). Specific activity of this fraction represents a 14-fold mitochondrial enrichment when compared to the intact cells (Table 2); mitochondrial purification was as high as 20-fold in some experiments. Millis and Pious (9) reported that mitochondria isolated from fibroblasts by a similar method appear to be functionally intact, demonstrating tight respiratory control and three sites of oxidative phosphorylation. However, cytochrome *c* oxidase assays indicated only a 10-fold mitochondrial purification in their preparation, and the distribution of marker enzymes for other subcellular organelles was not determined in their study.

Although the cytoplasmic contribution to the 15,000 × *g* pellet is minimal in our preparation as judged by glucose-6-phosphate dehydrogenase activity, there is some lysosomal contamination of the mitochondrial fraction as evidenced by the distribution of *N*-acetyl-β-glucosaminidase. However, the proportion of lysosomal contamination is lower than that observed in other procedures for isolating mitochondria from fibroblasts (16, 17).

Demonstration of Isovaleryl-CoA Dehydrogenase Deficiency in Mitochondria from Isovaleric Acidemia Cells. Activities of isovaleryl-CoA and butyryl-CoA dehydrogenases in mitochondria isolated from normal human fibroblasts and from isovaleric acidemia cells were determined by the tritium

Table 2. Specific activity of marker enzymes among subcellular fractions isolated from fibroblasts after protease VI treatment and homogenization

Fraction	Specific activity*		
	Glutamate dehydrogenase	Glucose-6-phosphate dehydrogenase	*N*-acetyl-β-glucosaminidase
700 × *g* pellet	3.2	9.8	28.0
15,000 × *g* pellet	52.0	1.3	76.0
15,000 × *g* supernatant	0.2	25.0	12.0

* nmol of product per min/mg of protein.

Table 3. Isovaleryl-CoA and butyryl-CoA dehydrogenase activities in mitochondria from fibroblasts of patients with isovaleric acidemia and from normal individuals

Origin of mitochondria	Specific activity*			
	Tritium release assay†		Dye reduction assay‡	
	Isovaleryl-CoA dehydrogenase	Butyryl-CoA dehydrogenase	Isovaleryl-CoA dehydrogenase	Butyryl-CoA dehydrogenase
Normal cells	310 ± 42	440 ± 91	1310 ± 208	738 ± 4§
Isovaleric acidemia cells	39 ± 3	475 ± 80	163 ± 68	714 ± 142
(% of normal)	(13%)	(108%)	(12%)	(97%)

* pmol of product per min/mg of protein. All results are expressed as mean ± SEM

† The assay mix (total volume = 100 μl) contained 25–150 μg of protein in 10 mM NaP_i, pH 7.5/18.8 mM phenazine methosulfate/100 μM [2,3-^{3}H]acyl CoA. The mix was incubated at 37°C for 15 min and the tritium released was quantitated. Four normal cell lines and five isovaleric acidemia cell lines were assayed a total of six and eight times, respectively, in duplicate.

‡ The assay mix (total volume = 470 μl) contained 70–350 μg of protein in 12.5 mM KP_i, pH 7.5/5.6 μg of pig liver electron transferring flavoprotein per ml/≈25 μM dichloroindophenol/50 μM acyl CoA; reduction rates were determined 2–4 min after addition of substrate. Four normal cell lines and five isovaleric acidemia cell lines were each assayed on a single occasion except as indicated in §.

§ Two cell lines were tested on a single occasion.

release method (Table 3). Isovaleryl-CoA dehydrogenase activity in mitochondria from isovaleric acidemia cell lines was about 13% of control values, whereas butyryl-CoA dehydrogenase activity in the isovaleric acidemia fibroblasts was approximately equal to that of controls. Assay of the two activities by the dye reduction method yielded almost identical results, with mitochondria from isovaleric acidemia cell lines demonstrating only 12% of control isovaleryl-CoA dehydrogenase levels (Table 3). Although the results of the two assay methods agree closely, the tritium release assay employed only 40% of the amount of mitochondrial sonicate used in the dye reduction method; in addition, the low levels of isovaleryl-CoA dehydrogenase activity observed in the isovaleric acidemia cell lines (≈1.5 times background for both methods) were easier to quantitate with the tritium release assay. Specific activities of both enzymes were found to be approximately 2- to 5-fold higher with the dye reduction assay than those obtained with the tritium release assay; this observation can be explained by a tritium isotope effect. Although primary isotope effects previously observed (18, 19) in both enzymatic and non-enzymatic dehydrogenations vary widely, the K_H-to-K_{3H} ratios of 2–5 observed in this study are similar to many reported values.

DISCUSSION

These results represent the direct *in vitro* demonstration of reduced isovaleryl-CoA dehydrogenase activity and preservation of normal butyryl-CoA dehydrogenase activity in tissue preparations from patients with isovaleric acidemia. These results give strong support to the hypothesis that isovaleryl CoA is dehydrogenated by a specific enzyme, isovaleryl-CoA dehydrogenase, and that its activity is deficient in patients with isovaleric acidemia due to a mutation (1, 2). It had been previously argued by Engel (20) that butyryl-CoA dehydrogenase was the enzyme responsible for isovaleryl CoA dehydrogenation and that the metabolic derangement in isovaleric acidemia may be explained by hypothesizing a point mutation that rendered butyryl-CoA dehydrogenase incapable of oxidizing isovaleryl CoA while retaining its activity towards its other substrate(s). Our present observations, as well as the metabolite analyses indicating that isovaleryl CoA dehydrogenation alone is functionally impaired in isovaleric acidemia, make Engel's hypothesis much less plausible. In addition, we have obtained preliminary evidence for isolation of a separate isovaleryl-CoA dehydrogenase from rat liver mitochondria that has little or no butyryl-CoA dehydrogenase activity (15). A similar observation has recently been made by another investigator who used pig liver as an enzyme source (C. L. Hall, personal communication). Thus, the accumulating evidence supports the original suggestion by Tanaka *et al.* (1) that isovaleryl CoA dehydrogenation *in situ* is catalyzed by a specific isovaleryl-CoA dehydrogenase and not by butyryl-CoA dehydrogenase.

The basis of the residual isovaleryl-CoA dehydrogenase activity in the isovaleric acidemia cell lines, when compared to the more markedly depressed [2-^{14}C]leucine oxidation (1–1.5% of control values) observed in whole fibroblasts (4), has not been fully elucidated. Two lines of evidence seem to indicate that part of this activity may be due to the relatively weak ability of butyryl-CoA dehydrogenase to release tritrium from [2,3-^{3}H]isovaleryl CoA by exchange with water, presumably without oxidizing the substrate. First, tritium is released from [2,3-^{3}H]isovaleryl CoA by pure butyryl-CoA dehydrogenase from pig liver in the absence of electron acceptor at 4–6% of the rate of tritium release from [2,3-^{3}H]butyryl CoA (W. Rhead, K. Tanaka, and C. Hall, unpublished observation). Second, when phenazine methosulfate is omitted as the electron acceptor in the tritium release assay, with fibroblast mitochondrial sonicate as an enzyme source, specific activities of isovaleryl-CoA dehydrogenase and butyryl-CoA dehydrogenase both fall substantially. However, the ratio of butyryl-CoA dehydrogenase activity to isovaleryl-CoA dehydrogenase activity in mitochondrial sonicates from the control cells increases approximately 2-fold, as does the apparent isovaleryl-CoA dehydrogenase activity in mitochondria from the isovaleric acidemia fibroblasts, to 25–30% of control levels (data not shown). In addition, because there is lysosomal contamination of the mitochondrial fraction employed in these experiments, peroxisomal β-oxidation (21) and microsomal ω-1 hydroxylation (22) may also account for part of the tritium release from [2,3-^{3}H]isovaleryl CoA observed in the isovaleric acidemia cell lines. However, the biochemical findings in isovaleric acidemia suggest that, among such possible alternative oxidative mechanisms, only ω-1 hydroxylation effectively catabolizes a portion of the isovalerate accumulated in the disease (22). It is also possible that, *in vivo*, isovaleryl-CoA dehydrogenase is located close to the branched-chain α-keto acid dehydrogenase complex where isovaleryl CoA is generated. The spatial location of the other dehydrogenase activities may not permit effective oxidation of the accumulated isovaleryl CoA. Whatever the contribution of these other activities is *in vitro*, isovaleryl CoA dehydrogenation *in vivo* is always markedly deficient in patients with isovaleric acidemia (1–3).

The residual isovaleryl-CoA dehydrogenase activity noted in the isovaleric acidemia cell lines in the dye reduction assay is presumably secondary to the action of ubiquitous thioesterases

on the acyl CoA substrates, followed by direct reduction of the dye by free CoASH (7), although it may be due, in part, to enzymatic reduction of the dye by the other activities discussed above.

We thank Drs. Vivian Shih, Lawrence Sweetman, Ingeborg Krieger, and Leon Rosenberg for the provision of the normal and mutant cell lines used in this study. We also thank Ms. Barbara Dubiel and Ms. Melanie Ball for their skilled technical assistance and Ms. Marilyn Feldman for her preparation of the manuscript. We greatly appreciate the constructive comments and advice given by Dr. Carole L. Hall, Georgia Institute of Technology, and by Drs. Dean J. Danner and Louis J. Elsas II, Emory University School of Medicine. This work was supported by a grant from the National Foundation–March of Dimes (1-378) and National Institutes of Health Grant AM 17453.

1. Tanaka, K., Budd, M. A., Efron, M. L. & Isselbacher, K. J. (1966) *Proc. Natl. Acad. Sci. USA* **56,** 236–242.
2. Budd, M. A., Tanaka, K., Holmes, L. B., Crawford, M. L. & Isselbacher, K. J. (1967) *N. Engl. J. Med.* **277,** 321–327.
3. Tanaka, K. (1973) in *Inborn Errors of Metabolism,* eds. Hommes, F. A. & Van den Berg, C. J. (Academic, London), pp. 269–278.
4. Tanaka, K., Mandell, R. & Shih, V. E. (1976) *J. Clin. Invest.* **58,** 164–172.
5. Beinert, H. (1962) *Methods Enzymol.* **5,** 546–557.
6. Osmundsen, H., Billington, D. & Sherratt, H. S. A. (1974) *Biochem. Soc. Trans.* **2,** 1286–1288.
7. Hall, C. L. (1978) *Methods Enzymol.* **53,** part D, 502–518.
8. Wieland, T. & Rueff, L. (1953) *Angew. Chem.* **65,** 186.
9. Millis, A. J. T. & Pious, D. A. (1973) *Biochim. Biophys. Acta* **292,** 73–77.
10. Danner, D. J., Wheeler, F. B., Lemmon, S. K. & Elsas, L. J., II (1978) *Pediat. Res.* **12,** 235–238.
11. Strecker, H. J. (1955) *Methods Enzymol.* **2,** 220–225.
12. Leaback, D. H. & Walker, P. G. (1961) *Biochem. J.* **78,** 151–156.
13. Yagil, G. & Feldman, M. (1969) *Exp. Cell Res.* **54,** 29–36.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
15. Rhead, W. & Tanaka, K. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 648 (abstr.).
16. Loverde, A. & Lehrer, G. (1973) *J. Neurochem.* **20,** 441–448.
17. Mahoney, M. J., Hart, A. C., Steen, V. D. & Rosenberg, L. E. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2799–2803.
18. Streitwieser, A., Jr. (1960) *Ann. N.Y. Acad. Sci.* **84,** 573–582.
19. Miller, W. W. & Richards, J. H. (1969) *J. Am. Chem. Soc.* **91,** 1498–1507.
20. Engel, P. C. (1974) *Nature (London)* **248,** 140–142.
21. Lazarow, P. B. (1978) *J. Biol. Chem.* **253,** 1522–1528.
22. Tanaka, K., Orr, J. C. & Isselbacher, K. J. (1968) *Biochim. Biophys. Acta* **152,** 638–641.