0031-3998/88/2404-0455$02.00/0
PEDIATRIC RESEARCH

Vol. 24, No. 4, 1988
Printed in U.S.A.

Oxidation of [U-^{14}C]Succinic Semialdehyde in Cultured Human Lymphoblasts: Measurement of Residual Succinic Semialdehyde Dehydrogenase Activity in 11 Patients with 4-Hydroxybutyric Aciduria

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ABSTRACT. The oxidation of [U-^{14}C]succinic semialdehyde to $^{14}CO_2$ has been investigated in cultured lymphoblasts to develop a whole cell assay for succinic semialdehyde dehydrogenase. We have previously demonstrated deficiency of this enzyme in extracts of white cells derived from 13 patients with 4-hydroxybutyric aciduria. Major goals were the demonstration of greater residual succinic semialdehyde dehydrogenase activity in patient cell lines and the better representation of physiology *in vivo*. In 18 control lymphoblast lines, the conversion of [U-^{14}C]succinic semialdehyde to $^{14}CO_2$ was 1579 ± 310 dpm. The mean value in lymphoblasts derived from 11 patients with deficiency of succinic semialdehyde dehydrogenase was 112 ± 36 dpm approximating 7% of the mean control value. Analysis of organic acids produced from [U-^{14}C]succinic semialdehyde in control lymphoblasts indicated that $^{14}CO_2$ emanated from the tricarboxylic acid cycle; the major metabolic products were succinic and lactic acids. In the presence of 5mM malonic and 2-propylpentanoic (valproic) acids, $^{14}CO_2$ production in a control lymphoblast line was decreased by 68 and 45%, respectively. The whole cell assay is less laborious than our previously described assay employing cell extracts, and the general trend was the demonstration of higher residual levels of activity for lymphoblasts derived from patients. (*Pediatr Res* 24: 455–460, 1988)

Abbreviations

SSADH, succinic semialdehyde dehydrogenase
SSA, succinic semialdehyde
GHB, 4-hydroxybutyric acid
GABA, 4-aminobutyric acid
EBV, Epstein-Barr virus
PBS, phosphate-buffered saline
TCA, trichloroacetic acid

Received January 25, 1988; accepted June 2, 1988.
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Supported in part by HL 07491 (P.P.P.) and Public Health Service New Investigator Research Award 23876, from the National Institute of Neurological and Communicative Disorders and Stroke (K.M.G.), National Institutes of Health, Bethesda, MD.

4-Hydroxybutyric aciduria is a rare inborn error in the metabolism of the neurotransmitter GABA (Fig. 1). This disease is unique in that it constitutes metabolic derangement in which a neuropharmacologically active compound accumulates in patient body fluids (1). The underlying defect is a deficiency of SSADH (E.C.1.2.1.24, Fig. 1). Under normal conditions, GABA enters the tricarboxylic acid cycle as succinic acid after transamination and oxidation catalyzed by 4-aminobutyric acid aminotransferase (E.C.2.6.1.19) and SSADH, respectively, In patients, SSA accumulates and is reduced to GHB by 4-hydroxybutyric acid dehydrogenase (E.C.1.1.1.61). The neuropharmacologic actions of GHB have been studied in detail (2).

In 13 documented patients with 4-hydroxybutyric aciduria the clinical picture was of mild to marked psychomotor retardation, muscular hypotonia, and nonprogressive ataxia. The urinary organic acid profile includes elevated concentrations of GHB, 3,4-dihydroxybutyric acid, and glycolic acid. There may be considerable clinical heterogeneity. Two patients did not display ataxia or convulsions (3, 4). Three patients, two of whom were siblings, manifested urinary glycinuria (3, 5). In one patient the urinary organic acid profile was that of 4-hydroxybutyric aciduria and also glutaric aciduria type II (Goodman SI, personal communication). Deficient activity of SSADH has been demonstrated in extracts of lymphocytes isolated from whole blood and/or cultured lymphoblasts transformed with EBV in each of the 13 patients (6).

We have investigated the oxidation of [U-^{14}C]SSA to $^{14}CO_2$ in intact EBV-transformed cultured human lymphoblasts in 11 patients with 4-hydroxybutyric aciduria. The objective of the study was the development of a whole cell assay in which greater residual activity might better represent physiology *in vivo* and distinguish variants with differing phenotypes.

MATERIALS AND METHODS

[U-^{14}C]GABA (sp. act. 231 mCi/mmol) was obtained from New England Nuclear (Boston, MA) and monoamine oxidase, used in the enzymatic conversion of [U-^{14}C]GABA to [U-^{14}C] SSA, was purchased from Sigma Chemical Co. (St. Louis, MO). The enzymatic preparation and purification of [U-^{14}C]SSA was performed as previously described (7). Septa and plastic center wells employed in trapping of CO_2 were obtained from Kontes (Vineland, NJ). The sodium salts of GHB and malonic acid were obtained from Sigma (St. Louis, MO) and valproic acid (2-propylpentanoic acid) was purchased from Aldrich (Milwaukee, WI).

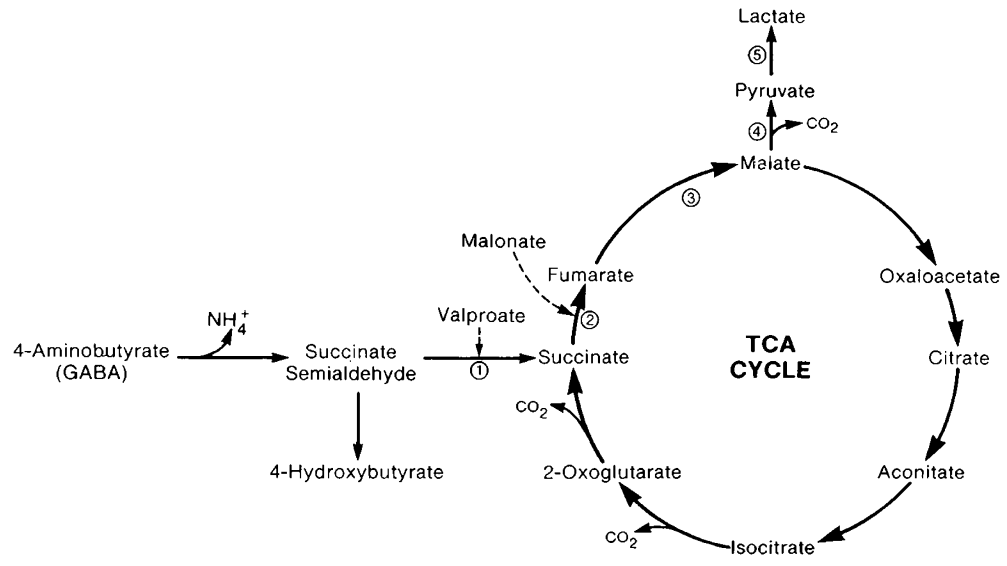

Fig. 1. Metabolic interconversion of succinate semialdehyde to intermediates of the tricarboxylic acid cycle. Numbered enzymes include: *1*, succinate semialdehyde dehydrogenase; *2*, succinate dehydrogenase; *3*, fumarase; *4*, malic enzyme; *5*, lactate dehydrogenase. The sites of action of two inhibitors, valproate (2-propylpentanoate) and malonate, are indicated by *dashed arrows*.

Lymphocytes isolated from whole venous blood (8) were transformed with EBV according to the procedure of Sly *et al.* (9). During the initial phase of transformation, lymphocytes were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum, penicillin, streptomycin, fungizone and glutamine. For maintenance of transformants, the culture medium was identical except that fungizone was omitted and the concentration of bovine serum was decreased to 10%. All cell lines were screened for mycoplasma contamination. Lymphoid lines were prepared from 18 control individuals, eleven patients with deficiency of SSADH and seven family members (six parents and a clinically unaffected male sibling).

The oxidation of [U-^{14}C]SSA (0.02 μCi/assay) was assessed in 15 ml plastic conical centrifuge tubes fitted with septa and center wells. Lymphoblasts in the log-phase of growth were harvested by centrifugation for 10 min at 800 × *g*. The cells were washed twice with PBS and reisolated. After the final wash, cells were resuspended in a total volume of 0.3 ml of PBS supplemented with 0.1% glucose and 0.4 mg/ml defatted bovine serum albumin (10) at a final concentration of 2 × 10^6 cells/0.3 ml. Tubes were incubated for 90 minutes in a shaking water bath at 37° C. Reactions were stopped by addition of 0.1 ml 4.2 M $HClO_4$ through septa with a Hamilton syringe, after which 0.1 ml of 3 N KOH was added to the center well for $^{14}CO_2$ collection. Trapping of $^{14}CO_2$ was carried out for 1 h on ice. The contents of center wells were transferred to 22 ml glass scintillation vials containing 10 ml of EcoLite scintillation fluid (West Chem, San Diego, CA). Wells were rinsed with 0.15 ml deionized water that was also added to vials. The vials were counted in a Beckman LS 3801 liquid scintillation spectrophotometer after storage for 24 h in the dark to facilitate decay of chemiluminescence.

The oxidation of [U-^{14}C]SSA in each cell line was measured in duplicate for two separate harvests of cells; the final $^{14}CO_2$ dpm values represent the mean of both harvests. For the analysis of organic acids, duplicate acidified reaction mixtures were pooled in 22 ml glass scintillation vials and neutralized with 6 N KOH, the insoluble K ClO_4 removed by centrifugation, and the supernatant fluids lyophilized to dryness. Each vial received 0.05 ml 28% methoxylamine hydrochloride, 0.03 ml 14% sodium hydroxide and 2.8 microequivalents nonradiolabeled carrier SSA (Sigma). In this way unreacted [U-^{14}C]SSA was converted to its methoxime. Radiolabeled organic acids were separated and quantified by liquid partition chromatography as previously described (11). In this system, unreacted [U-^{14}C]SSA-methoxime eluted at approximately 5 min and did not interfere with the quantification of other ^{14}C-organic acids.

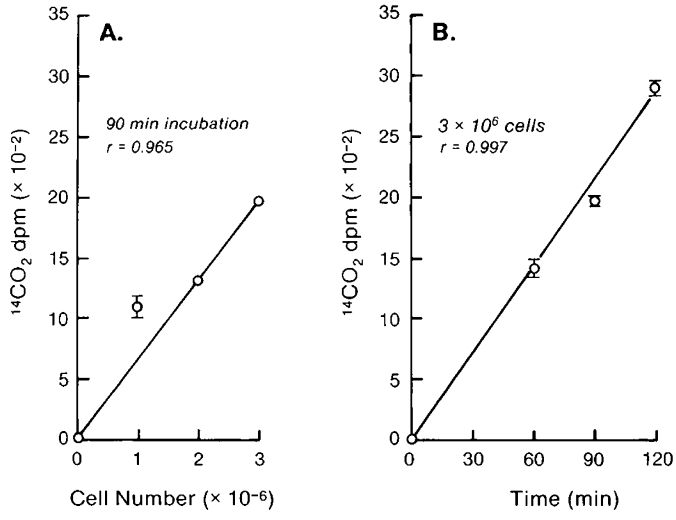

Fig. 2. Linearity of $^{14}CO_2$ production in an intact control lymphoblast cell line as a function of (*A*) cell number and (*B*) time.

RESULTS

The production of $^{14}CO_2$ was linear with up to 3 × 10^6 control cells incubated and with time up to 120 min (Fig. 2). The mean production of $^{14}CO_2$ from [U-^{14}C]SSA in 18 control lymphoblast lines was 1579 ± 310 dpm (Table 1). The mean value of cell lines derived from 11 patients with SSADH deficiency was 112 ± 36 dpm, approximately 7% of the mean control value. In seven family members, the mean $^{14}CO_2$ production in cultured lymphoblasts was 1428 ± 239 dpm approximating the mean control value. Furthermore, the range of values for family members was similar to the control range, and there was no evidence for two populations of family members.

Investigation of the production of ^{14}C-organic acids was undertaken in the presence and absence of different metabolic inhibitors in order to determine the metabolic sources of the $^{14}CO_2$ produced (Table 2). In the control reaction mixture, the major ^{14}C-organic acids were succinic and lactic acids; smaller

amounts of ^{14}C were found in fumaric, malic, and citric acids. These data suggested that the $^{14}CO_2$ production resulted from action of the TCA cycle. The inclusion of 5 mM unlabeled GHB did not result in a decrease in $^{14}CO_2$ production or in a significant alteration in the pattern of ^{14}C-organic acid production, suggesting that GHB was not involved in the metabolic transformation of [U-^{14}C]SSA in intact lymphoblasts. The inclusion of 5 mM malonic acid caused a 68% decrease in $^{14}CO_2$ production. Concomitantly, there was an approximately 10-fold increase in isotope in succinic acid, and decreases in the isotope content in fumaric, lactic, and malic acids. These data indicate that [U-^{14}C]SSA entered the TCA cycle at the level of succinic acid and was consistent with the expected inhibition by malonic acid of succinic dehydrogenase.

The effects of valproic acid were not so clear. $^{14}CO_2$ production from [U-^{14}C]SSA was decreased by 45%. However, there were increases in the isotope content of all of the organic acids, including an approximate 3-fold increase in malic acid.

The whole cell assay might be expected to yield higher residual SSADH activity in patient lymphoblast lines, and this was the case in each instance (Fig. 3). The mean oxidation of [U-^{14}C]SSA to $^{14}CO_2$ in intact lymphoblasts derived from eleven patients was 7.1 ± 2.3% (± 1 SD) (Fig. 3; Table 3) of the mean control value. In contrast, residual SSADH activity in extracts of lymphoblasts derived from the same 11 patients was 1.6% ± 1.8% in comparison to simultaneously assayed mean control values. Furthermore, the intact cell assay appeared of value in distinguishing distinct patient populations based on residual SSADH activity (4–5% for sibship a, 7–8% for sibship b, and 9–11% for sibship c) (Fig. 3).

DISCUSSION

Measurement of the oxidation of [U-^{14}C]SSA to $^{14}CO_2$ in intact cultured lymphoblasts has provided a convenient means of assessing residual activity of SSADH. Deficient activity of SSADH was readily documented in the eleven patients with 4-hydroxybutyric aciduria studied. The use of an intact cell assay appears intuitively to represent a more physiologic assessment of SSADH activity than an assay employing cell extracts. We (12) and others have demonstrated that the use of an intact cell assay permits the distinction of distinct variants with differing amounts of residual activity and good correlations between severity of clinical phenotype and severity of enzymatic deficiency. In contrast, cell free systems seldom permit such correlations because so many variants display essentially zero activity, presumably because mutant enzymes are inherently labile. Moreover, the coefficient of variation of less than 20% for 18 control cell lines was significantly smaller than the same value obtained with cell extract assays and far fewer control cell lines. The simplicity of the assay and the ease of production of [U-^{14}C]SSA from [U-^{14}C]GABA (7) provide advantages over our previously described assay for SSADH using cell extracts (6) and should facilitate the use of the intact cell oxidation assay in other laboratories.

Activities of SSADH in cell extracts have been previously reported (4, 5, 6, 13–15) for eight of 13 patients with 4-hydroxybutyric aciduria. The residual activity of SSADH in 11 patients in lymphoblast extracts ranged from 0–6% of control. The mean oxidation of [U-^{14}C]SSA to $^{14}CO_2$ in intact lymphoblasts derived from these patients was higher at approximately 7% of the control mean (Fig. 3). Moreover, the intact cell assay appears to be a more reliable method for assessing residual SSADH activity because the coefficient of variation for $^{14}CO_2$ production in control cell lines was appreciably smaller than found in cell extracts. In fact, in previously reported studies of SSADH activity in extracts of lymphoblasts the coefficients of variation of control SSADH activity were 16, 35, 38, and 49% (4, 13–15). These studies used four to five control lines. In the present study 18 control cell lines were used and the coefficient of variation was less than 20%.

The clinical and enzymatic phenotypes of patients with 4-hydroxybutyric aciduria are displayed in Table 3. There was little

Table 1. *Oxidation of [U-^{14}C]succinic semialdehyde to $^{14}CO_2$ in intact cultured lymphoblasts derived from controls, patients, and family members*

Subject	dpm $^{14}CO_2$ (mean ± 1 SD)	% of mean control
Control (n = 18)*	1579 ± 310† (range 1135–2082)	100
Patients (n = 11)	112 ± 36 (range 59–174)	7
Family members (n = 7)	1428 ± 239 (range 1089–1739)	90

* n denotes the number of cell lines studied.

† Each cell line was assayed in duplicate for two separate harvests, the mean production of $^{14}CO_2$ from each harvest being used to generate the final mean value.

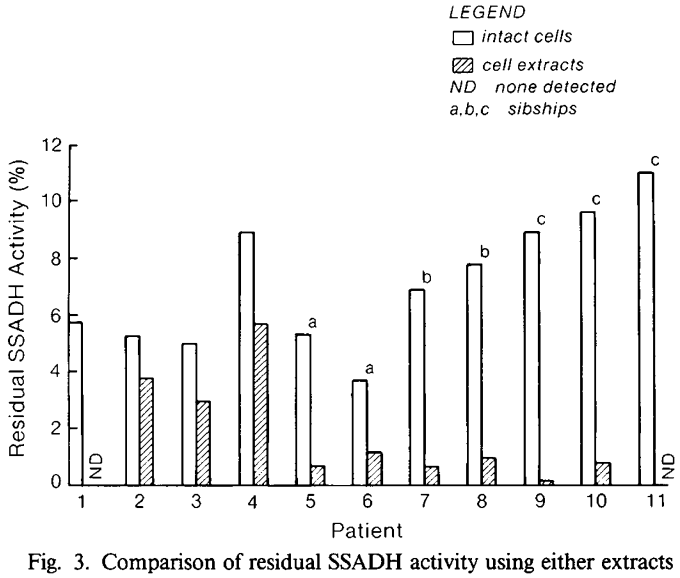

Fig. 3. Comparison of residual SSADH activity using either extracts of or intact cultured human lymphoblasts derived from eleven patients with 4-hydroxybutyric aciduria.

Table 2. *Production of ^{14}C-organic acids from [U-^{14}C]succinic semialdehyde in intact cultured lymphoblasts (dpm)*

Reaction mixture*	Fumaric	Lactic	Succinic	Malic	Citric	Total $^{14}CO_2$ ($\bar{x}$ duplicate)	% of control $^{14}CO_2$ production
Control	115	1931	1803	268	58	5818 (2909)	100
+ 5 mM GHB	159	1789	2680	336	157	6312 (3156)	108
+ 5 mM malonic	95	1311	17220	129	143	1718 (859)	32
+ 5 mM valproic	313	2614	2374	814	146	3144 (1572)	55

* All reaction mixtures contained 3 × 10^6 control cells and were incubated for 120 min. The data for $^{14}CO_2$ production are representative of similar results obtained in repeat studies, whereas the values for organic acids represent a single determination of pooled duplicate reaction mixtures. The sodium salts of GHB and malonic acid were used, and dilutions of valproic acid were prepared in 0.5 M potassium phosphate buffer, pH 7.

Table 3. *Correlation of clinical and enzymatic phenotypes in patients with 4-hydroxybutyric aciduria**

Patient	Sex	Age at presentation	Parental consanguinity	Nationality	Developmental delay	Hypotonia	Ataxia	Nystagmus	Oculomotor apraxia	Convulsions	Speech performance	Hyporeflexia	Associated findings	% residual SSADH activity†		Reference
														Cell extracts	Intact cells	
1	M	1.5 yr	Yes	Turkish	+	+++	+++	–	+	–	Delayed	–	–	ND‡	5.8%	21
2	M	1 yr	Yes	Algerian	+++	+++	+++	–	–	Present	–	–	–	3.8%	5.3%	21, 22
3	M	3 yr	First cousins	Maltese	Present	+	–	–	–	–	Delayed	Present	Mild autistic features increased plasma and urinary glycine	3%	5%	3
4	F	5.5 yr	NA§	Mexican	+	Present	–	–	–	–	Delayed	–	Microcephaly hyperkinesis	5.7%	8.9%	4
5[a]	F		Clinical details unavailable											0.7%	5.3%	
6[a]	F													1.2%	3.7%	
7[b]	F	6 yr	NA	NA	++	NA	Present	+	–	–	NA	Present	Macrocephaly increased urinary glycine‖	0.7%	6.9%	5
8[b]	M	1.5 yr	NA	NA	Present	NA	–	–	–	–	NA	Present	Macrocephaly increased plasma and urinary glycine‖	1%	7.8%	5
9[c]	F	9 mo	NA	NA	Present	NA	NA	NA	NA	NA	Delayed	NA	Elevated serum glycine urinary dicarboxylic aciduria	0.2%	8.9%	**
10[c]	F	NA	NA	NA	Present	NA	NA	NA	NA	NA	Delayed	NA	Elevated serum glycine	0.8%	9.6%	**
11[c]	F	NA	NA	NA	Present	NA	NA	NA	NA	NA	Delayed	NA	Elevated serum glycine	ND	11%	**
12[d]	F	11 yr	NA	Lebanese	+	+	++	–	+++	–	Slightly dysarthric	Present	Conjunctival telangiectasias	14.2%¶	–	7, 21
13[d]	M	9 yr	NA	Lebanese	+	–	++	–	Slight	–	Grossly retarded	Present	Conjunctival telangiectasias	19.4%¶	–	7, 21

* [a, b, c, d], sibships; sibship (*d*) not presented in Figure 3. +, mild; ++, moderate; +++, severe; –, absent.

† Residual SSADH activities based on comparison of simultaneously assayed mean control values.

‡ None detected.

§ Information not available.

‖ Both siblings noted as having nonspecific muscle atrophy.

¶ Lymphocyte extracts; all other values in this column were generated using lymphoblast extracts.

** Glasgow AM, personal communication.

Table 4. *SSADH activities in cell extracts and intact cells for family members of patients*

Family member	Sex	Status as heterozygote	Relationship*	% SSADH activity cell extracts†		% SSADH Activity intact cells	Reference
				Lymphocytes	Lymphoblasts		
1	M	obligate	Father (7, 8)	79	27	110	
2	F	obligate	Mother (7, 8)	35	49	76.6	
3	F	obligate	Mother (5, 6)	32	67	69	
4	M	obligate	Father (5, 6)	53	79	104.6	
5	F	suspected	Mother (3)		50	91.7	15
6	M	suspected	Father (3)		34	82.4	15
7	M	unknown	Sibling (5, 6)	49	45	98.5	
8	M	obligate	Father (9–11)	49			
9	F	obligate	Mother (9–11)	55			
10	M	obligate	Father (12, 13)	63.6			6
11	F	obligate	Mother (12, 13)	28.2			6
12	F	unknown	Sibling (12, 13)	128.5			6
13	M	unknown	Sibling (12, 13)	43.3			6
14	F	suspected	Mother (1)	30			14
15	M	suspected	Father (1)	58			14
16	F	unknown	Sibling (1)	102.2			14

* Values in parentheses refer to patient numbers in Table 3.

† During a period of extended studies of SSADH activity in control lymphocytes and lymphoblasts, there was no significant difference between control mean SSADH values for the two cell types.

variation between cell lines in the residual level of SSADH activity when intact cells derived from eleven patients were studied. In contrast, there were two distinct patient populations in terms of enzymatic phenotype when lymphocyte extracts were employed for determination of residual SSADH activities. In lymphocyte extracts derived from patients 1–3 and 7–11 SSADH activity ranged from 0–3.8% of parallel controls. Conversely, residual SSADH levels were 5.7, 14.2, and 19.4% of parallel controls in lymphocyte extracts derived from patients 4, 12, and 13. Despite inherent difficulties in grading the clinical presentation of patients with inherited metabolic defects, it would nonetheless appear that the latter three probands represent the least severely affected patients with 4-hydroxybutyric aciduria thus far described, an observation well correlated with the residual SSADH activities displayed in lymphocyte extracts.

The intact cell assay is not useful for heterozygote genotyping in family members (Table 4). The mean oxidation of [U-^{14}C] SSA to $^{14}CO_2$ in lymphoblasts derived from seven family members of patients was 90 ± 15% (± 1 SD) of the mean value in controls. Four of these seven individuals (two sets of parents) are obligate heterozygotes, since two affected offspring have occurred in each family. Conversely, heterozygosity has been readily documented in the assay of cell extracts (Table 4). For obligate heterozygotes depicted in Table 4, the mean SSADH in comparison to simultaneously assayed control values was 51 ± 18% (nos. 1–4, 8–11, ± 1 SD, range 27–79%) for both cell types. The activities of SSADH in extracts of lymphoblasts and lymphocytes derived from four additional parents (family members 5, 6, 14, and 15) (Table 4) ranged from 30–58% of parallel mean control values, suggesting their status as carriers of the mutant gene. For clinically unaffected siblings depicted in Table 4 (family members 7, 12, 13, and 16) the data suggested two as heterozygotes and two as homozygous unaffected.

The generation of $^{14}CO_2$ from [U-^{14}C]SSA in intact lymphoblasts was interpreted to emanate from the operation of the tricarboxylic acid cycle. Production of $^{14}CO_2$ was markedly curtailed in the presence of malonic acid, and isotope accumulated in succinic acid. Valproic acid has been reported to inhibit GABA-aminotransferase and SSADH in ox brain (16) and rat brain (17–20). We observed an inhibition of $^{14}CO_2$ production from [U-^{14}C]SSA in intact lymphoblasts in the presence of 5 mM valproic acid. Moreover, in an *in vitro* assay (6) using lymphoblast extract as source of enzyme we found that concentrations of 0.5–10 mM valproic acid inhibited SSADH by 39–58%, values similar to the 45% inhibition of $^{14}CO_2$ production observed in intact cells. However, the content of isotope in succinic and lactic acids was similar to control levels and even higher in fumaric and malic acids. If valproic acid had an inhibitory effect on malic enzyme, one might expect decreased production of ^{14}C-lactic acid, which was not the case.

The intact cultured cell assay is both rapid and simple. It should allow for more reliable assessment of residual enzyme activity in patients and will be of value in distinguishing distinct patient populations within families based on residual SSADH levels. However, the assay of SSADH using cell extracts remains at present the most reliable method of heterozygote detection in family members. For patients and family members yet to be discovered, it will be of value to use both types of assays.

Acknowledgments. The authors are indebted to Dr. R. A. Roesel, Dr. F. Hommes, Dr. A. M. Glasgow, Dr. J. Kneer, and Dr. C. Jakobs for collection and transport of blood specimens from patients and family members.

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