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Genetics

sar: A genetic mouse model for human sarcosinemia generated by ethylnitrosourea mutagenesis

(metabolic screening/inborn errors of metabolism/amino acid analysis/gas chromatography–mass spectrometry)

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ABSTRACT **A mouse mutant with sarcosinemia was found by screening the progeny of ethylnitrosourea-mutagenized mice for aminoacidurias. Paper chromatography, column chromatography, and gas chromatography–mass spectrometry identified high levels of sarcosine in the urine of the mutant mice. While sarcosine cannot be detected in the urine or plasma of normal mice, the urinary sarcosine level of 102 ± 58 mmol per g of creatinine in the mutant mice was at the upper range of the urinary levels (1.5–45 mmol of sarcosine per g of creatinine) observed in humans with sarcosinemia. Similarly, the plasma sarcosine level of 785 ± 153 μmol/liter in the sarcosinemic mice was at the upper range of the plasma sarcosine levels (53–760 μmol/liter) observed in affected humans. Sarcosine dehydrogenase [sarcosine:(acceptor) oxidoreductase (demethylating), EC 1.5.99.1] activity was deficient in sarcosinemic mice. The sarcosinuria phenotype in these mice was inherited as an autosomal recessive trait. This mouse mutant provides a useful genetic model for human sarcosinemia and for development of therapeutic approaches for genetic disease.**

Genetically modified mice are increasingly being used as models for diseases and for development of approaches for treatments such as gene therapy. Although some useful mouse strains arose spontaneously, two basic approaches have been developed to produce mouse mutants. When the gene is known beforehand, site-directed mutation induced by homologous recombination in embryo stem cells is a powerful approach for creating mouse mutants (1). When only the phenotype is known, germline mutagenesis followed by backcrossing and phenotypic screening has been successful. The latter approach utilizing ethylnitrosourea (ENU) mutagenesis has been used to develop hyperphenylalaninemic mice strains, deficient in phenylalanine hydroxylase or GTP cyclohydrolase activities (2, 3), and muscular dystrophic mice with defective dystrophin (4).

Mouse models exist for a few of the inborn errors of metabolism (IEM) (5). Many genes related to IEM in humans have been defined and could be targeted for disruption in mice by homologous recombination. Others, however, have not been identified to date. In many IEM, such as cystinosis or Hartnup disease, the defective protein has yet to be identified. Furthermore, several disorders with poorly defined metabolic defects such as lactic acidosis or 3-methylglutaconic aciduria also have poorly defined enzymatic deficiencies (6). Genetic mouse models for these IEM should be useful for assigning the chromosomal location and for eventual isolation of the relevant genes (7).

This report describes a mouse mutant with sarcosinemia found through metabolic screening of progeny of ENU-mutagenized mice. The breeding scheme and ENU mutagenesis protocol were identical to that used for creating the hyperphenylalaninemic mutants (8). The phenotype testing was based on the metabolic screens that have been developed for newborn screening (9, 10). Of 135 pedigrees evaluated, one mutant strain was found to have sarcosinemia, a rare inborn error of amino acid metabolism previously described in humans (for reviews, see refs. 11 and 12). Primarily, sarcosinemia results from a deficiency in sarcosine dehydrogenase [sarcosine:(acceptor) oxidoreductase (demethylating), EC 1.5.99.1] enzymatic activity, which is part of the one-carbon cycle and converts sarcosine (*N*-methylglycine) to glycine.

MATERIALS AND METHODS

Mutagenesis Protocol. Male BTBR inbred mice (13) were intraperitoneally injected with 200 mg of ENU (Sigma) per kg of body weight as described (8). After the mice regained fertility, each was bred to unrelated BTBR females to generate 10–15 G_1 males; 135 G_1 males were bred with females to generate G_2 offspring and then each of the G_1 males was back-crossed with two or three of the G_2 daughters to generate a total of 1296 potentially homozygous mutant G_3 mice, which were screened as indicated below.

Screening Protocol. For initial screening, weaned G_3 mice were placed on a high protein diet containing 40% (wt/wt) protein (Purina ferret chow) for at least 3 days before urine collection. For subsequent studies, mice were placed on normal mouse chow (Wayne mouse breeder Blox 8626), which contains 20% (wt/wt) protein. The mice were placed in a wire mesh cage (3 × 3 in) on top of filter paper (Whatman 3MM Chr) for 12–24 hr without food or water. Areas of stool contamination on the filter paper were excluded by its yellow appearance under long-wave UV illumination. Urine spots that appeared purple under UV illumination were eluted with 2 ml of 0.01 M NH_4OH (9).

Urine amino acids were examined with one-dimensional paper chromatography using butanol/acetic acid/water (12:3:5) solvent and detection with ninhydrin according to the method of Smith (14). Chromatograms were compared to a standard mixture of leucine, phenylalanine, valine, tyrosine, proline, glutamine, glycine, serine, lysine, and histidine. Quantitative urine and plasma amino acid analysis and quantitative urinary organic acid analysis were performed in any mouse that had an abnormal result by amino acid paper chromatography. Quantitative urine and plasma amino acids were analyzed on a Beckman system 6300 automatic amino acid analyzer according to the methods of Slocum and

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Abbreviations: ENU, ethylnitrosourea; IEM, inborn errors of metabolism; TBDMS, *tert*-butyldimethylsilyl; PMS, phenoxymethosulfate; GAII, glutaric aciduria type II; ETF, electron transfer flavoprotein.

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Cummings (15). Quantitative urine organic acids were measured by trimethylsilyl derivatization followed by gas chromatography–mass spectrometry (GC-MS) according to the method of Hoffman and Sweetman (16). Sarcosine, choline, betaine, ethanolamine, dimethylglycine, other amino acids, methylamine, and ethylamine were purchased from Sigma.

GC-MS Identification of Sarcosine. The amino acid analyzer fraction putatively containing sarcosine was further analyzed by GC-MS. *tert*-Butyldimethylsilyl (TBDMS) derivatives of the fraction were prepared for GC-MS analysis by the method of Kitson and Larsen (17); 0.25 ml each of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (Pierce) and *N,N*-dimethylformamide (Pierce) were added to the dried sample, capped, and heated at 60°C for 60 min. Electron impact mass spectra of the TBDMS derivatives were obtained on a Hewlett-Packard 5970B MSD (ionizing energy, 70 eV; scanned from 50–650 atomic mass units). Sample introduction was by gas chromatography using a Hewlett-Packard 5890 series II gas chromatograph equipped with a Durabond-5 (J & W Scientific, Rancho Cordova, CA) capillary column (i.d., 30 m × 0.32 mm; film thickness, 1.0 mm). The GC column was temperature programmed from 80°C to 300°C after an initial hold of 5 min. The injection port temperature was 250°C, and the capillary direct interface was held at 280°C.

Enzymatic Assays. Enzyme assays were performed in hepatic mitochondria that were isolated as reported (18). Approximately 2 g of liver was placed immediately in ice-cold 0.25 M sucrose and minced with scissors. The minced tissue was homogenized in 20 ml of ice-cold 0.25 M sucrose using a precooled, motor-driven Potter–Elvehjem tissue homogenizer (10 up–down strokes). The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C to remove cells and debris. The pellet was washed with 10 ml of 0.25 M sucrose and recentrifuged, and the two supernatants were combined. The mitochondria were collected from the supernatant by centrifugation at 13,800 × *g* for 10 min at 4°C. The pellet was resuspended in 1.5 ml of ice-cold 0.5 mM potassium phosphate buffer (pH 7.0). Protein concentration was determined by a bicinchoninic acid procedure (Pierce) (19). Mitochondrial preparations were diluted to 5 mg of total protein per ml in 0.5 mM potassium phosphate buffer before assay. Mitochondria were frozen and thawed before analysis.

Sarcosine dehydrogenase activity was measured by following the reduction of 2,6-dichlorophenolindophenol at 600 nm (ε_{600} = 21 $mM^{-1} \cdot cm^{-1}$) according to the method of Hoskins and Bjur (18). Components of the assay in a total vol of 1 ml were 20 μmol of Tris·HCl (pH 7.8), 8 μmol of potassium cyanide, 0.032 μmol of 2,6-dichlorophenolindophenol, and 500 μg of mitochondrial protein. The mixture was incubated in a water bath for 10 min at 37°C. The reaction was initiated by the addition of 4 μmol of sarcosine (Sigma). The background rate of reduction of 2,6-dichlorophenolindophenol in the absence of sarcosine is 0 over at least 15 min. Ten microliters of 1% (wt/vol) phenoxymethosulfate (PMS) was added to the reaction mixture as an electron carrier in the initial experiments. No significant difference in sarcosine dehydrogenase activity with or without PMS was detected in liver mitochondria from either control or sarcosinemic mice. PMS was omitted in subsequent experiments.

Mitochondrial cytochrome *c* oxidase (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1) activity was measured by following the oxidation of ferrocytochrome *c* (horse heart, type III; Sigma) at 550 nm (ε_{550} = 18.5 $mM^{-1} \cdot cm^{-1}$) according to the method of Wharton and Tzagoloff (20). The reaction mixture in a total vol of 1 ml contained 10 μmol of potassium phosphate buffer (pH 7.0) and 70 μl of 1% (wt/vol) ferrocytochrome *c*. The mixture was incubated at 37°C for 10 min, and the reaction was initiated by the addition of 50 μg of enzyme extract. The background oxidation rate of ferrocytochrome *c* without the addition of enzyme extract was negligible over at least 15 min.

Statistical Analysis. All means are reported ± sample SD. A two-tailed Student's *t* test was used to compare means of all data. Significance was assumed at $P < 0.05$.

RESULTS

Initial Detection of Mice with Sarcosinemia. The urine of 1296 G_3 mice within 135 pedigrees was screened by amino acid paper chromatography over a period of 1 year. In one pedigree, the urine of two male siblings had an abnormal amino acid that migrated with a R_f of 27 and appeared brownish-purple on staining with ninhydrin. This abnormal brownish-purple spot was never observed in the urine from any other mice, including BTBR, C57BL/6J, and BALB/c mice. Purified sarcosine also appeared as a brownish-purple spot with an R_f of 27, suggesting that the abnormal spot present on urine chromatograms from the G_3 siblings represented sarcosine. The intensity of the spot was not attenuated after reducing dietary protein from 40% to 20% (wt/wt). Subsequent studies of these two siblings and their offspring were performed on the normal, 20% protein diet.

Column Chromatography and GC-MS Analysis. Further confirmation of the identity of the elevated amino acid in affected mice was obtained by high-pressure liquid chromatography analysis of blood and urine on the amino acid analyzer. Urine and blood from affected mice gave a large peak with a retention time of 21.6 min (Fig. 1), which was identical to the retention time of 21.6 min for pure sarcosine (data not shown). The position of this peak relative to other amino acids is identical to that previously reported for sarcosine (21). Control BTBR or C57BL/6J mice never demonstrated this abnormal peak on the amino acid analyzer (Fig. 1). Fractions corresponding to this abnormal peak were collected and further analyzed by GC-MS to more definitively identify it. The sarcosine standard and the abnormal amino acid fraction from the two mice had almost identical retention times of 31.2 and 31.3 min, respectively, on GC. The mass spectra of both compounds were also identical (Fig. 2), indicating that the amino acid abnormally excreted by these mice is in fact sarcosine.

After further breeding of the sarcosinemic mice, quantitative amino acid analysis using the amino acid analyzer was performed on presumptively homozygous sarcosinemic mice (*sar/sar*), obligate heterozygotes (+/*sar*), and normal mice (+/+) consuming the normal mouse diet (Table 1). The minimal amount of sarcosine that can be detected with the Beckman 6300 amino acid analyzer is ≈5 μmol/liter in urine and plasma. Sarcosine was not detected in the urine or plasma of any of the normal or heterozygotic mice, but both the urine and plasma of the sarcosinemic mice contained substantially elevated levels of sarcosine. In the homozygotic sarcosinemic mice, mean sarcosine levels were 785 ± 153 μmol/liter in plasma (n = 8) and 102 ± 58 mmol per g of creatinine in urine (n = 23).

Except for the concentrations of plasma glycine and plasma and urinary methionine, the levels of other plasma and urinary amino acids were similar in sarcosinemic and normal mice (data not shown). The mean plasma glycine level of 430 ± 100 μmol/liter was significantly greater ($P < 0.005$) in the sarcosinemic mice than the levels of 263 ± 21.1 μmol/liter in normal mice and 286 ± 33 μmol/liter in heterozygotes (Table 1). The plasma methionine of 112 ± 29.4 μmol/liter in sarcosinemic mice (n = 8) was significantly greater ($P < 0.03$) than the level of 75.7 ± 6.2 μmol/liter in normal mice. Likewise, the urinary methionine of 0.294 ± 0.078 mmol per mg of creatinine in sarcosinemic mice (n = 23) was significantly greater ($P < 0.003$) than the level of 0.143 ± 0.111 mmol per mg of creatinine in normal mice (n = 4).

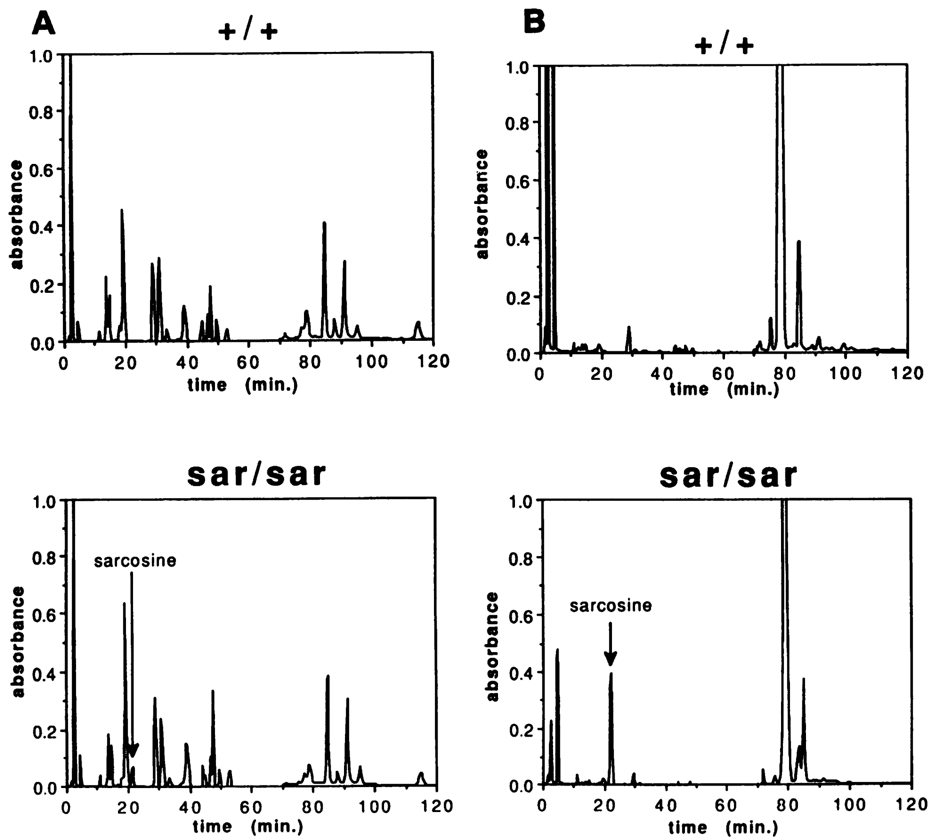

FIG. 1. Column chromatography of plasma (*A*) and urine (*B*) amino acids from sarcosinemic (*sar*/*sar*) and normal (+/+) mice. Arrows indicate location of sarcosine.

A small, unidentified, ninhydrin-reactive biochemical with a retention time of 83.5 min appeared in the urine amino acid analysis of sarcosinemic mice. This peak did not appear in the plasma amino analysis and has not been reported in sarcosinemic patients. This substance has been tentatively identified as methylamine from its position on the column chromatogram. The analysis of dimethylglycine, betaine, choline, or ethanolamine that are part of the one-carbon cycle indicated that none of these compounds was contained in the unidentified peak. Collection of the amino acid analyzer fraction containing the suspected methylamine yielded insufficient material for TBDMS derivatization and analysis by GC-MS.

The urine of both sarcosinemic and normal mice was analyzed for organic acids by GC-MS and contained similar concentrations of organic acids. In particular, abnormal organic acids associated with glutaric aciduria type II (also known as multiple acyl-CoA dehydrogenase deficiency), such as glutaric, 2-hydroxyglutaric, ethylmalonic, adipic, suberic, and sebacic acids, were not detected in either normal or sarcosinemic mice. The sensitivity of this GC-MS method for these organic acids is ≈1 mmol per mol of creatinine.

Enzymatic Analysis. Mitochondria isolated from the livers of normal (+/+), obligate heterozygotic (*sar*/+), and homozygotic sarcosinemic (*sar*/*sar*) mice were analyzed for sarcosine dehydrogenase and cytochrome *c* oxidase activity

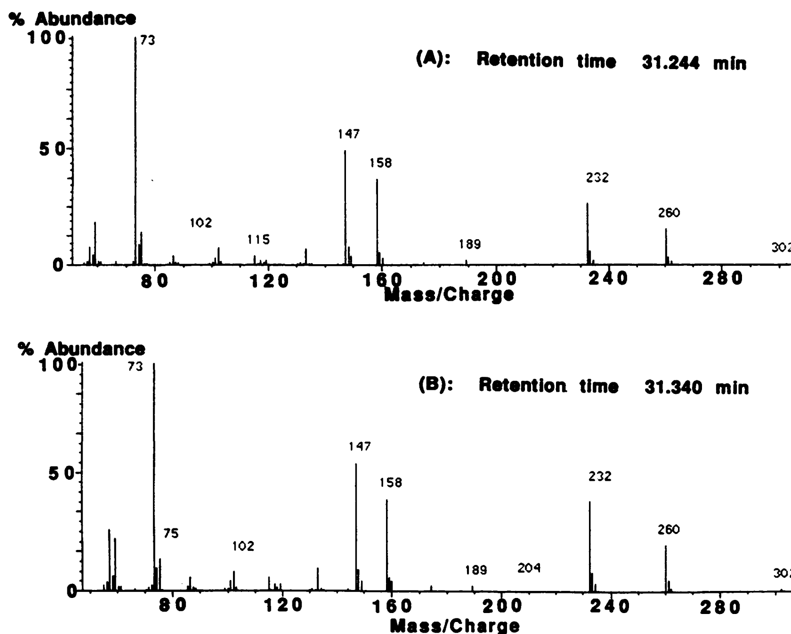

FIG. 2. Electron impact mass spectra of the TBDMS derivative of the amino acid analyzer fraction containing the suspected sarcosine from the affected mouse (*A*) and the TBDMS derivative of authentic sarcosine (*B*). Ordinate indicates percentage abundance relative to the fragment present in the greatest amount.

Table 1. Comparison of plasma and urine amino acid levels in normal mice (+/+) and heterozygotes (*sar*/+) or homozygotes (*sar*/*sar*) for sarcosinemia

	Plasma, μmol/liter			Urine, mmol per g of creatinine		
Genotype	Sarcosine	Glycine	Methionine	Sarcosine	Glycine	Methionine
+/+	ND	263 ± 21.1	75.7 ± 6.24	ND	0.78 ± 0.17	0.14 ± 0.11
	(n = 5)	(n = 5)	(n = 5)	(n = 4)	(n = 4)	(n = 4)
sar/+	ND	286 ± 33.0	87.1 ± 18.3	ND	1.32 ± 0.42	0.08 ± 0.07
	(n = 7)	(n = 7)	(n = 7)	(n = 4)	(n = 4)	(n = 4)
sar/*sar*	785 ± 153	430 ± 99.9	112 ± 29.4	102 ± 58.3	1.09 ± 0.54	0.29 ± 0.08
	(n = 8)	(n = 8)	(n = 8)	(n = 23)	(n = 23)	(n = 23)

ND, not detected. Lower limit of detection for sarcosine is 5 μmol/liter.

(Table 2). Mean sarcosine dehydrogenase activity was 0.54 ± 0.61 nmol per min per mg of mitochondrial protein in *sar*/*sar* mice, while it was 3.14 ± 1.20 and 2.98 ± 1.53 activities in the +/+ and *sar*/+ mice, respectively. The ≈83% decrease in sarcosine dehydrogenase activity in *sar*/*sar* mice as compared to normal mice was statistically significant ($P < 0.001$). Since the assay is inaccurate at these low levels, the exact percentage of normal activity in the *sar*/*sar* mice was indeterminate. The slight decrease in the enzymatic activity in the *sar*/+ mice as compared to the +/+ mice was not statistically significant. In a 1:1 mixture of the mitochondria preparations from +/+ and *sar*/*sar* mice, sarcosine dehydrogenase activity was 45% of the activity in pure +/+ extract (data not shown).

Among all three groups of mice, +/+, +/*sar*, and *sar*/*sar*, there was no significant difference in cytochrome *c* oxidase activity. Therefore, the deficiency in sarcosine dehydrogenase activity in the *sar*/*sar* mice represents an isolated defect and is not an artifact of mitochondrial preparation.

Genetic Analysis. We tested 202 progeny of 22 +/*sar* × +/*sar* crosses by paper chromatography for the presence of sarcosine in their urine: 43 were sarcosinuric. This number was not significantly different from the 50 expected for autosomal recessive inheritance [$\chi^2 = 1.5$; degrees of freedom (df) = 1]. Five *sar*/*sar* × +/*sar* crosses yielded 17 sarcosinuric mice of 35 total progeny. This fraction is nearly identical to that expected for autosomal recessive inheritance ($\chi^2 = 0.029$; df = 1). The *sar*/*sar* mice are also being bred to congenicity on the C57BL/6J background.

Phenotype Analysis. Sarcosinemic patients have been reported to have growth retardation (11), but normal and *sar*/*sar* mice weighed about the same at 1 and 4 months (data not shown). Also, the livers, brains, and kidneys weighed about the same in 6-month-old normal and sarcosinemic mice. Microscopic examination of brain and liver tissue slices stained with hematoxylin and eosin did not reveal any abnormalities (data not shown).

DISCUSSION

A mouse mutant was shown by amino acid paper chromatography to have large amounts of sarcosine in the urine. The identity of the elevated metabolite as sarcosine was confirmed by column chromatography and GC-MS. Sarcosine was also detected by column chromatography in plasma from the mutant mice. The plasma sarcosine level of 785 ± 153 μmol/liter in the homozygotic mice was at the upper range of the plasma sarcosine levels (53–760 μmol/liter) observed in humans with sarcosinemia (11). Similarly, the urinary sarcosine level of 102 ± 58 mmol per g of creatinine in the homozygotic mice was at the upper range of the urinary levels (1.5–45 mmol of sarcosine per g of creatinine) observed in affected patients (11). The slightly elevated levels of plasma glycine and plasma and urine methionine that were noted in the mutant mice have not been noted in sarcosinemic humans and remain unexplained. The urinary levels of glycine, proline, and hydroxyproline, which are reabsorbed by the same renal tubule transport system as that of sarcosine (22), were normal, as they are in human patients.

Table 2. Comparison of mitochondrial enzymatic activities in liver extracts of normal (+/+) mice and heterozygotes (*sar*/+) or homozygotes (*sar*/*sar*) for sarcosinemia

Genotype	Sarcosine dehydrogenase	Cytochrome *c* oxidase
+/+	3.14 ± 1.23	53.5 ± 30.8
	(n = 12 assays, 3 mice)	(n = 9 assays, 3 mice)
sar/+	2.98 ± 1.53	80.4 ± 55.5
	(n = 6 assays, 2 mice)	(n = 6 assays, 2 mice)
sar/*sar*	0.540 ± 0.614	92.3 ± 76.5
	(n = 27 assays, 8 mice)	(n = 24 assays, 8 mice)

Mean values ± SD are given in nmol per min per mg of mitochondrial protein.

Elevated levels of sarcosine have been described in humans with folate deficiency (11, 23), "primary sarcosinemia" due to sarcosine dehydrogenase deficiency (11), and glutaric aciduria type II (GAII, also known as multiple acyl-CoA dehydrogenase deficiency) due to deficiency of electron transfer flavoprotein (ETF) or ETF dehydrogenase (24–26). The sarcosinemia phenotype in these mice was inherited as an autosomal recessive trait like that observed in people with primary sarcosinemia or GAII (11). Folate deficiency is also unlikely because folate intake was normal in these mice and folate supplementation did not correct the sarcosinuria (data not shown).

The decreased sarcosine dehydrogenase activity in *sar*/*sar* mice could be due to deficiency either in the sarcosine dehydrogenase itself or in its electron acceptor, the ETF, and ETF-ubiquinone oxidoreductase (ETF dehydrogenase) proteins. However, the use of 2,6-dichlorophenolindophenol and PMS as electron acceptors in the assay conditions negates the requirement of ETF and ETF-ubiquinone oxidoreductase for sarcosine dehydrogenase activity. A primary defect in sarcosine dehydrogenase is most likely. The lack of any elevated levels of lysine, glutaric acid, 2-hydroxyglutaric acid, and dicarboxylic acid as analyzed by the relatively sensitive GC-MS technique suggests that these mice do not have GAII. In summary, the deficiency in sarcosine dehydrogenase enzymatic activity and the lack of metabolites characteristic in GAII suggest that the *sar*/*sar* mice have the same genetic defect as humans with primary sarcosinemia. Further definition of the genetic defect must await the cloning of the gene for sarcosine dehydrogenase.

The normal plasma and urinary levels of sarcosine in the heterozygote (*sar*/+) mice are consistent with the normal levels of sarcosine in the blood and urine of some but not all obligate human carriers, even after sarcosine loading (11). Interestingly, *sar*/+ mice were not deficient in sarcosine dehydrogenase activity. Sarcosine dehydrogenase activity is unknown in human heterozygotes because it mostly resides in the liver with little activity in the kidneys and no activity in other tissues. Equal mixing of liver extracts from normal and sarcosinemic mice yielded 45% of normal sarcosine

dehydrogenase activity, indicating that the particular assay used in this study would have been able to detect a possible reduction in activity in the carriers. The mixing results also indicate that there is no inhibitor of enzymatic activity in the livers of sarcosinemic mice as was described in a particular strain of hyperphenylalaninemic mice (27).

No obvious symptoms or pathology have been observed in the sarcosinemic mice after a limited search that included assessment of growth and gross and microscopic examinations of tissues including brain and liver. In humans, it is unclear whether sarcosinemia causes pathology. Sarcosinemia was initially described by Gerritsen and Waisman in a child with mental retardation after screening patients at the Central Wisconsin Center, a state institution for the mentally retarded (28). This patient also had hypertonia, tremors, and failure to thrive. Subsequently, five more patients with sarcosinemia and neurologic symptoms have been described (23, 28–30). Several other patients with sarcosinemia have been described without neurologic symptoms but with a variety of symptoms that included short stature, hepatomegaly, or vomiting (10, 22, 31–33). The causal relationship between sarcosinemia and disease has been questioned because of the observation of sarcosinemia in several patients without symptoms (10). More sensitive behavior testing would be required to determine whether the mice have mental or neurologic deficits. Although the exact degree of enzymatic deficiency is unknown in these mice, more severe alleles can be obtained by ENU mutagenesis and easier breeding schemes. The presence of any deficit in sarcosinemic mice would provide support for a causal relationship between symptoms and sarcosinemia in humans. However, the lack of any symptoms in the mice would not necessarily exclude a causal relationship since mouse genetic models may be imperfect models of their disease in humans (34).

This mouse model will have immediate use for further studies of the one-carbon cycle in mammals (35). In addition, the breeding of the defective sarcosinemia gene onto a congenic background would enable this mouse model to be used for studies of liver and hepatocyte cell transplantation. Also, the eventual cloning of the gene for sarcosine dehydrogenase would enable this mouse model to be used for the development of approaches for gene therapy. The ability to easily and quickly analyze sarcosine levels in blood and urine increases the utility of this model for such purposes. A variety of mouse models will provide useful assays for different gene therapy approaches, just as a variety of reporter genes and assay systems such as chloramphenicol acetyltransferase, luciferase, β-galactosidase, and human growth hormone have provided different measures of gene transfer and expression *in vitro* and *in vivo*. This sarcosinemic mouse will be particularly useful for gene therapy approaches attempting to correct hepatic IEM in which disease is caused by an inability to clear a circulating toxic metabolite. The lack of any phenotype and the normal breeding of the sarcosinemic mice simplifies the care and handling for this purpose.

This study confirms the ability of the combination of ENU mutagenesis, back-crosses, and screening to generate genetic mouse models for IEM. It suggests that this approach will be especially useful for creating genetic mouse models for those IEM in which the genetic defects are currently unknown.

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