Homocystinuria due to Cystathionine Synthase Deficiency

STUDIES OF NITROGEN BALANCE AND SULFUR EXCRETION

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ABSTRACT Apparent nitrogen balances and urinary sulfur excretions were determined for normal subjects, seven cystathionine synthase-deficient patients, and a single cystathioninuric patient on semisynthetic diets containing low-adequate amounts of methionine and very low amounts (12 mg daily, or less) of cystine. The amounts of supplemental cystine required to prevent abnormally high nitrogen or sulfur losses were determined. The five cystathionine synthase-deficient patients who had low residual activities of this enzyme detected in fibroblast and/or liver extracts did not lose more nitrogen or sulfur on diets virtually devoid of cystine than did the normal subjects. These results suggest that the widely expressed opinion that cystine is an essential amino acid for cystathionine synthase-deficient patients requires modification. Residual enzyme activity of only a few percent of normal may obviate such a cystine requirement. These results are compatible with, and lend support to, the working hypothesis which states that the pyridoxine response in cystathionine synthase-deficient patients is mediated by an increase in the residual activity of the affected enzyme.

INTRODUCTION

The transsulfuration pathway in mammals provides a route whereby the sulfur of methionine becomes the sulfur of cyst(e)ine. Methionine is demethylated via two intermediates to form homocysteine, which is combined with serine by the action of cystathionine synthase (EC 4.2.1.22) to yield the thioether, cystathionine ($HOOC(NH_2)CHCH_2CH_2SCH_2CH(NH_2)COOH$)

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(reaction 1). Cystathionine is cleaved by γ-cystathionase (EC 4.4.1.1) to form cysteine and α-ketobutyrate (reaction 2):

$$\text{homocysteine} + \text{serine} \xrightarrow[\text{synthase}]{\text{cystathionine}} \text{cystathionine} + H_2O \quad (1)$$

$$\text{cystathionine} + H_2O \xrightarrow{\gamma\text{-cystathionase}} \text{cysteine} + \alpha\text{-ketobutyrate} + NH_3 \quad (2)$$

$$\text{homocysteine} + \text{serine} \rightarrow \text{cysteine} + \alpha\text{-ketobutyrate} + NH_3 \quad (1) + (2)$$

Humans have been discovered with genetically determined deficient activity of either cystathionine synthase (1, 2) or γ-cystathionase (3–5), the hallmark excretory abnormalities of which are, respectively, homocystinuria and cystathioninuria.

Early in the study of homocystinuria, it was pointed out that a block in the transsulfuration pathway would be expected to decrease the ability to synthesize cysteine (1, 6). Normal humans do not require exogenous cyst(e)ine (7, 8). In contrast, it seemed possible that patients with sufficiently severe cystathionine synthase deficiency would require an exogenous supply of this amino acid. For these patients, cyst(e)ine would have become an essential amino acid (1, 6). This theoretical expectation was apparently confirmed when Brenton, Cusworth, Dent, and Jones carried out nitrogen balance studies on a single homocystinuric patient. This patient went into negative nitrogen balance when daily cystine intake was decreased to 85 mg. Nitrogen balance was restored when the cystine intake was increased to 1,085 mg (9). In subsequent reviews and texts it has

TABLE I
Patients

Patient	Sex	Age	Admission weight	Demonstration of enzyme deficiency	Responsive to pyridoxine
		yr	*kg*		
Cystathionine synthase deficient					
Wi. Cr.	M	15	79.3	Fibroblast assay (patient 16, reference 18)	Yes*
Bu. Ha.	M	8	29.3	Fibroblast assay (patient 32, reference 18)	No‡
Jo. Ho.	F	31	69.5	Liver, fibroblast assays (patient 31, references 18, 19)	Yes (19)
Ja. Is.	F	13	62.4	Fibroblast assay (patient 1, reference 18)	Yes§
Ro. Kr.	F	20	63.9	Fibroblast assay (patient 6, reference 18)	Yes§
Th. Kr.	M	31	79.5	Fibroblast assay (patient 8, reference 18)	Yes (19)
Ol. Ma.	F	19	55.9	Fibroblast assay (patient 34, reference 18)	No‖
Ca. Me.	F	20	64.5	Fibroblast assay¶	Yes§
Fr. Mi.	M	12	54.5	Fibroblast assay¶	No**
Ba. Re.	F	31	67.6	Fibroblast assay (patient 35, reference 18)	No§
Vi. Sw.	F	17	62.0	Fibroblast assay¶	No‡‡
Cystathioninuric					
Ge. Po.	M	21	74.9	Indirect evidence only§§	Yes‖‖

* Personal communication from Dr. A. Sass-Kortasak. Patient previously reported (20).
‡ Personal communication from Dr. N. Holtzmann.
§ Personal communication from Dr. V. McKusick.
‖ Patient's urinary and plasma homocystine and methionine values remained essentially unchanged despite the administration of 500 mg pyridoxine·HCl daily for 11 days. Serum folate was normal.
¶ Previously unpublished specific activities of cystathionine synthase, assayed in fibroblast extracts without addition of pyridoxal phosphate, or in the presence of pyridoxal phosphate added at final concentrations of 0.1, 1.0, and 5.0 mM were as follows: Ca. Me., 0.46, 0.64, 0.83, 0.73; Fr. Mi., 0.59, 0.70, 0.96, 0.65 (mean of two assays); Vi. Sw. 0, 0, 0, 0 (i.e., <0.10). Activities are in nmol cystathionine/mg protein/135 min; control mean, 31.7; range, 3.7–60.0 (18).
** Personal communication from Dr. G. Morrow, III. Morrow's observation was confirmed during the present studies. Hypermethioninemia, homocystinemia, hypocystinemia, and homocystinuria all remained relatively constant during 10 days of treatment with a daily oral dose of 500 mg pyridoxine·HCl.
‡‡ After taking 3 mg folic acid and 300 mg pyridoxine·HCl daily for several months, the patient had elevated urinary homocystine and methionine levels. 1 mo after decreasing the folic acid to 2 mg twice weekly and discontinuing the pyridoxine, no significant change in urinary homocystine or methionine was noted.
§§ Cystathionine was elevated in plasma and urine. Patient was free of nongenetic conditions known to cause cystathioninuria (21).
‖‖ The classification of Ge. Po. as a responder to B_6 is based upon the response of his cystathioninuric brother (21).

been widely and generally stated that cystine is an essential amino acid for cystathionine synthase-deficient patients (2, 10–17).

Recent indications of genetic heterogeneity in cystathionine synthase-deficient patients (18), in conjunction with an increasing realization of the possible functional significance of small residual activities of cystathionine synthase in deficient patients (19), led us to question whether the generally accepted opinion of the essential nature of cyst(e)ine for such patients were not an oversimplification. As explained more fully in the Discussion, it seemed possible that patients with as little as 1–2% of the normal activity of cystathionine synthase might *not* require cyst(e)ine. To test this hypothesis, we determined the amount of cystine required to maintain short-term nitrogen balance in a series of cystathionine synthase-deficient patients. As will be reported, we found that under the conditions employed those cystathionine synthase-deficient patients with low, but detectable, residual enzyme activities had no greater need for exogenous cystine than did normal volunteer subjects.

METHODS

Subjects. The control subjects were normal volunteers aged 18–24 yr, in good health as indicated by medical histories, physical examinations, and laboratory tests, including urinary amino acid analyses.

Patients. Information regarding the patients is summarized in Table I. Presently, certain vitamin B_6-responsive cystathionine synthase-deficient patients are maintained, as a therapeutic measure, on large supplemental doses of pyridoxine (16, 17, 22). To study such patients in "basal" states, their vitamin B_6 intakes were limited to that contained in their diets for a minimum of 1 mo before the beginning of the present studies. This was sufficient time

for the urinary homocystine excretions of responsive patients previously under treatment with pyridoxine to return to pretreatment levels (19) and for urinary 4-pyridoxic acid excretions to return to normal (see Results). The cystathioninuric patient had not recently received pyridoxine treatment.

Experimental plan. During the present work, several studies were performed, each requiring a different diet. (*a*) When nitrogen balance and sulfur excretion studies were performed concomitantly, each subject received a constant natural diet (the "equilibration diet") until nitrogen balance was attained (approximately 6–15 days), after which he was placed on a semisynthetic diet (the "experimental diet") containing approximately the same amount of nitrogen as the equilibration diet. For a period of 0–3 days after switching to the experimental diet, adjustments were made in caloric intake and the other dietary components until the subject's weight remained stable and the diet was acceptable to him. Thereafter, the experimental diet became constant, and was divided into 6-day dietary periods, during each of which a specified amount of L-cystine was added to the diet. During all studies the subjects were inpatients at the Clinical Center of the National Institutes of Health. They were instructed to maintain reasonably constant levels of activity, avoiding strenuous exercise. 24-h urine collections were made under toluene and were refrigerated until volumes were measured and samples frozen. Stool collections were pooled for each 72-h period. Fasting blood specimens were obtained during the latter half of each dietary period. (*b*) For sulfur partition studies, subjects received constant natural diets calculated to provide half of the dietary sulfur in the form of methionine and half in the form of cystine. These diets provided approximately 0.14 mmol of methionine and 0.07 mmol of cystine (i.e., 0.14 mg-atom of cystine sulfur)/kg body wt.

Diets. All of the diets were prepared and served under the supervision of a dietician trained in the techniques of metabolic balance studies. The equilibration diets provided between 10.0 and 11.2 g of nitrogen (as indicated by calculation and analyses), 1,175–1,563 mg of methionine, and 912–1,197 mg of cystine. These dietary amino acid intakes were calculated from published tables (23).

The semisynthetic experimental diet consisted of foods and beverages low in methionine and cystine, including cookies, muffins, and pancakes prepared from Cellu Low Protein Baking Mix, Cellu Pasta Imitation Macaroni (both Cellu products from Chicago Dietetic Supply House, Inc., La Grange, Ill.), butter oil, jelly, air mints, sour balls, vinegar, oil, various vegetables, fruits, and fruit juices, carbonated beverages, coffee, tea, and sugar. Controlyte (The Doyle Pharmaceutical Company, Minneapolis, Minn.), a high calorie dietary supplement with trace protein content (1.4 mg methionine and 0.7 mg cystine/100 g) was used in the form of a fruit-flavored beverage as a source of calories and as a vehicle for the supplemental amino acids. Together, these materials provided 0.4–1.2 g of nitrogen (calculation and analysis), approximately 14–55 mg of methionine (calculation), and 3–12 mg of cystine (calculation). To these materials were added supplemental amino acids as shown below and in the individual studies.

Amino acids. The eight essential amino acids plus arginine, histidine, and tyrosine were added to the experimental diet in their pure L-forms in the approximate quantities found in 20 g of whole egg protein. The amounts added were (in grams per day): L-arginine, 1.31 (as equivalent quantity of hydrochloride salt); L-histidine, 0.48; L-isoleucine, 1.33; L-leucine, 1.76; L-lysine, 1.28 (as equivalent quantity of hydrochloride salt); L-methionine, 0.63; L-phenylalanine, 1.16; L-threonine, 1.00; L-tryptophan, 0.33; L-tyrosine, 0.86; L-valine, 1.49. For patient Fr. Mi. an additional 300 mg of L-lysine and 170 mg of L-methionine were added to meet the published amino acid requirements for his age group (24). Otherwise, methionine and cystine were added as specified in the individual studies. Glycine was added to bring the total supplemental amino acid nitrogen to 10.0 g. Thus, total nitrogen content of the experimental diet (food nitrogen plus supplemental amino acid nitrogen) ranged from 10.4 to 11.2 g/day, depending on each subject's weight and caloric requirements.

Vitamin and mineral supplements. While on the experimental diet all subjects received one 0.5-mg pyridoxine·HCl capsule daily and one especially formulated combination vitamin and mineral capsule which supplied 10,000 U vitamin A, 400 U vitamin D, 5 mg thiamine mononitrate, 5 mg riboflavin, 25 mg niacinamide, 2 μg cyanocobalamin, 5 mg calcium pantothenate, 100 mg ascorbic acid, 10 mg ferrous sulfate, 1 mg copper sulfate, 150 μg calcium iodate, 1 mg manganese sulfate, 5 mg magnesium oxide, and 1.5 mg zinc sulfate. During initial studies, five subjects (Wi. Cr., Ba. Ha., Ba. Re., Ka. Ro., and An. To.) did not receive these two capsules, but instead one commercial multivitamin tablet (Dayalets-M, Abbott Laboratories, North Chicago, Ill.) daily. The contents of this tablet differed from those of the especially formulated combination vitamin and mineral capsule only in that the commercial tablet provided a total of 2.0 mg of pyridoxine·HCl and approximately twice the amount of each of the minerals. Throughout all of the studies, each subject received 2 mg of folic acid twice weekly.

Medications. Patients Jo. Ho., Ca. Me., and Ba. Re. each necessarily received certain medication(s) during the present studies. Patient Jo. Ho. received 60 mg isoxsuprine·HCl daily throughout all of the studies. Patient Ca. Me. received 30 mg phenobarbital twice daily and 100 mg diphenylhydantoin twice daily throughout the studies. Patient Ba. Re. received 75 mg of amitriptyline·HCl and 50 mg hydrochlorthiazide daily throughout her first two studies. Urinary sulfur partition (Table VI) was measured during the first study, and nitrogen balances on 109, 309, and 509 mg cystine (Table IV) were determined during the second. During her second study she was also given 300 mg and subsequently 400 mg of sulfinpyrazone daily. During her third study (nitrogen balance on 8 and 1,008 mg cystine [Table IV]) she was no longer on any of the above medications, and she received 50 mg spironolactone and 400 mg of dipyridamole daily.

Methionine loads. For methionine loading studies, the patient was placed on the constant natural diet used for sulfur partition studies. After the total sulfur output had remained relatively constant for several days, L-methionine was given in gelatin capsules, each containing 0.25 g of L-methionine. The subject was fasted from 10 p.m. the previous night, and the amino acid was administered at 8 a.m. in a dose of 0.5 mmol/kg body wt. Breakfast was fed at 9 a.m. 24-h urines were collected before and after the methionine load.

Analytical methods. Nitrogen analyses were performed by use of a micro-Kjeldahl method (25). Values obtained for fecal nitrogen excretions over 72 h were divided by 3 to give mean daily fecal nitrogen outputs. Urinary creatinines were determined by the method of Chasson, Grady, and Stanley (26). Urinary and plasma amino acids were determined with an automatic amino acid analyzer with the buffer system of Spackman, Stein, and Moore (27). 4-Pyridoxic acid concentrations in the urine were determined

by the microprocedure of Woodring, Fisher, and Storvick (28). Serum folate concentrations were determined by the procedure of Harper (29).

Differential analyses of urinary inorganic sulfate, total sulfate (i.e., inorganic sulfate + "ethereal" sulfate), and total sulfur were performed according to the general procedure of Rosenheim and Drummond, as specified in Hawk, Oser, and Summerson (30). A detailed description is given, since the methods we are currently using involve a number of modifications of the original method.

For phosphate removal, up to 12.5 ml of urine was diluted with water to a volume of approximately 25 ml in a 50-ml volumetric flask, and the solution made basic with NH_4OH (approximately 5 N) with phenolphthalein as indicator. After addition of 5 ml 5% NH_4Cl and an aqueous slurry containing 0.75 g $MgCO_3$ (Baker reagent: $4MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$) in 5 ml, the preparation was diluted to volume and inverted five times. The mixture was inverted at 3-min intervals for 15 min, allowed to stand for 15 min, mixed again, and centrifuged. Aliquots of the supernatant fluid were taken for analyses.

For analysis of inorganic sulfate, an aliquot (usually 5 ml) of the phosphate-free supernatant fluid was brought to a total volume of 10 ml in a 50-ml beaker and acidified with HCl to bromphenol blue. To precipitate sulfate ions, the sample was chilled for 15 min at 4°C, and mixed with 2 m cold benzidine reagent (5 g benzidine dihydrochloride and 50 ml 1 N HCl in a total volume of 250 ml, made fresh each week). After 5 min at 4°C, 5 ml 95% acetone was added, and the mixture allowed to stand an additional 15 min at 4°C. Precipitate was isolated by filtration through glass fiber filter paper in Gooch crucibles and washed twice with 2-ml portions of cold 95% acetone and once with a 5-ml portion. The precipitate, together with the filter paper, was transferred to a 250 ml Erlenmeyer flask. Boiling water was used to wash the crucible to ensure complete transfer and to suspend the filter paper and precipitate. The precipitate dissolved slowly during the subsequent titration. Titration was carried out with 0.01 N NaOH at approximately 100°C with constant mechanical stirring to a lasting phenol-red end point. The normality of the NaOH was determined at each use by titration against commercial standard HCl.

For analysis of total sulfate (inorganic and ethereal), an aliquot (5–10 ml) of the phosphate-free supernatant fluid was subjected to acid hydrolysis to convert ethereal sulfate to inorganic sulfate. The aliquot was mixed with 1 ml 1 N HCl in a porcelain evaporating dish and taken to dryness over boiling water. (Unless otherwise specified, heating and drying were routinely done on a boiling water bath.) The residue was transferred to a 50-ml beaker with two 5-ml portions of distilled water. No acidification of this sample was required before the subsequent steps, which were the same as those described above for the acidified aliquot analyzed for inorganic sulfate.

For analysis of total sulfur, an aliquot (5–10 ml) of the phosphate-free supernatant fluid was subjected to oxidation to ensure that all sulfur would be in the form of inorganic sulfate. The aliquot was taken to dryness in an evaporating dish together with 0.25 ml Benedict's reagent (20% Cu $[NO_3]_2 \cdot 3H_2O$, 5% $KClO_3$), reconstituted with 1 ml distilled H_2O and 0.25 ml Benedict's reagent, and again taken to dryness. The residue was heated to redness with a Bunsen burner for 3 min. The carbon-free residue was reconstituted with 1 ml 2 N HCl and taken to dryness. One drop of 2 N HCl was added to the evaporating dish, and the dry residue was transferred with two 5-ml portions of distilled H_2O to a 50-ml beaker. A clear blue solution should result from this procedure. Subsequent steps were the same as those used for the 10-ml acidified aliquot analyzed for inorganic sulfate except that the precipitate was washed once with 2 ml cold 50% acetone and then with 2 ml and 5 ml cold 95% acetone.

Further modifications were required for samples very low in sulfur because of low dietary intake. Initial samples of approximately 1% of a daily urinary volume were diluted to 21 ml. 10 ml of the diluted specimen was treated for phosphate removal with half the reagent quantities previously described and ending with a volume of 25 ml. Appropriate aliquots, usually 7.5 ml, were then treated as usual except that precipitates were titrated with 0.005 N NaOH.

Precipitates containing less than 5 μmol or more than 25 μmol sulfate often gave unsatisfactory results, and sample sizes were adjusted accordingly. It was also found that total sulfur results tended to be spuriously low if the starting sample exceeded 1–2% of the total daily urine volume. Nonsulfate sulfur was calculated as total sulfur *minus* total sulfate. Since this value and that for ethereal sulfate (total sulfate *minus* inorganic sulfate) usually depend upon relatively small differences between two larger experimentally determined quantities, it was important to perform the three determinations (inorganic sulfate, total sulfate, total sulfur) for a given specimen in parallel during a single analytical run. All determinations were done in duplicate, and the mean result is reported. Usually four urine samples could be handled in this way during a single day. Determinations were repeated if disagreement between duplicates was more than 10%.

Fecal specimens were assayed for total sulfur only by a modification of the above method. After collection, samples were homogenized with approximately 2 weights H_2O and stored frozen. Aliquots of 3 g total weight were mixed with further H_2O to a total volume of 10 ml. 0.5 ml Benedict's reagent was added, and the suspension was dried in an evaporating dish over boiling H_2O and heated for 3 h in a muffle oven, attaining a temperature of 300°C within an hour and a final temperature of 650–700°C. After cooling, the residue was dissolved in 1 ml 2 N HCl and dried. The cooled residue was reconstituted with 1 drop 2 N HCl and two 5-ml portions of H_2O and transferred to a 25-ml volumetric flask. Phosphate removal, as previously described, was carried out by using the appearance of the intense blue color of the copper-ammonia complex ion as indicator, corresponding to a pH of 7.0–7.5. 10 ml of the phosphate-free supernatant fluid was then assayed according to the usual procedure for inorganic sulfate. In 13 runs using this procedure the mean recovery of an added mixture containing equal amounts of methionine sulfur and cyst(e)ine sulfur was 56.8±3.3% (SE) (range, 34–74%).

Growth of fibroblasts and sensitive assay of cystathionine synthase activity. These procedures were performed essentially as previously described (18).

Treatment of the data. In calculating nitrogen balances, only those days were considered during which the patients were on the experimental diets and adjustments of caloric intakes had been completed. From these, all days in which urine collections were lost or known to be incomplete were eliminated, leading to the omission of five of a potential total of 204 subject-days for the studies reported here. The urinary creatinine excretions for the remaining days for each patient were then analyzed by the methods of Grubbs to identify any statistical outliers (31). Excretions for these outlying days (a total of 11) were considered suspect and were eliminated from the subsequent analyses. Nitrogen bal-

ances were then obtained by subtracting the daily values for urinary and fecal nitrogen from the total nitrogen intake provided by food, vitamin and mineral capsules, supplemental amino acids, and medications.

Excess urinary sulfur was calculated as dietary sulfur intake *minus* urinary sulfur excretion. The shift from the equilibration diets to the experimental diets involved decreases in total sulfur intakes (unlike nitrogen intakes, which remained relatively constant). Thus, sulfur excretions usually decreased during the first 2 days on the experimental diets and assumed more or less constant values from the 3rd day through the end of the period. For this reason, only those days eligible after the statistical analyses described above and included among the last 3 days on the experimental diets were used in calculating excess urinary sulfur values.

RESULTS

The homocystine excretion of cystathionine synthase-deficient patients is known to be affected by two vitamins, pyridoxine (16, 17, 19) and folic acid (32, 33). Some patients respond to large doses of B_6 with decreases in plasma and urinary homocystine and methionine, whereas other, unrelated patients show no such response (16, 17, 19). These responses to B_6 are thought to be genetically determined manifestations of heterogeneity in cystathionine synthase-deficient patients (18). Therefore, each patient was classified with respect to his pyridoxine responsiveness (Table I), and the experiments were designed so that each patient would be in his basal state during the present studies, rather than in pyridoxine-induced response. To determine relative pyridoxine intakes, urinary 4-pyridoxic acid excretions were measured. The excretion of this compound provides an index of B_6 intake, being equivalent to approximately 50% of the vitamin ingested by subjects on normal diets or receiving small supplements (34). For the present studies, 4-pyridoxic acid excretions were measured during the latter half of both the equilibration and experimental diet periods (Table II). During the equilibration periods no vitamin supplements were given, and 4-pyridoxic acid excretions were close to or within the ranges reported for humans on normal diets by other workers using the same method of determination (0.27–1.08 mg/day [34]; 0.65–1.32 mg/day [28]). During the experimental dietary periods the first five subjects to be studied (Wi. Cr., Ba. Ha., Ba. Re., Ka. Ro., and An. To.) received 2 mg pyridoxine daily in commercial multivitamin tablets and had slightly higher urinary excretions of 4-pyridoxic acid than during the preceding equilibration periods when no vitamin supplement was given. Under no circumstances, however, did urinary 4-pyridoxic acid excretion exceed 1.65 mg, a value only slightly above the normal range. In subsequent studies the pyridoxine·HCl supplement was decreased from the 2.0 mg supplied by the commercial multivitamin tablets to 0.5 mg. On this regimen all subjects had lower urinary 4-pyridoxic acid excretions during the experimental periods than during the preceding equilibration periods.

TABLE II
Urinary 4-Pyridoxic Acid Excretions

Subject	4-Pyridoxic acid excretion		Pyridoxine·HCl supplement on experimental diet*
	Equilibration diet	Experimental diet	
	mg/day		*mg/day*
Normal volunteers			
Ba. Ha.	0.75	1.13	2.0
Ka. Ro.	0.80	1.25	2.0
An. To.	0.55	0.83	2.0
Re. Da.	0.75	0.68	0.5
An. Na.	1.15	0.50	0.5
St. Po.	0.50	0.50	0.5
Cystathionine synthase deficient			
Vi. Sw.	1.10	0.40	0.5
Ba. Re.	1.03	0.50	0.5
Fr. Mi.	0.58	0.30	0.5
Wi. Cr.	1.37‡	1.65‡	2.0
Jo. Ho.	0.95‡	0.57‡	0.5
Ro. Kr.	0.98	0.40	0.5
Ca. Me.	0.88	0.35	0.5
Cystathioninuric (presumptive γ-cystathionase deficient)			
Ge. Po.	1.20	0.35	0.5

* Supplied as either 2.0 mg in Dayalets-M tablet or as 0.5 mg in separate capsule.
‡ Value represents average of two measurements.

Cystathionine synthase-deficient subjects tend to develop abnormally low serum folate concentrations when receiving normal folate intakes (32). To ensure that all subjects were replete with folate during the present experiments, each was routinely given supplemental folic acid (2 mg twice weekly). Several patients had subnormal serum folic acid concentrations upon admission to hospital (lowest, 3 ng/ml; normal, 5–21 ng/ml), but repeat determinations before, or during, the experimental diet periods were in all cases within or above the normal limits.

Nitrogen balance, excess urinary sulfur, and sulfur balance

Normal controls. A typical balance study for a normal subject is shown in Fig. 1. At the outset of this experiment the subject was changed from an equilibration diet to an isonitrogenous experimental diet on which the total methionine intake was 685 mg and the total cystine intake 10 mg. After the first 2 days, because of an apparent weight gain, the calorie intake was reduced from 49.0 to 45.1 cal/kg, and the weight was effectively stabilized. On day 9 the methionine intake was increased to 1,155 mg by the addition of further supplemental free amino acid. The urine collections for days 9 and 14 were known to be incomplete. The creatinine excretion for day 7 indicated this collection

TABLE III

*Apparent Nitrogen Balance and Excess Urinary Sulfur: Normal Volunteers on Cystine-Poor Diet**

Subject‡	Sulfur amino acid intake		Apparent nitrogen balance	Excess urinary	
	Met	Cys		Nitrogen	Sulfur
	mg/day		*g/day* (±*SE*)	*g/day* (±*SE*)	*mg-atom/day* (±*SE*)
Females					
Ba. Ha.	662	9	+0.31±0.30	+1.32±0.24	−0.3±0.1
St. Po.§	661	3	+0.01±0.22	+0.72±0.22	−1.7±0.2
Ka. Ro.	670	11	−0.15±0.27	+0.54±0.22	−1.6±0.6
An. To.	659	10	+1.32±0.49	+1.64±0.49	−1.1±1.3
Males					
Re. Da.	683	11	+0.22±0.38	+1.43±0.42	−1.6±0.4
Fr. Li.	667	8	−0.53±0.13	+0.36±0.13	−2.1±0.1
	1,137	8	+0.39±0.36	+1.03±0.40	+0.1±0.2
An. Na.	670	9	+2.30±0.45	+2.71±0.44	−0.1±0.5
Jo. Ro.	685	10	+0.34±0.45	+1.16±0.40	−2.6±0.4
	1,155	10	−0.43±0.24	+0.24±0.22	−1.8±0.5

* Experimental plan, diet, and methods of treating the data are described in Methods. The actual data from which these balances were calculated have been deposited with the National Auxiliary Publications Service (NAPS document no. 02536; ASIS/NAPS, Microfiche Publications, New York). The nitrogen intakes ranged from 10.49–11.07 g/day. Caloric intakes varied from 39.0–54.8 cal/kg body wt and for each subject had been adjusted before the experimental period and were then kept constant, so that during these periods weight changes were small. Ba. Ha. was exceptional in that the initial caloric intake was excessive and was decreased on day 3 of the study. During the study this subject gained 1.4 kg.

‡ All subjects were normal volunteers between 18 and 24 yr old.

§ The equilibration diet for this subject was unusual and provided approximately 450 mg of methionine and 375 mg of cystine, with additional nitrogen furnished by supplemental L-glycine, bringing the total dietary nitrogen to approximately 10.9 g.

to be an outlier (31). These days were eliminated from consideration. It is seen that on these virtually cystine-free dietary regimens this normal control had mean nitrogen excretions which deviated from absolute balance by less than 5% of the total intake of 11.0 g of nitrogen. That is, the balances fell within what has been defined by other workers as the "zone of nitrogen equilibrium" (35, 36).

The total daily sulfur intake on the equilibration diet for this experiment was 19.4 mg-atom sulfur, and the daily urinary excretion was 18.7±0.6 (not shown in Fig. 1). The intake was decreased to 4.67 mg-atom sulfur on the experimental diet, whereupon the urinary sulfur excretion decreased for 2 days, then remained relatively constant. The excess urinary sulfur for this experimental period was calculated using the values for days 6 and 8, day 7 being eliminated as a possible outlier. For these days the mean urinary sulfur excretion was 7.25 mg-atom/day. Excess urinary sulfur thus was equal to $4.67 - 7.25 = -2.6$ mg-atom/day. Note that when urinary sulfur exceeds sulfur intake, the excess urinary sulfur has been assigned a negative value, indicative of the fact that total bodily sulfur has decreased by at least this amount. The increase in the methionine supplement on day 9 raised the total intake to 7.82 mg-atom. The excess urinary sulfur decreased somewhat to −1.8±0.5 mg-atom for the last 3 days of this period included in the analysis.

In Table III is presented a summary of all similar experiments on normal volunteers. Each of the four male and four female subjects was either within the zone of equilibrium or in positive nitrogen balance on dietary regimens containing 659–685 mg methionine and 3–11 mg cystine. Urinary nitrogen excretions generally reflect the metabolic system and are not complicated by the difficulties inherent in obtaining accurate estimates of fecal nitrogen output during short-term metabolic balance periods. Therefore, data on the "excess urinary nitrogen," (calculated as total dietary nitrogen − urinary nitrogen) are also compiled in Table III. These subjects excreted from 0.36 to 2.71 g less nitrogen in their daily urine than their daily intakes on diets containing 659–685 mg methionine and 3–11 mg cystine. On the same regimens, the urinary sulfur losses of most subjects slightly exceeded their dietary intakes, the maximum loss being −2.6 mg-atom sulfur/day. Increasing the methionine intake decreased (Jo. Ro.) or entirely ablated (Fr. Li.) the excess urinary sulfur loss.

To gain a preliminary indication of the relationship between excess urinary sulfur and the sulfur balance

and how these parameters are affected by limitation of amino acids other than methionine and cystine, a further experiment was carried out with two normal control subjects (De. Wa. and Ma. Sw.). After equilibration periods, these subjects were given experimental diets analogous to the experimental diets used for the studies reported in Table III, except that the methionine intakes were increased to 1,209–1,219 mg in an effort to avoid excessive urinary sulfur losses. On this diet one subject (De. Wa.) was in nitrogen equilibrium (+0.30±0.13 g/day), whereas, unexpectedly, the second subject (Ma. Sw.) was below the zone of equilibrium but by less than 1 SEM (−0.65±0.11 g/day). Under these conditions, the excess urinary sulfurs were +0.8 and +0.1±0.6 mg-atom/day. Fecal sulfurs were also measured, and sulfur balances were calculated as total dietary sulfur − (urinary sulfur + fecal sulfur). Sulfur losses in the stools were small relative to urinary losses,[1] and the resulting sulfur balances were +0.1 and −1.6± 0.5 mg-atom/day.

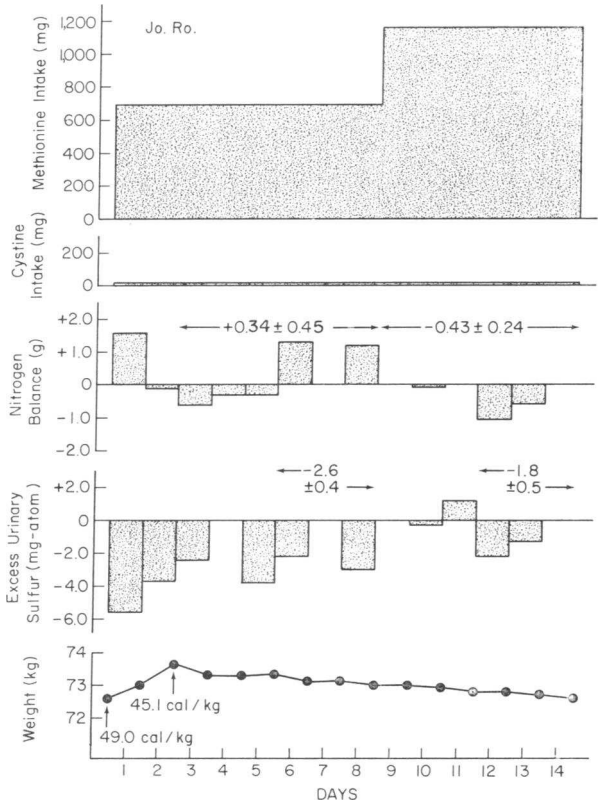

FIGURE 1 A representative study of a normal volunteer subject (Jo. Ro.). Daily methionine intakes were 685 and 1,155 mg. Daily intakes of cystine and nitrogen were 10 mg and 11.0 g, respectively, throughout. The figures for apparent nitrogen balance and excess urinary sulfur excretions are indicated as mean±1 SE in g nitrogen/day and mg-atom sulfur/day, respectively. See text for further explanation.

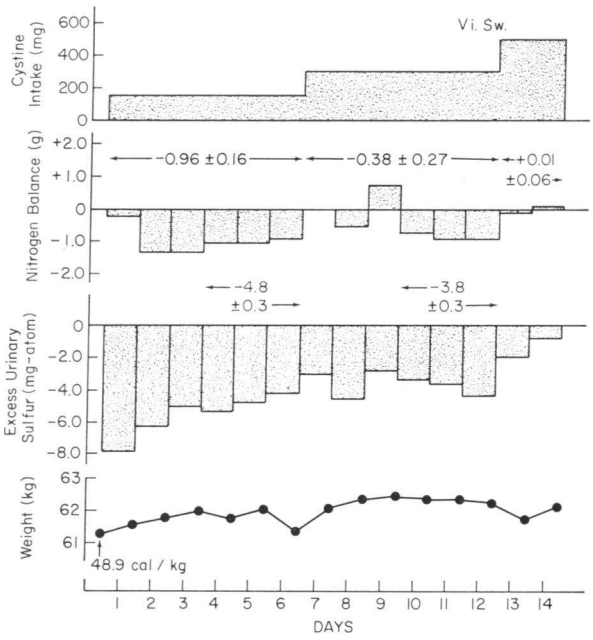

FIGURE 2 A study of a cystathionine synthase-deficient, B_6-nonresponsive patient (Vi. Sw.) on daily cystine intakes of 155, 305, and 505 mg. Daily intakes of methionine and nitrogen were 655 mg and 10.36 g, respectively, throughout. Only 2 days of study could be completed on the intake of 505 mg of cystine. See text and Fig. 1 for further explanation.

After 6 days on the experimental diet, the phenylalanine supplement was decreased so that the subjects were receiving only 100 mg daily of phenylalanine, a marginal amount for maintenance of nitrogen equilibrium on the tyrosine intake of these subjects (37). As expected, on this diet both subjects were near the lower end of the zone of nitrogen equilibrium (−0.51±0.13 and −0.63±0.24 g/day). On the same regimen, sulfur losses increased so that the excess urinary sulfur values were −1.5±0.3 and −2.5±0.1 mg-atom/day, and the sulfur balances were −2.0±0.3 and −4.6±0.5 mg-atom/day.

Cystathionine synthase-deficient patients. A study of a cystathionine synthase-deficient, B_6-nonresponsive patient (Vi. Sw.) is shown in Fig. 2. This study was similar in design to those carried out with the control subjects, except that each of the experimental dietary regimens contained some supplemental cystine. The total daily intake of this amino acid was increased progressively during the three periods from 155 to 305,

[1] This finding was typical. The fecal sulfur content of 21 stool samples from normal control subjects and cystathionine synthase-deficient patients on equilibration or sulfur partition diets averaged 13.2±1.3% of the total urinary sulfur (range 3.4–30.0%). The samples from the patients did not differ significantly from those for normal controls.

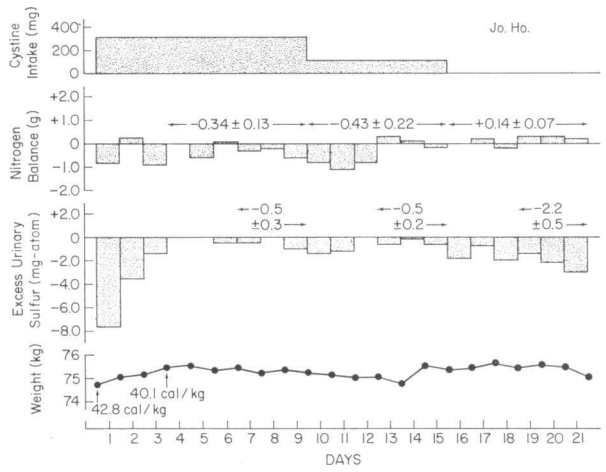

FIGURE 3 A study of a cystathionine synthase-deficient, B_6-responsive patient (Jo. Ho.) on daily cystine intakes of 312, 112, and 12 mg. Daily intakes of methionine and nitrogen were 677 mg and 10.64 g, respectively, throughout. The urinary creatinine excretion for day 4 indicated this collection to be a statistical outlier. See text and Fig. 1 for further explanation.

then to 505 mg. On the lowest cystine intake the nitrogen balance of this patient was -0.96 ± 0.15 g/day, a value significantly below the zone of equilibrium. The value for excess urinary nitrogen was less positive than the value for any control subject (Table III). The excess urinary sulfur for this period was -4.8 ± 0.3 mg-atom/day, a much more negative value than was observed for any control subject, even on lower cystine intakes (Table III). When the cystine intake was increased to 305 mg, the nitrogen balance for patient Vi. Sw. was in the lower region of the zone of equilibrium. The excess urinary nitrogen remained somewhat low. The excess urinary sulfur decreased to -3.8 ± 0.3 mg-atom/day, a value still outside the range observed in control subjects. The increase in cystine intake to 505 mg apparently decreased the excessive losses of nitrogen and sulfur, although only 2 days could be completed on this dietary regimen. Thus, as expected on the basis of the results of Brenton et al. (9), this cystathionine synthase-deficient, B_6-nonresponsive patient failed to remain in balance unless supplied with a substantial amount of dietary cystine.

The results obtained in two studies of a second nonresponsive subject, Ba. Re. were less clear-cut. In the initial balance study (Table IV, study II) this patient was below the zone of nitrogen equilibrium and lost excessive urinary nitrogen and sulfur on the lowest cystine intake (109 mg).[2] The urinary losses were reduced to acceptable levels by increased cystine intake, but the nitrogen balance remained equivocally below the zone of equilibrium. During a second balance study (Table IV, study III), similar observations were recorded with respect to urinary sulfur loss, but the nitrogen balances remained low and the urinary nitrogen losses appeared to be inconsistent with the results of study II. During these studies the two periods of lowest cystine intakes had to be discontinued during the 5th and 4th days, respectively, because of nausea, irritability, lassitude, and one episode of vomiting. These symptoms have been noted in subjects deprived of essential amino acids (7). Ba. Re. tolerated well the diets containing more cystine. Thus, the clinical results, as well as most of the chemical measurements, suggest that this patient has a cyst(e)ine requirement, but the present studies do not conclusively demonstrate this requirement because of the failure of high cystine intakes to bring all parameters unequivocally within the control ranges. The clinical status of Ba. Re. was such that she required multiple medications during these studies, and it is possible that one or more of these medications may have affected the results obtained.

For the additional five cysthathionine synthase-deficient patients studied (Table IV, and Fig. 3) the results were quite different. For none of these patients was the nitrogen balance significantly below the zone of nitrogen equilibrium, even on diets as low in exogenous cystine content as could be experimentally attained. The urinary nitrogen losses for four of the five were within the control range.

A parallel conclusion may be drawn from the results on sulfur excretion. On the lowest cystine intakes, the excess urinary sulfur excretions of these five patients ranged from $+1.1$ to -2.3 mg-atom/day. Thus, under these conditions the excess urinary sulfurs of these patients were less than those of Vi. Sw. or Ba. Re. but similar to the excess urinary sulfurs of most control subjects on similar dietary regimens. Measurements of fecal sulfur were performed during the studies of Ro. Kr. and Ca. Me. The calculated sulfur balances were -4.9 ± 0.2 and -3.4 ± 0.1 mg-atom sulfur/day, respectively.

γ-Cystathionase-deficient patient. Included in Table IV are the results of a study of Ge. Po., a patient with a presumptive block at another step in the transsulfuration pathway, the γ-cystathionase reaction (21). On a diet virtually free of cystine this cystathioninuric, B_6-re-

[2] After these dietary studies had been completed, we became aware of the not uncommon practice in the formulation of medications of adding calcium sulfate as an excipient. Being regarded as "inert," this ingredient is not specified in descriptions of the medications. Therefore, all medications taken by any patient during the course of this work were analyzed for inorganic sulfate and total sulfur. The major resulting uncertainty occurred in the case of Ba. Re., who received as much as 1.5 mg-atom inorganic sulfate and 0.2 mg-atom organic sulfur in her medications. It is difficult to correct for these intakes because the portions absorbed are not known (see footnote ¶ of Table IV).

TABLE IV

*Apparent Nitrogen Balance and Excess Urinary Sulfur: Cystathionine Synthase-Deficient or Cystathioninuric Patients on Cystine-Poor Diet with Varying Supplements**

Subjects‡	Sulfur amino acid intake		Apparent nitrogen balance	Excess urinary	
	Met	Cys		Nitrogen	Sulfur
	mg/day		*g/day(±SE)*	*g/day(±SE)*	*mg-atom/day(±SE)*
Cystathionine synthase deficient					
Vi. Sw.	655	155	−0.96±0.16	0.00±0.17	−4.8±0.3
	655	305	−0.37±0.27	+0.04±0.30	−3.8±0.3
	655	505	+0.01±0.06§	+0.52±0.06	—
Ba. Re. (study II)	667	109	−0.88±0.77‖	+0.02±0.64	−5.9 to −7.6‖¶
	667	309	−0.22±0.51	+0.56±0.50	−3.8 to −4.0±0.8¶
	667	509	−0.67±0.30	+0.45±0.86	−0.1 to −0.3±1.5¶
(study III)	662	8	−0.68±0.49‖	+0.58±0.49	−5.7 to −7.3‖¶
	662	1,008	−0.95±0.30	−0.10±0.34	+1.1 to −0.4±1.0¶
Fr. Mi.	822**	5	+1.07±0.14	+1.79±0.15	+1.1±0.2
	822	80	+2.16±0.33	+2.76±0.35	+1.9±0.3
	822	155	+0.99±0.41	+1.82±0.43	−0.6±0.6
Wi. Cr.	682	7	+0.58±0.21	+1.48±0.19	−2.3±0.3
	682	107	+2.21±0.29	+3.13±0.29	−1.2±0.3‡‡
Jo. Ho.	677	12	+0.13±0.07	+0.75±0.05	−2.2±0.5
	677	112	−0.43±0.22	+0.34±0.22	−0.5±0.2
	677	312	−0.34±0.13	+0.39±0.19	−0.5±0.3
Ro. Kr.	659	3	−0.60±0.14	+0.14±0.13	−2.3±0.2
Ca. Me.	644	4	+0.87±0.19	+1.49±0.14	−1.3±0.1‖‖
Cystathioninuric (presumptive γ-cystathionase deficient)					
Ge. Po.	673	6	−0.11±0.37	+0.71±0.38	−3.8±0.0

* See Table II and Methods. The actual data from which these balances were calculated have been deposited with the National Auxiliary Publication Service (NAPS document no. 02536). Nitrogen intakes ranged from 10.36 to 11.20 g/day. Caloric intakes varied from 36.5 to 59.5 calories/kg body wt and were adjusted as described in Table III. The initial intake for Wi. Cr. was excessive and during the period on 107 mg cystine this subject gained 1.9 kg.

‡ See Table I for further details on these subjects.

§ Based on 2 days only, after which study was discontinued because of a death in the patient's family.

‖ The studies of Ba. Re. on 8 mg and 109 mg cystine had to be discontinued during days 4 and 5, respectively, because of nausea, vomiting, and weakness. The excess urinary sulfur values are those for the last complete days of these studies.

¶ This patient was receiving some sulfur in her medications. The values for excess urinary sulfur were calculated on the alternative assumptions that (*a*) all of this sulfur was absorbed (yielding the less negative values for each study) or (*b*) none of this sulfur was absorbed (more negative values).

** An additional 170 mg of L-methionine was added to the diet to meet the requirement for this 15 yr-old boy (24).

‡‡ This study was the initial patient study, and the experimental protocol differed slightly from the standard one adopted subsequently and described in Methods. This patient was gaining significant weight for the first 4 days on the experimental diet. His weight was stabilized after caloric adjustments on days 5 and 7. The excessive caloric intake may have accounted for the relatively high nitrogen retention during the dietary period he was receiving 107 mg cystine.

‖‖ For this patient, in contrast to others studied, the daily urinary sulfur excretion did not reach a constant value until the 5th day on the experimental diet. Therefore, the mean excess urinary sulfur excretions averaged over the last 4, 3, and 2 days decreased progressively, being equal to −3.6±1.5, −2.2±0.9, and −1.3±0.1 mg-atom sulfur, respectively. The last value is regarded as the most valid, and has been entered in the table.

sponsive patient remained within the zone of nitrogen equilibrium with a balance of −0.11±0.37 g/day (Fig. 4). His urinary nitrogen loss was acceptable. His excess urinary sulfur was −3.8 mg-atom/day, slightly more negative than the values observed for normal control subjects (Table III).

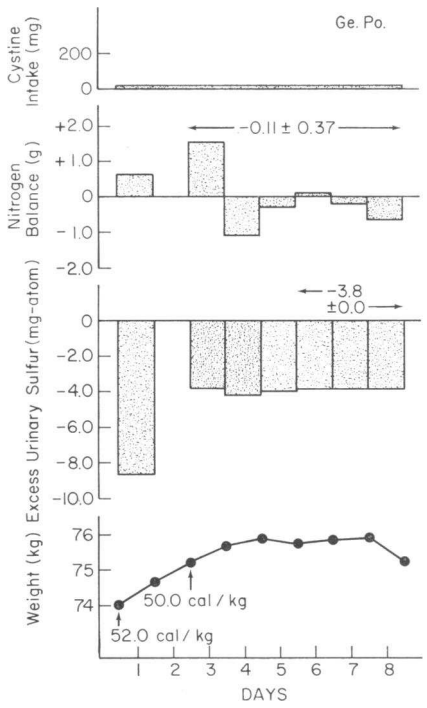

FIGURE 4 A study of a cystathioninuric, B_6-responsive patient (Ge. Po.) on a daily cystine intake of 6 mg. Daily intakes of methionine and nitrogen were 673 mg and 10.86 g, respectively, throughout. See text and Fig. 1 for further explanation.

Plasma and urine amino acids

Plasma and urine amino acid analyses were performed at least once during the latter half of each dietary balance period. In the normal controls, no changes were noted in either methionine or cystine levels, all being within normal limits on both the equilibration and the experimental diets (data not shown). The values for patients are shown in Table V. The most notable observations were the following: After the change from the equilibration diets to the experimental diets which contained less methionine, the concentrations of methionine and homocystine in plasma and the urinary excretions of homocystine decreased in cystathionine synthase-deficient, B_6-responsive patients Wi. Cr., Ro. Kr., and Ca. Me. but not in responder Jo. Ho. Similar changes did not occur in nonresponders Vi. Sw. and Ba. Re. Fr. Mi. was atypical among the B_6-nonresponsive patients and displayed changes similar to those found among the responders. All responsive patients maintained or increased their plasma cyst(e)ine concentrations, even on the lowest cystine intakes. Fr. Mi. manifested a similar tendency, although no plasma cystine was detected in one sample taken during a period of relatively low cystine intake. The cystathioninuric patient, Ge. Po., also increased his plasma cyst(e)ine concentration in the face of severe cystine restriction.

Urinary sulfur partition studies

The partition of total urinary sulfur between sulfate and nonsulfate sulfur should provide an index of the extent to which organic sulfur ingested as either methionine or cystine has been metabolized to the common end product sulfate. Accordingly, urinary sulfur partition studies were performed on five normal volunteers, three cystathionine synthase-deficient, pyridoxine responders, and two cystathionine synthase-deficient, pyridoxine nonresponders who did not have residual cystathionine synthase activities in their fibroblast extracts (18). Each subject received a constant natural diet calculated to provide half the dietary sulfur as methionine and half as cystine. Urinary inorganic sulfate, ethereal sulfate, and total sulfur were measured daily. Nonsulfate sulfur was then calculated as total sulfur *minus* total sulfate. In most cases values were obtained with the subjects receiving only normal dietary intakes of B_6 and also on high doses of pyridoxine (500 mg orally daily) (Table VI).

In their basal states (i.e., while receiving no supplemental pyridoxine) normal control subjects excreted 8.4–10.6% of their total urinary sulfur as nonsulfate sulfur. The pyridoxine-responsive patients excreted 15.3–25.9% as nonsulfate sulfur, values that in each case were significantly higher than the highest value found for a control subject ($P < 0.01$ for Jo. Ho. and Ja. Is.; $P < 0.05$ for Th. Kr.). The percent nonsulfate sulfur excretions of the non-B_6-responsive patients (27.5 and 31.7%) were elevated above the control values but, surprisingly, were not significantly higher than the corresponding values found for two of the responders (Jo. Ho. and Ja. Is.). Among the responders, only Th. Kr. excreted significantly less nonsulfate sulfur than did the nonresponsive patients, Ol. Ma. and Ba. Re.

Pyridoxine administration did not cause a statistically significant change in the percent nonsulfate sulfur excretion of any normal control. On the other hand, each of the three responsive patients showed a significant decrease in nonsulfate sulfur excretion during pyridoxine treatment ($P < 0.01$). Under these conditions, the percent nonsulfate sulfur excreted by responders Jo. Ho. and Th. Kr. fell to values not significantly higher than the highest control value. The nonsulfate sulfur excretion of responder Ja. Is. remained marginally elevated ($0.05 < P < 0.10$). For the nonresponders, pyridoxine therapy did not bring about a significant decrease in nonsulfate sulfur excretion. As a result, while the patients were on pyridoxine, the percent nonsulfate sulfur excretion of each nonresponsive patient was significantly higher than the corresponding value for each responder.

TABLE V

Plasma and Urinary Amino Acids during Equilibration and Experimental Diets

Subject	Diet*	Dietary intake		Plasma amino acids‡			Urinary amino acids‡	
		Met	Cys	Met	½ Cystine	Homocystine	Met	Homocystine
		mg		*μm/ml*			*μm/day*	
Cystathionine synthase deficient								
Vi. Sw.	Equilibration	1,451	924	—	—	—§	354	368
	Experimental	655	155	—	—	—	299	520
	Experimental	655	305	—	—	—	340	932
	Experimental‖	655	505	—	—	—	355	1,118
Ba. Re.	Equilibration	1,613	1,021	0.462	ND	0.120	194	429
	Experimental	667	509	0.412	<0.015	0.118	155	348
	Experimental	667	309	0.616	ND	0.122	216	432
	Experimental	667	109	—	—	—	227	421
	Equilibration	1,559	990	0.888	0.034	0.094	507	496
	Experimental	663	8	—	—	—	—	—
	Experimental	663	1,008	0.748	ND	0.069	88	147
Fr. Mi.	Equilibration	1,473	1,124	0.140	ND	0.148	204	297
	Experimental	822	155	0.064	0.106	0.010	126	25
	Experimental	822	80	0.070	ND	0.014	57	25
	Experimental	822	5	0.070	0.098	0.012	94	50
Wi. Cr.	Equilibration	1,511	912	0.400	0.034	0.062	185	220
	Experimental	682	107	0.076	0.112	0.008	65	25
	Experimental	682	7	0.070	0.066	0.007	108	41
Jo. Ho.	Equilibration	1,469	1,005	0.098	0.028	0.056	67	333
	Experimental	663	312	0.104	0.070	0.024	83	165
	Experimental	663	112	0.146	0.090	0.046	49	254
	Experimental	663	12	0.112	0.056	0.036	54	248
Ro. Kr.	Equilibration	1,444	995	0.064	0.112	0.010	91	106
	Experimental	659	3	0.050	0.126	ND	49	14
Ca. Me.	Equilibration	1,505	984	0.608	ND	0.070	303	443
	Experimental	644	4	0.090	0.042	0.028	91	156
Cystathioninuric (presumptive γ-cystathionase deficient)								
						Cystathionine		Cystathionine
Ge. Po.	Equilibration	1,451	965	0.028	ND	trace	104	585
	Experimental	677	6	0.028	0.118	trace	83	370

* The equilibration diets were composed of natural foodstuffs isonitrogenous with the experimental diets which were semisynthetic as described in Methods. The results are listed in the sequence that the studies were actually carried out.

‡ Sample from near the end of specified dietary regimen. ND, not detected; dash (—), sample not available.

§ Patient refused venipuncture.

‖ This experimental period lasted 2 days only (see Table IV).

DISCUSSION

The chief aim of the present work was to evaluate as quantitatively as possible the capacities of various patients with defects in the transsulfuration pathway to biosynthesize cysteine from methionine. To this end, we measured nitrogen balances of patients and controls maintained on semisynthetic diets containing low-adequate amounts of methionine but virtually free of cyst(e)ine. The amount of supplemental cystine required to achieve nitrogen equilibrium was then determined. The experiments were designed to eliminate virtually all cystine from the basal experimental diet, a consideration that dictated the use of a semisynthetic diet in which most of the amino acid intake was provided in the form of the pure compounds. The diet chosen was modelled after that often used in studies of essential amino acid requirements (35, 38–40) and contained the essential amino acids plus arginine, histi-

TABLE VI

*Total Urinary Sulfur and Percent Urinary Organic Sulfur on Sulfur Partition Diet: Effect of Pyridoxine**

Subject	Total urinary sulfur		Organic sulfur	
	Pyridoxine supplement		Pyridoxine supplement	
	None	500 mg	None	500 mg
	μg-atom/kg (±SE)		*Percent (±SE)*	
Normal volunteers				
Ba. Ha.	299±10	—	8.4±1.2	—
Ja. Ho.‡	356±35	354±34	10.3±1.5	8.1±0.9
Cr. Ki.‡	328±35	341±38	10.6±1.7	8.4±1.4
Ka. Ro.	334±15	—	9.0±1.4	—
An. To.	294±40	275±19	9.5±1.0	12.2±1.3
Cystathionine synthase-deficient B_6-responders§				
Jo. Ho.	265±12	276±11	25.9±1.7	11.9±0.9
Ja. Is.	313±4	293±24	25.5±1.8	16.0±1.2
Th. Kr.	304±32	294±35	15.3±1.3	8.4±0.9
Cystathionine synthase-deficient B_6-nonresponders‖				
Ol. Ma.	304±24	266±26	31.7±2.4	31.2±0.9
Ba. Re.¶	283±12	280±5	27.5±1.8	26.3±2.5

* Each subject was given a constant diet estimated to contain methionine and cystine so that each contributed 140 μg-atom sulfur/kg body wt.

‡ 20 yr-old male.

§ Residual cystathionine synthase activity was detected in fibroblast extracts from each of these patients (18).

‖ No residual cystathionine synthase activity was detected in fibroblast extracts of either of these patients (18).

¶ Corrected for sulfur content of medications taken by this subject. The maximum uncertainty in organic sulfur due to these corrections is 1%.

dine, and tyrosine in the approximate amounts found in 20 g whole egg protein. Supplemental glycine was added to keep the experimental diets isonitrogenous with the equilibration diets (35, 38–40). This level and pattern of amino acid intake satisfied an additional desideratum to keep the methionine intake reasonably low to avoid insofar as possible the accumulation of what might be considered "toxic" concentrations of methionine metabolites in the patients with transsulfuration defects. As has been pointed out (41) this diet satisfies the minimum essential amino acid requirements proposed by Rose and Wixom (42) and others (43, 44). However, recent results suggest that this regimen may be marginally inadequate for maintenance of true nitrogen balance (41, 45, 46). Although, in practice, our normal subjects on the basal semisynthetic diet containing virtually no cystine remained within the zone of nitrogen equilibrium (Table III), these relatively short-term studies do not prove the complete, long-term nutritional adequacy of the diets employed. Longer studies, or adoption of "corrected" rather than "apparent" nitrogen balances (47) might have indicated that the basal diet used was inadequate.

An additional criterion for comparison of normals and patients is afforded by the measurements of excess urinary sulfur performed in the course of our studies. Again, it is noted that these measurements do not test the long-term adequacy of any particular dietary regimen. For this purpose, true balances taking into account fecal and other losses would be needed.

In Table VII the results on our present patients, as well as the patient studied by Brenton and coworkers

TABLE VII

*Patients with Transsulfuration Defects: B_6-Responsiveness, Presence of Detected Residual Enzyme Activity, and Presence of Excessive Loss of Nitrogen or Sulfur on Lowest Cystine Intake Studied**

Patient	Defective enzyme	B_6-responsive	Residual enzyme detected	Excessive loss of:	
				Nitrogen	Sulfur
Stuart M.‡	Cystathionine synthase	No	Unknown	Yes	Unknown
Vi. Sw.	Cystathionine synthase	No	No	Yes	Yes
Ba. Re.	Cystathionine synthase	No	No	?	Yes
Fr. Mi.	Cystathionine synthase	No	Yes	No	No
Wi. Cr.	Cystathionine synthase	Yes	Yes	No	No
Jo. Ho.	Cystathionine synthase	Yes	Yes	No	No
Ro. Kr.	Cystathionine synthase	Yes	Yes	?	No
Ca. Me.	Cystathionine synthase	Yes	Yes	No	No
Ge. Po.	γ-Cystathionase	Yes	Unknown	No	Slight

* Data on B_6-responsiveness and enzyme assays are recorded or referred to in Table I. The nitrogen and sulfur losses are those for the lowest cystine intake tested for each patient (Table IV).

‡ Studied by Brenton et al. (9). This patient was subsequently shown to be unresponsive to pyridoxine (D. C. Cusworth, personal communication).

(9), are summarized. It has previously been shown that there is a strong correlation between the presence or absence of detected residual cystathionine synthase activity in cystathionine synthase-deficient patients and their clinical responsiveness or nonresponsiveness to B_6 administration. Residual cystathionine synthase activities were not detected in fibroblasts of 11 of 13 B_6-nonresponsive patients (18 and our unpublished results), nor in liver of two of two nonresponders (48). Among the patients in Table VII, Vi. Sw. and Ba. Re. fall into the category of B_6-nonresponsive patients without detected residual cystathionine synthase activity. Stuart M. is B_6-nonresponsive. Although he has not yet been categorized with respect to residual enzyme activity, the results described above make it statistically likely that he lacks such activity. Each of these three patients evidently lost excessive nitrogen and/or sulfur when placed on low cystine intakes. These losses were reversed by increasing the cystine intakes (except, questionably, for the nitrogen loss of Ba. Re.).

In contrast to the results with most B_6-nonresponsive patients, sensitive assays did detect measurable residual cystathionine synthase activities (0.1–10% of the mean control value) in liver and/or fibroblasts extrasts of each of the 25 responsive patients we studied (18, 19, 49), as well as seven of seven B_6-responders studied by others (48, 50, 51). Wi. Cr., Jo. Ho., Ro. Kr., and Ca. Me. are in this category of B_6-responsive cystathionine synthase-deficient patients with detected residual enzyme activity. None of these patients were significantly below the zone of nitrogen equilibrium or lost more sulfur than did our control subjects on diets virtually free of cystine.

Fr. Mi. falls into the relatively infrequently encountered class of cystathionine synthase-deficient B_6-nonresponders with detected residual enzyme activity (18). Such patients illustrate that the presence of residual cystathionine synthase activity is a necessary but not sufficient condition for B_6-responsiveness. Fr. Mi. had no excessive loss of either nitrogen or sulfur, even when his cystine intake was lowered to 5 mg daily.

Thus, among the eight cystathionine synthase-deficient patients studied to date, the five with residual enzyme activities could not be shown to require more cystine than do normal subjects. There is no indication that for these patients cystine is an essential amino acid. In contrast, for the three patients without detected residual enzyme activities, the evidence clearly suggests a requirement for exogenous cystine in two, and the clinical signs and sulfur losses of the third patient are suggestive of such a requirement. It appears that the generalization that cystine is an essential amino acid for cystathionine synthase-deficient patients (2, 10–17) is not universally correct and that in all likelihood the presence of a residual few percent of the normal control cystathionine synthase activity is sufficient to obviate this requirement.

How much cyst(e)ine must be generated endogenously to avoid a requirement for exogenous cyst(e)ine in a cystathionine synthase-deficient patient without residual activity of this enzyme? The nitrogen balance of the B_6-nonresponder studied by Brenton et al. on a daily cystine intake of 85 mg was -1.12 ± 0.23 g/day (estimated from Fig. 13 of reference 9). Nitrogen balance was restored with a cystine intake of 1,085 mg. Intermediate intakes of cystine were not studied. The nitrogen balance of our patient Vi. Sw. was -0.96 ± 0.16 g/day on a daily cystine intake of 155 mg. Nitrogen equilibrium was restored and excessive urinary nitrogen loss was prevented by an intake of 505 mg. The urinary sulfur loss of Vi. Sw. remained slightly excessive on 305 mg cystine. These studies suggest a cystine requirement of between 305 and 505 mg for Vi. Sw. The results with patient Ba. Re. are more difficult to interpret because nitrogen equilibrium was not restored with certainty even on a cystine intake as high as 1,008 mg. Judging by the clinical responses of Ba. Re. to various cystine intakes and by her urinary sulfur losses, it may be tentatively estimated that 309–509 mg of cystine was adequate for this patient. Also pertinent are studies of Bu. Ha., another cystathionine synthase-deficient B_6-nonresponsive patient without significant detected cystathionine synthase activity in fibroblast extracts (18). The results obtained with this 8-yr-old boy were not included in Table IV because he could not be persuaded to accept the semisynthetic experimental diet. Accordingly, a normal food diet was devised, calculated to provide 577 mg of methionine, 307 mg of cystine, and 4.13 g of nitrogen. Supplemental glycine was added to bring the total nitrogen intake to 10.7 g. On this diet, the nitrogen balance was $+1.98\pm0.46$ g and excess urinary sulfur was -1.4 mg-atom/day. All of these observations together suggest that 310–510 mg cystine is adequate for patients with virtually no residual cystathionine synthase activity detected by present methods. Because of the small number of patients studied, it should be clear that *at best* these estimates are approximations of the amount of cystine needed to prevent excessive nitrogen or sulfur losses under our conditions.

If cystathionine synthase-deficient patients without detected residual enzyme required 310–510 mg cystine under these conditions, it follows that the patients with residual cystathionine synthase activities who remained in equilibrium on diets containing adequate methionine, but at most 12 mg cystine, were forming endogenously at least 300–500 mg (i.e., 2.5–4.0 mmol) cysteine. The sulfur for this cysteine must ultimately have derived from methionine, since there was no other major dietary sulfur source. The values of 2.5–4.0 mmol (which are minimum estimates since our experiments would not

detect the formation of more cysteine that would be required to sustain equilibrium) are equivalent to 20–50% of the normal dietary intake of methionine of 8–12 mmol. The hepatic cystathionine synthase activities of our B_6-responsive patients in their basal states (i.e., on B_6 intakes provided by normal diets only) were 1–2% of the mean control value (19, 49). Thus, it appears that a residual activity of 1–2% of this enzyme endows the patient with the capacity to convert at least 20–50% of the normal methionine intake to cysteine. Our working hypothesis as to the mechanism of action of B_6 in responsive patients is that vitamin administration brings about an increase of 2–4-fold in the basal enzyme activity of such patients (18, 19). The present demonstration that the basal residual enzyme activity is capable of metabolizing 20–50% of the normal methionine intake is clearly compatible with and supports this working hypothesis. To be able to metabolize the normal methionine intake without undue homocysteine accumulation it may be necessary to possess no more than a relatively small portion of the mean control cystathionine synthase activity.

Cystathionine synthase catalyzes also the sulfhydration of serine to form cysteine (52, 53). If cystathionine synthase-deficient patients retain a disproportionate share of the normal serine sulfhydrase activity, it is possible that reaction accounts for the residual capacity to form cysteine demonstrated during the present work. This possibility, which in our opinion is not very likely, could be evaluated by assays of serine sulfhydrase in cells of patients who do not require cystine and by determination of cystathionine concentrations before and during response to B_6.

The nutritional approach used in the present work to determine the residual metabolic capacities of patients with cystathionine synthase deficiency might usefully be extended to other enzymopathies. One example, of such an extension is provided by the study reported here of a single B_6-responsive cystathioninuric patient (Table IV).[3] Enzyme assays and nutritional studies of γ-cystathionase-deficient patients not responsive to B_6 (5, 54) would be of interest, as would a correlation of residual enzyme activities of phenylalanine hydroxylase and the tyrosine requirements of patients with phenylketonuria and its variants.

Note added in proof. Two full-length reports especially pertinent to this paper have recently appeared. The results of Gaull, Sturman, and Schaffner (55) generally support the hypothesis that B_6 treatment brings about increases in residual hepatic cystathionine synthase activities of responsive patients. The mean basal activity in four responsive patients under the conditions employed was 6% of the mean control value; the mean activity during response, 18%. Porter, Grishaver, and Jones (56) found that extracts of fibroblasts from a B_6-responsive patient retained 8% residual capacity to form cystathionine from homocysteine and serine, whereas no residual capacity to form cysteine from Na_2S and serine was detected.

[3] The results of a methionine loading study with Ge. Po. are consistent with his possessing some residual γ-cystathionase activity. In the 24 h following an oral dose of L-methionine, 36.9 mmol, this patient excreted in his urine an increment of 17.1 mmol of total sulfur. The increment in daily excretions of cystathionine and total *sulfate* were, respectively, 4.0 and 11.3 mmol. Thus, Ge. Po. had converted a major portion of the methionine sulfur to sulfate within 24 h.

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