Homocystinuria: Studies in Tissue Culture

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Extract

Cystathionine synthase activities are reported for extracts of fibroblasts grown from 39 control subjects, 47 homocystinuric individuals, and 10 parents of cystathionine synthase-deficient patients. Among the group with homocystinuria, fibroblast extracts from 38 had specific activities of cystathionine synthase below the control range. A number of considerations indicate that these 38 patients excrete homocystine because of cystathionine synthase deficiency. Fibroblasts from nine patients with homocystinuria had specific activities of cystathionine synthase within the control range. This group of nine was shown to be comprised of two individuals with cystathionine synthase deficiency, three with deficient activity of methylenetetrahydrofolate reductase, three with deficient activity of N^5-methyltetrahydrofolate-homocysteine methyltransferase, and one in whom homocystine excretion found by others in earlier studies could not be confirmed during the present investigation. The specific activities in fibroblasts of parents of cystathionine synthase deficient patients in most cases fall near the low end of the control range.

An assay of increased sensitivity was used to measure the cystathionine synthase activities in extracts of fibroblasts from cystathionine synthase-deficient patients at several concentrations of added pyridoxal phosphate. Of 25 cystathionine synthase-deficient patients judged to be clinically responsive to pyridoxine treatment, 24 had detectable cystathionine synthase activities in fibroblast extracts when the assays were performed without added pyridoxal phosphate. These activities ranged from approximately 0.1% to 10% of the mean control value, and generally were stimulated no more by the addition of pyridoxal phosphate than were extracts from normal cells. Of 10 cystathionine synthase-deficient patients judged not to be responsive to pyridoxine, 9 had no cystathionine synthase activity in fibroblast extracts detected by the method employed. The cystathionine synthase in the cell extracts of the single nonresponsive patient with significant activity was stimulated much more by in vitro addition of pyridoxal phosphate than was control cystathionine synthase.

Genetic heterogeneity in cystathionine synthase-deficient patients and some of the genetic implications of the demonstrated $\alpha_2\beta_2$ subunit structure of mammalian cystathionine synthase are discussed.

Speculation

Knowledge of enzyme activities, which includes cystathionine synthase, N^5-methyltetrahydrofolate-homocysteine methyltransferase and methylenetetrahydrofolate reductase, in cultured fibroblasts is of diagnostic value in distinguishing between the several genetic abnormalities which may lead to excessive excretion of homocystine in the urine.

Clinical response of cystathionine synthase-deficient patients to pyridoxine therapy is thought to be due to a several-fold increase of residual cystathionine synthase activity. This increase occurs in the patient by an unknown mechanism, but in general is not caused by an unusual stimulation of residual enzyme activity by high concentrations of pyridoxal phosphate.

Introduction

Patients with a genetically determined deficient activity of cystathionine synthase, the enzyme which catalyzes the condensation of homocysteine and serine to form cystathionine (reaction 4, Fig. 1), excrete excessive amounts of homocystine in their urine [4, 7, 14, 26, 30, 38]. The term "homocystinuria" has often been used to designate this genetic disorder, but this restricted use of the term is no longer appropriate because it is now known that excessive homocystine excretion (i.e., homocystinuria) may occur in a number of other situations. For example, homocystinuria accompanies any condition in which the rate of homocysteine methylation catalyzed by N^5-methyltetrahydrofolate-homocysteine methyltransferase (reaction 8, Fig. 1) is sufficiently decreased. There are conditions known to decrease pathologically the rate of this methyl transfer reaction. (1) Failure to form N^5-methyltetrahydrofolate, the methyl donor for the enzyme in question, decreases the rate of transfer. This failure is due to deficient activity of methylenetetrahydrofolate reductase (reaction 18, Fig. 1) [35]. (2) Failure to form methyl-B_{12}, a cofactor required by N^5-methyltetrahydrofolate-homocysteine methyltransferase may also be responsible. This failure may be the result of either B_{12} deficiency, occurring, for example, in familial gastrointestinal malabsorption of B_{12} [15, 17], or the result of impaired cellular uptake or metabolism of B_{12} (deficiency in reaction pathway 16, Fig. 1) [23, 33, 34, 36]. In addition to these genetic conditions, another situation leading to excessive homocystine excretion is the administration of 6-azauridine triacetate [16]. The mechanism of this drug effect has not been clarified.

From the above considerations, and in accord with a suggestion first published in 1964 [4], we prefer to use the term "homocystinuria" not as the name of a particular disease, but rather "to denote excretion of [excessive] homocystine in the urine, without etiologic connotation." The specific diseases which include homocystinuria among their manifestations may then be termed "cystathionine synthase deficiency," "methylenetetrahydrofolate reductase deficiency," and so on.

For the sake of gaining further understanding of the various diseases in question, and in the interest of rational patient management [12], it is now necessary to define the etiology of each case of homocystinuria.

Of the many lines of evidence which may assist in distinguishing between the various causes of homocystinuria, the least equivocal is a direct demonstration of deficient activity of a specific enzyme. Unfortunately, studies of cystathionine synthase activity in humans have been limited by the restricted distribution of this enzyme, which is found in liver and brain, but not in more accessible tissues [30, 31, 42]. To meet the need for a readily available experimental system, we turned to the use of tissue culture and found that fibroblasts grown from biopsies of normal human skin contain easily detectable concentrations of cystathionine synthase, whereas those grown from several homocystinuric patients had markedly decreased, or absent, activity of this enzyme [42]. This tissue culture system has provided a convenient means to study cystathionine synthase from normal and homocystinuric subjects. We have subsequently investigated lines of fibroblasts from more than 80 individuals, including 47 homocystinuric patients. In this paper we report our findings.

Methods

Methods for obtaining skin biopsies, initiating tissue cultures, and growing and harvesting fibroblasts have been described [36]. Preparation of cell extracts, assays of N^5-methyltetrahydrofolate-homocysteine methyltransferase and methylenetetrahydrofolate reductase activities, and determination of protein concentrations were performed according to methods previously used [35, 36], as were experiments to determine the ability of cells to grow in media supplemented with either methionine or homocystine. Cystathionine synthase activity in cell extracts was usually assayed as described [36]. All enzyme assays were carried out with freshly prepared cell extracts. A unit of cystathionine synthase activity is defined as the amount of enzyme required to synthesize 1 nmol of cystathionine in 135 min. With the standard assay, little or no activity was
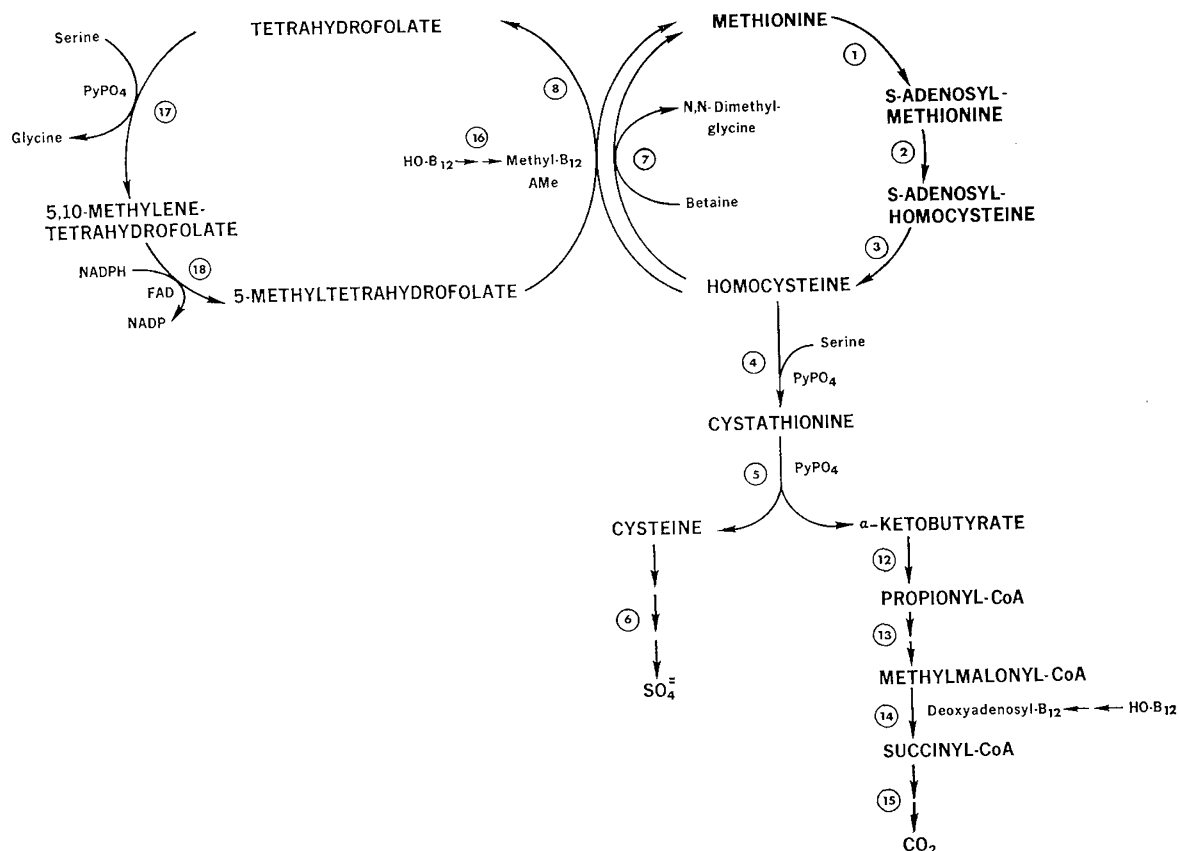
Fig. 1. Metabolic relationships of interest in a consideration of homocystinuria. No attempt has been made to include minor reactions of the sulfur-containing amino acids or major pathways for other compounds not immediately pertinent to the discussion in this paper. $PyPO_4$: Pyridoxal phosphate; B_{12}: the cobalamin moiety, with the prefix indicating the substituent "above" the ring; *FAD*: flavine-adenine dinucleotide; *AMe*: S-adenosylmethionine. Homocysteine participates in most metabolic reactions in the reduced form. The disulfide, homocystine, formed by mild oxidation, is the compound which predominates in the urine. The latter term will be used here in referring to measured values.

detected in lines of cystathionine synthase-deficient cells. To obtain better quantitation of any residual cystathionine synthase activity which might be present in such cell lines, a more sensitive assay was used in which the cystathionine-containing fractions eluted from Dowex 50 were oxidized and subjected to further purification by paper chromatography [31]. In these experiments the specific radioactivity of the substrate serine-3-^{14}C was increased about 10 times above that used in the standard assay and an amount of cell extract containing 1–2 mg protein was added to each experimental reaction mixture. After termination of the reaction with trichloroacetic acid, aliquots of the deproteinized, diluted reaction mixtures were chromatographed, as described [36], on small columns of Dowex 50. The columns were repoured with fresh resin after use in no more than two of these experiments. Cystathionine was eluted from the columns

with 3.9 ml 3 N NH_4OH and the eluate was brought to a final volume of 4.5 ml. A small aliquot of this eluate was analyzed for radioactivity and a 4.4 ml-aliquot was evaporated to dryness with a Rotary Evapo-Mix [44]. The residue was dissolved in 0.1 ml freshly prepared performic acid (1 ml 30% H_2O_2 to 9 ml 88% formic acid incubated before use for 30 min at 27° [10]). The dissolved residue was incubated at 27° for 60 min, and again evaporated to dryness. The residue was dissolved in 100 μl 88% formic acid and an aliquot (up to 75 μl) was spotted on Whatman no. 1 paper before chromatography with isopropanol, formic acid (88%), water, 70/10/20 (v/v). Radioactivity on the developed chromatogram was located and quantified as described [31], by means of a Packard Tri-Carb [45] or a Beckman LS-250 Soft-Beta counting spectrometer [46]. After appropriate corrections for apportionment of sample volumes and efficiency of counting, the radioac-
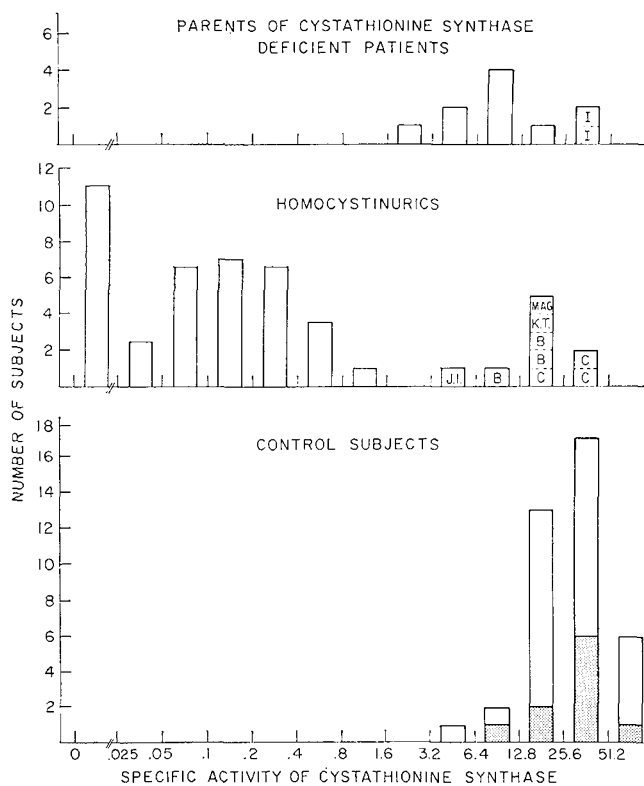
Fig. 2. Distributions of specific activities of cystathionine synthase. A single value is entered for the fibroblasts from each individual studied. In some instances these are mean values for assays of extracts from several preparations of fibroblasts grown from one or more skin biopsies. For purposes of grouping, when a specific activity fell on the dividing line between two groups, 0.5 unit was assigned to the lower group, 0.5 unit to the higher group. The stippled areas in the lower histogram represent values from normal volunteers. The values represented here were measured with 1.0 μm/ml pyridoxal phosphate added to each reaction mixture. Measurements for homocystinuric patients with specific activities below the control range were obtained by means of the sensitive assay. These values have not been corrected for loss of cystathionine during the oxidative and paper chromatographic steps described in *Methods*.

tivity in the area of cystathionine sulfone [47] was taken as a measure of cystathionine-^{14}C formation. The value from a blank incubated without enzyme was subtracted (see Figs. 3 and 4). Control experiments with authentic cystathionine-3-^{14}C demonstrated that approximately 50% of this material in reaction mixtures not supplemented with pyridoxal phosphate was recovered on the chromatograms as cystathionine sulfone. The recoveries in reaction mixtures supplemented with 0.1, 1.0, and 5.0 μm/ml pyridoxal phosphate were, respectively, 50%, 40%, and 30%. Corrections for these recoveries were made as specified in the legends to the figures and tables.

Results

Cystathionine Synthase Activities in Extracts of Fibroblasts from Control Subjects and Individuals with Homocystinuria

The results of our assays of the specific activities of cystathionine synthase in extracts of fibroblasts grown from control subjects, patients with homocystinuria, and parents of cystathionine synthase-deficient patients are diagrammed in Figure 2. The mean specific activity of cystathionine synthase in the group of 39 control fibroblast lines was 31.7 $\pm$ 14.2 units/mg protein (mean $\pm$ 1 SD) with a range from 3.7 to 60.0. This control group was comprised of three subgroups: 10 normal volunteers, 24 patients or relatives of patients with diseases not known to involve abnormalities of amino acid metabolism, and 5 patients with hypermethioninemia. There was no significant difference between the means for the total control group and those for the three subgroups which, respectively, had mean specific activities of 33.8 $\pm$ 13.8, 30.1 $\pm$ 15.0, and 35.3 $\pm$ 12.2. Plotted on a logarithmic scale as in Figure 2, the values for the control group form a reasonably symmetrical distribution [48].

The distribution of specific activities of cystathionine synthase in cells grown from homocystinuric individuals (Fig. 2) differs clearly from the distribution of the control values. Of 47 such fibroblast lines, 38 had specific activities of cystathionine synthase below the lowest control value, whereas 9 had specific activities within the control range. The same distribution is obtained using values corrected for loss of cystathionine in the sensitive assays. A number of considerations, developed more fully under *Discussion*, indicate that the individuals from whom the fibroblast lines with activities below the control range were derived are indeed homocystinuric on the basis of cystathionine synthase deficiency. Of the nine homocystinuric individuals with cystathionine synthase activities within the control range, two have cystathionine synthase deficiency, seven do not (see *Discussion*).

Cystathionine Synthase in Extracts of Fibroblasts Grown from Heterozygotes

Also shown in Figure 2 are the results of assays of cystathionine synthase activity in extracts of fibroblasts grown from the parents of cystathionine synthase-deficient children. On the basis of studies of the mode of inheritance of homocystinuria [24] and the results of assays of liver enzymes [4, 8, 21], such parents may be presumed to be heterozygous for cystathionine syn-

Table I. Cystathionine synthase activity at different pyridoxal phosphate (PP) concentrations in extracts of fibroblasts from cystathionine synthase-deficient subjects[1]

| Patient No. | Patient | Sibship | Cystathionine synthase activity, nmol/mg protein/135 min[2] | | | | Response to pyridoxine | Citation |
			PP, 0 μm/ml	PP, 0.1 μm/ml	PP, 1.0 μm/ml	PP, 5.0 μm/ml		
1	JI		2.7		3.4	2.6	Yes	3
2	DS		1.8		3.2		Yes	3
3	IM		0.94	0.98	1.0	1.3	Yes	3
4	MD	I	0.88		1.8		Yes	3
5	DD	I	0.70	0.86	1.1	1.4	Yes	3
6	RK	II	0.70		1.3		Yes	3
7	JK	II	0.68	0.74	0.75	0.67	Yes	3
8	TK	II	0.66	0.98	0.88	0.83	Yes	[29]
9	DH	III	0.48	0.48	0.70	0.60	Unknown	3
10	RH	III	0.20	0.34	0.53	0.37	Unknown	3
11	RJ	IV	0.36	0.38	0.18	0.37	Yes	[14]
12	DJ	IV	0.20	0.24	0.25	0.33	Yes	[14]
13	BJ	IV	0.16	0.24	0.23	0.30	Yes	[14]
14	MJ	IV	0.12	0.18	0.30	0.27	Yes	[14]
15	MC	V	0.34	0.30	0.35	0.53	Yes	3
16	WC	V	0.18	0.26	0.35	0.27	Yes	3
17	TC	V	0.14	0.14	0.23	0.17	Yes	3
18	TY		0.24	0.36	0.40	0.57	Yes	[43]
19	WS		0.18	0.24	0.40		Yes	3
20	LG		0.16	0.06	0.25	0.27	Yes	3
21	KB		0.12	0.14	0.18	0.10	Yes	[29]
22	RF		0.10	0.22	0.23	0.40	Yes[4]	3
23	DF		0.08	0.08	0.10	0.13	Yes	3
24	SB	VI	0.08	0.12	0.58	0.80	Yes	3
25	JB	VI	0.08	0.18	0.43	0.43	Yes	3
26	JB		0.06	0.18	0.73	1.0	No	[25]
27	DA		0.04	0.06	0.06	0.06	Unknown	3
28	CT		0.04	0.06	0.10	0.12	Yes[4]	[2]
29	DR	VII	0.02	0	0	0	No	3
30	MR	VII	0	0	0	0.03	No	3
31	JH		0	0.10	0.05	0.10	Yes	[29]
32	BH		0	0	0.03	0.07	No	3
33	RM	VIII	0	0	0.03	0.03	Unknown	3
34	OM	VIII	0	0	0	0.03	No	3
35	BR		0	0	0	0	No	3
36	MH	IX	0	0	0	0	No	3
37	SH	IX	0		0		No	3
38	PN		0	0	0	0	No	3
39	BO		0	0	0	0	No	3

[1] An extract of fibroblasts from each patient was assayed in a single experiment with the indicated additions of pyridoxal phosphate. In some instances, an assay was repeated with an extract from a different fibroblast preparation from the same patient, and the results averaged. For purposes of this survey, all cells were grown in medium supplemented with the same batch of fetal calf serum. Generally three or four extracts were assayed in a given experiment. These extracts were chosen so that extracts from members of any one sibship were assayed in different experiments. In this table the patients are grouped in sibships which are listed in rank order according to the highest value within a sibship for the specific activity of cystathionine synthase (assayed with no added pyridoxal phosphate).

[2] Values obtained by means of the sensitive assay have been corrected for loss of cystathionine during the oxidative and paper chromatographic steps as specified in *Methods*.

[3] Personal communications from the following physicians Dr. V. A. McKusick (*patients 1–7, 9, 10, 22, 27, 29, 30*, and *35*); Drs. A. Sass-Kortsak and S. H. Jackson (*patients 15–17*); Dr. V. E. Shih (*patients 19, 23, 38*, and *39*); Dr. C. R. Scott (*patient 20*); Dr. R. W. Townley (*patients 24* and *25*); Dr. R. R. Howell (*patient 32*); Dr. S. I. Goodman (*patient 33*); Dr. J. Poole, (*patients 34* and *35*); and Dr. H. L. Levy (*patients 36* and *37*).

[4] Response was transient.

thase deficiency. Most of the specific activities of fibroblasts from these parents fell near the low end of the control range. A statistical distinction between the parents and the control group would be possible, although it is clearly not yet feasible to determine whether a given individual is normal or heterozygous for cystathionine synthase deficiency merely by assay of the activity of this enzyme in his cultured fibroblasts.

More Detailed Studies of Cystathionine Synthase Activity in Extracts of Fibroblasts from Cystathionine Synthase-deficient Individuals

Assays of cystathionine synthase activity were carried out at several concentrations of pyridoxal phosphate with each fibroblast extract from a patient with homocystinuria with lower than control activity of this enzyme. The results are summarized in Table I. It is apparent that there is a good deal of variation between residual cystathionine synthase activities in fibroblast lines from different deficient patients. One line (*JI*) has about 10% of the mean control activity. Many

lines have lower, yet clearly detectable activities (*patients 2–28,* inclusive). Ten lines of fibroblasts, (*patients 29, 30,* and *32–39*) have no detectable activity. The sensitivity of the assays may be judged by reference to a representative experiment, shown in Figure 3. Illustrated in this figure are portions of paper chromatograms resulting from a nonenzyme "blank" incubation, and incubation with comparable fibroblast extracts from patients MR (*patient 30*), BH (*patient 32*), CT (*patient 28*), and DS (*patient 2*). In the blank experiments, or in the experiments with extract from fibroblasts from MR, there was no peak of radioactivity in the area of the chromatograms to which cystathionine sulfone migrates. The extract from fibroblasts from DS yielded a relatively large peak of radioactivity in the cystathionine sulfone area, whereas the extract from fibroblasts from CT yielded a much smaller peak. The corrected specific cystathionine synthase activities calculated from these results were 1.8–3.2 for DS and 0.04–0.12 for CT. The latter values represent approximately the lower limit of activity detectable with this
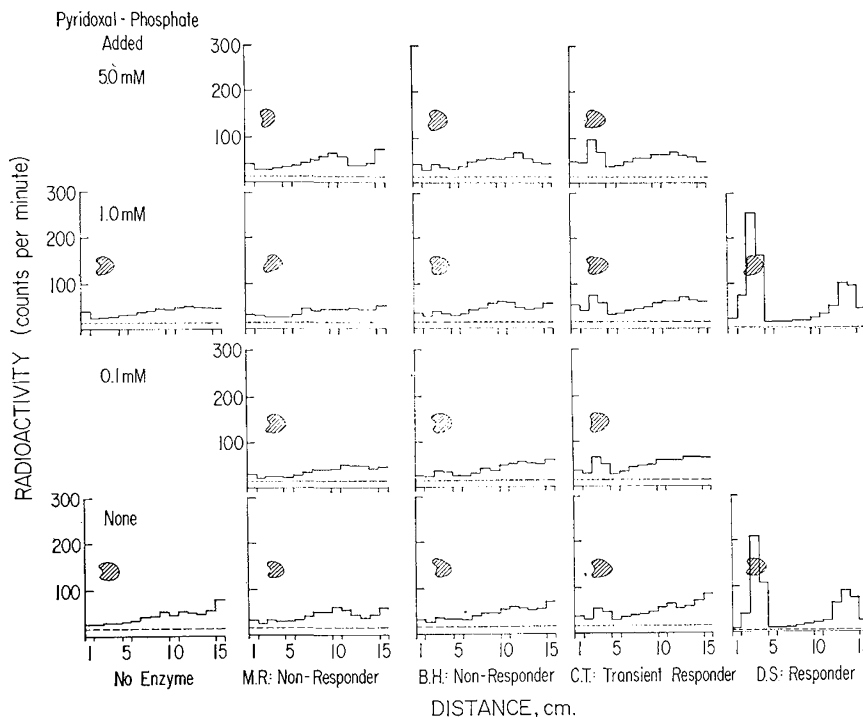

Fig. 3. The results of a representative assay of cystathionine synthase activities in extracts of fibroblasts from four cystathionine synthase-deficient patients. Extracts were incubated with serine-3-^{14}C and homocysteine as described in *Methods*. The amount of pyridoxal-phosphate added to each reaction mixture is indicated at the left. After termination of the reaction, aliquots were subjected to preliminary purification on Dowex 50 to separate the product, cystathionine-^{14}C, from unreacted serine-3-^{14}C. The column eluates containing cystathionine were oxidized and chromatographed on paper. Represented here are the distributions of radioactivity in the areas of the resulting chromatograms to which cystathionine sulfone moved. The *dashed lines* (– – –) indicate background radioactivity. The *cross-hatched areas* indicate the location of authentic carrier cystathionine sulfone. The amount of radioactivity in this area is a measure of cystathionine synthase activity.

assay. The extract from *BH* yielded very small peaks in the cystathionine sulfone area which corresponded to calculated corrected specific activities of 0.00–0.07. These values we view as of only questionable significance.

Correlation between Responsiveness to Pyridoxine and Presence of Residual Cystathionine Synthase Activity in Fibroblasts

In Table I each patient is categorized with respect to his clinical response to treatment with large doses of pyridoxine. Positive responses are characterized by very marked decreases in the concentrations of plasma and urinary homocyst(e)ine, methionine, and other metabolites accumulating proximal to the block in cystathionine synthesis. Concomitantly, the cyst(e)ine concentration of the plasma increases [1, 29]. Patients not responsive to pyridoxine manifest little or no change during administration of this vitamin. The classifications of the patients in Table I as to pyridoxine responsiveness are based upon published evidence, as indicated in the last column of Table I, or upon the findings of the physician who had studied the patient in question. In the latter cases the physician was asked simply to rate the patient as "responsive" or "not responsive." This dichotomous classification is likely to be an oversimplification since both *a priori* expectations and available evidence suggest the presence of considerable genetic heterogeneity among cystathionine synthase-deficient patients ([3, 8, 38] and our findings discussed below). Nevertheless, a rather striking correlation is apparent between the presence of detectable cystathionine synthase activity in fibroblasts and responsiveness to pyridoxine. This correlation is made more clear by grouping the patients as in Table II. Of those patients for whom data is available, 25 were judged responsive to B_6 and 10 were judged not responsive. In the assays without added pyridoxal phosphate significant cystathionine synthase activity was detected in fibroblasts from 24 of the 25 B_6-responsive patients. *CT* (*patient 28* and Fig. 3) was the responder with the lowest cystathionine synthase activity judged to be significant in all assays. One responding patient (*JH, patient 31*) had traces of activity, apparently significant in some assays, but not in others. This patient had been studied previously by assay of cystathionine synthase in liver tissue and had been shown to possess approximately 2% of the mean hepatic cystathionine synthase activity [32]. Thus, even this patient has significant residual cystathionine synthase activity. We

Table II. Correlation between presence of detectable cystathionine synthase activity in fibroblast extracts and responsiveness to pyridoxine[1]

	Number of patients with cystathionine synthase activity[2]	
	Detected	Not detected
Responsive to B_6[3]	24	1
Not responsive[3]	1	9

[1] In compiling Tables I and II results obtained with extracts of fibroblasts from *MAG* were omitted because of her uncertain genetic status, as discussed in the text. This patient falls into the category of cystathionine synthase-deficient, B_6-responsive individuals with detectable cystationine synthase activity in fibroblast extracts [2].

[2] Grouped according to the results of assays performed without added pyridoxal phosphate. If the results of the assays performed in the presence of 1 $\mu m/ml$ pyridoxal phosphate had been used to construct this table, the only change would have been the detection of significant cystathionine synthase activity in the fibroblasts from each of the 25 patients responsive to B_6.

[3] The probability that responsiveness to B_6 and the presence of detected enzyme activity are independent is $P < 0.001$, calculated by the chi-square test.

conclude that each patient who is responsive to B_6 has at least a small amount of residual cystathionine synthase activity. On the other hand, of the 10 patients who do not respond to B_6, 9 had no significant cystathionine synthase activity in fibroblast extracts. Of those 9, *BH* (*patient 32* and Fig. 3) was the one with the most suggestive traces of possible cystathionine synthase activity. A single exceptional nonresponsive patient (*JB, patient 26*) had definite activity in his fibroblasts. In this case the cystathionine synthase was unusual in that the activity was enhanced about 10-fold by *in vitro* addition of pyridoxal phosphate. This is illustrated in Figure 4 which shows the results of an experiment in which pyridoxal phosphate was added to extracts from fibroblasts from *JB*, or to fibroblasts from responsive patient *DD* (*patient 5*). A progressive increase in the measurable cystathionine synthase activity in extracts from *JB* occurred as the concentration of pyridoxal phosphate was raised in the assay medium. The extract from *DD* exhibited only a slight stimulation, in this respect being similar to the extracts from most B_6-responsive patients and to extracts from control subjects [49]. Thus, the fibroblasts from patients who do not respond to B_6 treatment either have no significant cystathionine synthase activity, or, in one case, have activity which is unusually stimulated by the addition of pyridoxal phosphate.
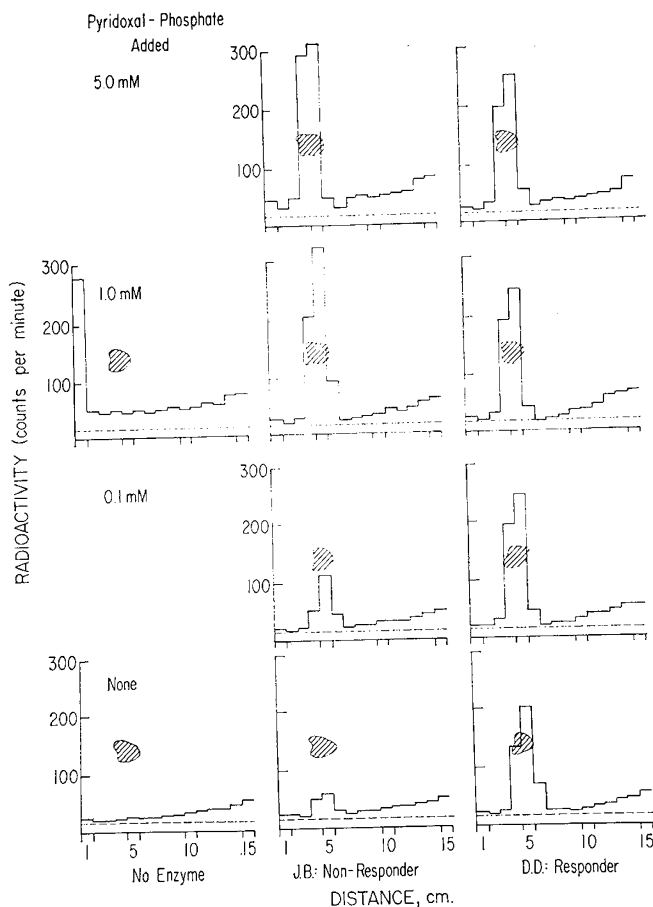
Fig. 4. The results of an assay of cystathionine synthase activities in extracts of fibroblasts from *JB*, a B_6-nonresponsive patient, and *DD*, a typical B_6-responsive patient. See legend to Figure 3 for further explanation.

Discussion

Diagnostic Value of Cystathionine Synthase Activities in Fibroblast Extracts

A major goal of the present work was to evaluate the extent to which cystathionine synthase activities in fibroblast extracts can indicate the cause in specific cases of excessive homocystine excretion. In making this evaluation, it is useful to examine initially the homocystinuric patients whose fibroblasts have activities of cystathionine synthase below the control range. To what extent does additional evidence confirm or disprove that these patients are indeed cystathionine synthase deficient? Such evidence may be of several types, as follows.

Measurement of cystathionine synthase activity in liver extracts. Seven homocystinuric patients whose fibroblasts have cystathionine synthase activity below the

control range have been examined by assay of hepatic cystathionine synthase [14, 26, 30, 43, 28]. Each had a subnormal activity of this enzyme in his liver tissue.

Measurement of rate at which sulfur of a large oral dose of methionine is converted to inorganic sulfate. As might be expected (see Fig. 1), cystathionine synthase-deficient patients carry out this conversion at a less than normal rate [20, 29]. This conversion rate has been measured in eight homocystinuric patients whose fibroblasts have cystathionine synthase activity below the control range [20, 29, 28]. Each had an impaired ability to convert methionine sulfur to inorganic sulfate.

Measurement of methionine in plasma and/or urine. Presumably because of methylation of accumulated homocysteine via *reactions 7* and *8* (Fig. 1), cystathionine synthase-deficient patients often have elevated methionine concentrations in plasma and urine. We are aware of measurements of methionine in plasma and/or urine for 19 homocystinuric patients whose fibroblasts have cystathionine synthase activity below the control range [2, 13, 14, 29, 37, 39, 43, 28]. In 16 cases definite elevations of methionine were noted. In the three remaining cases only equivocal elevations were observed [37, 28], but, for each of these cases convincing evidence is available, based on studies of a homocystinuric sibling, that the homocystinuria in these families is due to cystathionine synthase deficiency.

This evidence may be summarized as follows. Many fibroblast lines from individuals with homocystinuria have specific activities of cystathionine synthase below the range of activities found in a series of control subjects without homocystinuria. When independent evidence is available, it invariably indicates that the homocystinuria of these individuals is indeed caused by a deficient activity of cystathionine synthase and not by some other metabolic abnormality. Thus, an activity of cystathionine synthase in cultured fibroblasts below the control range provides very strong evidence for the diagnosis of cystathionine synthase deficiency in an individual with homocystinuria.

We may now look at the other side of this question and ask: "What of the nine homocystinuric patients whose fibroblasts have specific activities of cystathionine synthase falling within the control range"? A variety of evidence, including enzyme assays of fibroblast extracts, indicates that the three patients of *group B* in Figure 2 are homocystinuric because of deficiency in N^5-methyltetrahydrofolate-homocysteine methyltransferase activity, secondary to failure to accumulate normal amounts of methyl-B_{12}, the cofactor for this reac-

tion (*step 16*, Fig. 1) [12, 22, 23, 33, 34, 36]. The three patients of *group C*, Figure 2, are homocystinuric because of deficiency in methylenetetrahydrofolate reductase activity [35]. This leaves three exceptional patients.

KT. In 1964, Gerritsen and Waisman [9] reported that this patient was homocystinuric. These authors noted that *KT* did not have an elevation of plasma methionine. These, and other findings, led them to suggest that *KT* might have a different abnormality than that present in a second patient with homocystinuria studied at the same time who was later shown by direct assay of brain tissue to be cystathionine synthase deficient [32]. When *KT* was studied at the National Institutes of Health in 1966, no homocystine was detected in his urine. He converted methionine sulfur to inorganic sulfate at a normal rate [28]. Fibroblasts from this patient have normal specific activities of N^5-methyltetrahydrofolate-homocysteine methyltransferase and methylenetetrahydrofolate reductase, as well as cystathionine synthase. We conclude that, if *KT* ever excreted excessive homocystine, he did so only transiently. Such homocystinuria may have been related to an environmental rather than to a genetic cause.

JI. This patient had specific activities of cystathionine synthase in fibroblast extracts which, in some experiments, fell somewhat below the control range, in other experiments, just above the lowest observed control value [48]. She has dislocated ocular lenses, elevated plasma methionine concentrations, and fails to convert methionine sulfur to inorganic sulfate at a normal rate [28]. Although cystathionine synthase activity in her liver has not been assayed, these findings in combination appear to establish that the homocystinuria of *JI* is due to some form of cystathionine synthase deficiency. However, not only is the cystathionine synthase activity in fibroblasts from *JI* high relative to activities in other cystathionine synthase-deficient patients, but so also are the activities of her parents' fibroblasts high relative to activities in parents of other cystathionine synthase-deficient patients (Fig. 2), which suggests that *JI* may have an unusual form of cystathionine synthase deficiency.

MAG. The final patient with homocystinuria with a specific activity of cystathionine synthase within the control range for fibroblasts is *MAG*, cousin of a patient with proven cystathionine synthase deficiency (*i.e., CT patient 28*, Table I). At age 24, *MAG* was clinically unaffected. She was only mildly homocystinuric, excreting 44–125 μmol/24 hr of this amino acid

(*case 2* [40]). Her plasma methionine was marginally elevated (*patient 4*, [2]; *case 2*, [40]), and her urinary and plasma cystine concentrations were not depressed below normal. She converted methionine sulfur to inorganic sulfate at a partially impaired rate [20]. Activity of cystathionine synthase in her liver was about 12% of the mean control value [4], well below the control range, yet higher than the activity we have detected in the liver of any other cystathionine synthase-deficient individual [26]. The fibroblasts from this patient have a normal activity of N^5-methyltetrahydrofolate-homocysteine methyltransferase. Further indication that the N^5-methyltetrahydrofolate-dependent conversion of homocysteine to methionine occurs at an adequate rate [35] in these cells is provided by the finding that they grow as well as control fibroblasts on a medium in which methionine has been replaced by homocystine. These results suggest that *MAG* is homocystinuric due to a deficiency of cystathionine synthase activity which, by all parameters, is more mild than that usually reported. Her genetic status remains undetermined (see *Discussion* below) [50].

To summarize, a relatively small portion of the patients with homocystinuria studied have, in their fibroblasts, cystathionine synthase activities within the control range. Six such patients have been shown conclusively to have metabolic abnormalities other than cystathionine synthase deficiency. In one patient, the homocystinuria reported by others [9] could not be confirmed on later examination. One, possibly two, patients appear to be homocystinuric on the basis of cystathionine synthase deficiencies less severe than those usually encountered.

Mechanism of pyridoxine effect in cystathionine synthase deficiency. As was first demonstrated by Barber and Spaeth [1], some patients with cystathionine synthase deficiency respond to large doses of pyridoxine with decreases in urinary excretions and plasma concentrations of homocystine and other metabolites. Other cystathionine synthase-deficient patients do not so respond. References to early studies of this phenomenon will be found in a previous publication [29], and a summary of more recent clinical studies has been reported by Brenton and Cusworth [3]. The mechanism whereby pyridoxine exerts this effect has been the subject of several investigations. Although pyridoxal phosphate is almost certainly necessary for the activity of mammalian cystathionine synthase [18, 27], neither administration of pyridoxine to the responding patient, nor addition of pyridoxal phosphate *in vitro* to extracts of liver obtained from such patients restores

activity of cystathionine synthase to values even close
to the normal range [6, 7, 14, 29, 30, 32, 51]. Neverthe-
less, a variety of lines of evidence previously led us to
suggest [29] that pyridoxine might indeed act by bring-
ing about an increase in residual cystathionine syn-
thase activity to about 3–4% of normal. It was sug-
gested that this activity might be sufficient to metabo-
lize the normal dietary load of methionine without
undue accumulation of homocystine and its metabo-
lites. The results of our investigations of the mecha-
nism of the pyridoxine effect by the use of fibroblasts
grown in tissue culture provide further support for
this hypothesis. It has now been shown (Table II) that
there is an excellent correlation between the presence
of residual cystathionine synthase activity in cultured
fibroblasts and responsiveness to pyridoxine. The re-
sidual activity need not be very great. Less than 1% of
the mean control value may be indicative of a capacity
to respond. Patients whose fibroblasts lack this small
activity do not respond to pyridoxine [52]. This pat-
tern, of course, suggests that the B_6 response occurs by
an effect on cystathionine synthase itself, and not by
an effect on another enzyme catalyzing some alternate
metabolic reaction.

The mechanism whereby pyridoxine administration
might lead to such enhancement of activity remains to
be established. The results of our studies of cystathion-
ine synthase activity offer no support to the possibility
that clinical responsiveness to B_6 correlates with the
existence of an abnormal enzyme which requires an
unusually high concentration of pyridoxal phosphate
for catalytic activity (as has been suggested in some
cases of other B_6-responsive enzyme abnormalities [5,
27, 41]). The residual cystathionine synthase activities
in extracts of fibroblasts from responsive patients are,
in most cases, stimulated by in vitro addition of pyri-
doxal phosphate no more than are the activities in
extracts of control fibroblasts (which are stimulated
30–50% by addition of 1 mM pyridoxal phosphate).
Conversely, the enzyme activity which shows the most
notable enhancement upon addition of pyridoxal
phosphate is found in tissue from a nonresponder (JB,
patient 26). An intermediate degree of stimulation is
shown by activities from two siblings, SB and JB (pa-
tients 24 and 25), who are responsive to pyridoxine.
Altogether, this evidence strongly suggests that clinical
responsiveness to B_6 correlates not with the in vitro
stimulation of residual cystathionine synthase activity
by pyridoxal phosphate addition, but rather with the
presence of traces of residual cystathionine synthase

activity which is, in most instances, normally stimu-
lated by addition of pyridoxal phosphate.

Genetic heterogeneity in cystathionine synthase defi-
ciency. It appears that, at a minimum, there is a ge-
netic difference between the B_6 responders and the B_6
nonresponders. The correlation between B_6 responsive-
ness and the presence of residual cystathionine syn-
thase activity, as well as the fact that the members of a
given sibship are all either responsive or nonrespon-
sive, provide compelling evidence in this regard. We
think it likely that there is further genetic heterogene-
ity within both the responsive and the nonresponsive
groups. Evidence supporting this suggestion is found in
Table I. JB (patient 26) differs from all other nonre-
sponders studied in that he alone among this group has
detectable cystathionine synthase activity, and this ac-
tivity is exceptional in regard to its stimulation by the
addition of pyridoxal phosphate. The unusually high
specific activities in fibroblasts from JI and MAG have
been discussed above. Both members of sibships VI
(patients 24 and 25) appear unusual with respect to
the degree of stimulation of cystathionine synthase ac-
tivity by addition of pyridoxal phosphate. Further-
more, the analysis of variance test, applied to the data
in Table I for patients with detectable fibroblast cysta-
thionine synthase, demonstrated that the differences in
this activity between sibships are greater than the dif-
ferences within sibships. For example, in the case least
favorable to this hypothesis (i.e., sibships I–V only),
the probability is less than 0.005 that the greater varia-
tion between than within these sibships is due to
chance. Within any of the sibships in question, each
affected member may be expected to have inherited
the same two mutant genes determining cystathionine
synthase activity. The variation of this enzyme activity
within any one sibship then provides an indication of
the scatter encountered under our experimental condi-
tions between individuals in whom these genetic fac-
tors are held invariant. The observation that there is
more variation between than within these sibships can,
in our opinion, best be explained by the hypothesis
that these sibships differ from one another in their
genetic determinants of cystathionine synthase. These
suggestions are in accord with the postulations by
other authors of genetic heterogeneity in B_6-responsive
cystathionine synthase-deficient patients [8, 38]. Quan-
titative differences in residual cystathionine synthase
activities may possibly account for differences in the
dose-response curves of individual patients to B_6. Such
differences were observed by Seashore and her col-

leagues [38] in their studies of two pyridoxine-responsive, cystathionine synthase-deficient patients. The postulate of a qualitative difference in the mechanism of B_6 response [38] is not supported by compelling evidence at this time.

Evidence has recently been presented that native rat liver cystathionine synthase consists of four subunits: two identical components which move relatively rapidly during acrylamide gel disc electrophoresis, and two identical components which move relatively slowly [19]. This finding suggests to us that human cystathionine synthase has a composition of the $\alpha_2\beta_2$ type; i.e., mammals possess at least two pairs of structural genes for cystathionine synthase, each pair determining a polypeptide subunit. If this is so, within a population there may be multiple alleles at the A locus (A, A', A'', etc., where A represents the normal allele, and A' and A'' represent mutant alleles), and/or multiple alleles at the B locus (B, B', B''). In addition to individuals homozygous for the same mutant allele (sample genotype: A'A', BB), the population may then contain individuals who are genetic compounds with two different mutant alleles at the same locus (sample genotype: A'A'', BB). The population may contain also individuals who are double heterozygotes, carrying single mutant alleles at each of two loci (sample genotype: AA', BB'). Detailed interpretations of the relations between genotypes and phenotypes of patients with cystathionine synthase deficiency are not possible at this time. For example, although the evidence on B_6-responsiveness suggests that at least two mutations affecting cystathionine synthase activity are present in the population, it is not certain whether these two mutations (1) are allelic, (2) affect both loci A and B, or (3) involve other loci (for example, a regulatory gene). For a simple model compatible with available data we postulate the existence of allelic "responder" and "nonresponder" mutations. According to this model, fibroblasts from individuals homozygous for the "nonresponder" gene would have no cystathionine synthase activity detected with assays of the present sensitivity. Fibroblasts from persons homozygous for the "responder" gene would have detectable activity. Probability then suggests that among the patients studied in Table I occur some genetic compounds with one "responder" and one "nonresponder" gene. The fibroblasts from these individuals might be expected to have intermediate activities of cystathionine synthase. The fact that all patients (except JB, patient 26) whose fibroblasts have detectable cystathionine synthase are

B_6 responsive suggests that such genetic compounds are, phenotypically, B_6 responders. Additional mutations must be postulated to accommodate exceptional patients such as JB (patient 26).

Heterozygotes for cystathionine synthase deficiency tend to have less than 50% of the normal specific activity of this enzyme. Four parents of affected children studied in this laboratory had specific activities of liver cystathionine synthase of 33–43% of the mean control value [4, 21]. The range for the four parents reported by Gaull and Sturman [8] was 22–47%. The mean for these eight parents was 34.0% (SD 7.8; SE 2.8%). The data presented in Figure 2 for extracts of fibroblasts from parents are not inconsistent with this suggested trend. The $\alpha_2\beta_2$ structure of cystathionine synthase permits a tentative explanation of these observations. Assume that parents are simple heterozygotes (genotype: AA', BB, or AA, BB', etc.). If each locus accounts for equal numbers of enzyme subunits and subunits are randomly assorted, parents of the first genotype will have three types of enzyme molecules: $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, and $\alpha'_2\beta_2$, constituting respectively 25%, 50%, and 25% of the cystationine synthase population. Molecules of the type $\alpha_2\beta_2$ are normal and will have a normal activity, designated here as 1.0. Molecules of the type $\alpha'_2\beta_2$ are the same as those to be found in homozygous deficient individuals and therefore will contribute little or no activity. The $\alpha\alpha'\beta_2$ molecules are unique to heterozygotes. A model which assumes that such a molecule, in which one-half the α subunits are normal, will have a relative activity of 0.50, predicts an overall specific activity of cystathionine synthase of 50% of normal. To fit more closely to the 34% actually observed, one may assume subunit interaction such that the $\alpha\alpha'\beta_2$ molecule has a relative activity of 0.25. The predicted overall specific activity in heterozygotes is then 37–38%. According to the same assumptions, the double heterozygote (genotype: AA', BB') would have a specific activity of 14% of the mean control. Possibly MAG falls in this category.

In order to analyze further genotype-phenotype relationships in cystathionine synthase-deficient patients, additional information is needed. More detailed studies of structural and functional aspects of mutant cystathionine synthases, more accurate quantitation of the relation between doses of pyridoxine and clinical responses in individual patients, and hybridization studies between fibroblasts from different deficient individuals, may all be helpful in gaining further insight into this aspect of cystathionine synthase deficiency.

Summary

The genetic abnormality underlying excessive homocystine excretion may be established by assays of the activities of cystathionine synthase, N^5-methyltetrahydrofolate-homocysteine methyltransferase, and methylenetetrahydrofolate reductase in extracts of fibroblasts grown in tissue culture. The present study, which includes results obtained on 40 homocystinuric, cystathionine synthase-deficient individuals, indicates that in 38 of these cases cystathionine synthase activities in fibroblast extracts were below the control range, whereas the activity in one case came just within the control range [48], and in one exceptional case of unknown genetic status the activity fell within the control range.

The presence of clinical responsiveness of cystathionine synthase-deficient patients to pyridoxine treatment correlates not with the *in vitro* stimulation of residual cystathionine synthase activity by pyridoxal phosphate addition, but rather with the presence of traces of residual cystathionine synthase, the activity of which is, in most instances, stimulated no more than control activity by addition of pyridoxal phosphate. This evidence strongly supports our working hypothesis that the pyridoxine effect is due to an enhancement of residual cystathionine synthase activity in the patient to a few percent of normal.

The presence of genetic heterogeneity among cystathionine synthase-deficient patients is suggested by the demonstrated genetic determination of responsiveness to pyridoxine, as well as by a detailed consideration of the properties of residual cystathionine synthase activities in such patients. The proven $\alpha_2\beta_2$ subunit structure of mammalian cystathionine synthase suggests that populations with genetic heterogeneity among the structural genes determining this enzyme will contain not only mutants homozygous for defective alleles, but also genetic compounds and double heterozygotes. Postulation of negative interaction between normal and mutant subunits provides a tentative explanation for the observation that heterozygotes for cystathionine synthase deficiency tend to have less than 50% of the normal specific activity of this enzyme.

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47. The product of oxidation of cystathionine under the conditions used has not been unequivocally identified. Preliminary data (J. Giovanelli and S. H. Mudd, unpublished) indicate that the product is cystathionine sulfone, and, for convenience, it will be so referred to in this paper.

48. The lowest observed control value was 3.7 units/mg protein, only one-third the next lowest observed value of 10.6. The patient whose fibroblasts had the specific activity of 3.7 suffered from primary amyloidosis. It is not possible to be certain that he was not heterozygous for cystathionine synthase deficiency. These considerations serve to emphasize that the present control range for fibroblast extracts may extend further in the direction of low specific activities than would the actual normal range.

49. It should be apparent that, for extracts with low specific activities of cystathionine synthase detected with the sensitive assay, the results obtained are subject to more experimental error than are those for extracts of higher specific activities measured by the standard assay. Thus, variations such as those encountered, for example, as the pyridoxal phosphate concentrations were raised in the extracts from RJ (patient 11) are not considered significant, nor can it be concluded surely that extracts such as those from the fibroblasts of RH (patient 10), or WS (patient 19) are stimulated more than control extracts by the addition of pyridoxal phosphate.

50. It may be supposed that there is a quantitative discrepancy between the fact that MAG, with 12% of the mean control hepatic cystathionine synthase activity, excretes homocystine in her urine, and the postulate that restoration of 3-4% of the mean control activity of this enzyme is sufficient to account for the response of typical cystathionine synthase-deficient patients to pyridoxine. However, it may be most valid to compare MAG in her basal state to more typical patients in states of B_6-induced response, inasmuch as, under these circumstances, the quantitative extent of the biochemical abnormalities are similar [29, 40]. Thus, in our opinion, the possible discrepancy emphasized here is not sufficient to negate our basic hypothesis that relatively minor amounts of cystathionine synthase activity can bring about major alleviations in the biochemical abnormalities which are secondary to deficiency of this enzyme activity.

51. A single, possibly exceptional, case has been reported [43].

52. These suggestions are consistent with previous published data concerning cystathionine synthase activity in livers of homocystinuric patients. All of the patients in whom we assayed hepatic cystathionine synthase activity [26] have subsequently been shown to be responsive to B_6, and we detected significant hepatic activity in all cases but one, CT (patient 28), the initial patient studied. In that early study a relatively insensitive assay was used, one which would have failed to detect 10% or less of the mean cystathionine synthase activity. Other studies of cystathionine synthase activity in liver [7, 14, 43] or fibroblasts [38] of homocystinuric patients responsive to B_6

have, in some cases detected residual cystationine synthase activity [38, 43]. In those studies in which no activity was detected, the sensitivity of the assay used was not mentioned [7, 14], so it is difficult to judge whether a few percent of normal activity would have been detected. We are not aware of any assay of cystathionine synthase activity in the liver of a non-B_6-responsive patient, performed with sensitivity sufficient to establish whether or not there is a small residual hepatic activity in such a case. The published data on cystathionine synthase activities in short term lymphocyte cultures [11] are as yet insufficient to establish whether the presence or absence of residual cystathionine synthase activity in these cells will also correlate with clinical responsiveness or nonresponsiveness to pyridoxine therapy.

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