CYSTATHIONINE SYNTHASE DEFICIENCY: HETEROZYGOTE DETECTION USING CULTURED SKIN FIBROBLASTS*

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SUMMARY: An assay for cystathionine synthase in cultured fibroblasts, of high sensitivity and not rate-limited for substrate, is described. Enzymatic activity was found to be highest in controls (mean ± SEM = 20.97 ± 1.81 nmole cystathionine/mg protein/hour), intermediate in obligate heterozygotes for synthase deficiency (4.40 ± 0.92), and lowest in patients (0.77 ± 0.42), with no overlap between controls and heterozygotes. One clinically and biochemically atypical patient had a synthase activity at the low end of the heterozygote range. Thus, this method is effective for the detection of heterozygotes for cystathionine synthase deficiency.

The most extensively studied of the diseases of sulfur amino acid metabolism is homocystinuria due to a deficiency of cystathionine synthase activity. This enzymatic deficiency is inherited as an autosomal recessive. An absence of synthase activity in affected individuals has been demonstrated in liver and brain (1,2), and more recently in cultured skin fibroblasts and amniotic fluid cells (3), long-term lymphoid cell lines (4), and phytohemagglutinin (PHA***)-stimulated lymphocytes (5). Distinguishing heterozygotes on the basis of loading tests has not proved reliable, and thus it is generally attempted by assay of synthase activity in liver biopsy material. However, tissue culture provides a readily available source of material for enzyme assay and this has prompted workers to seek an in vitro method for heterozygote detection.

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***Abbreviations used: PHA, phytohemagglutinin; PLP, pyridoxal 5'-monophosphate

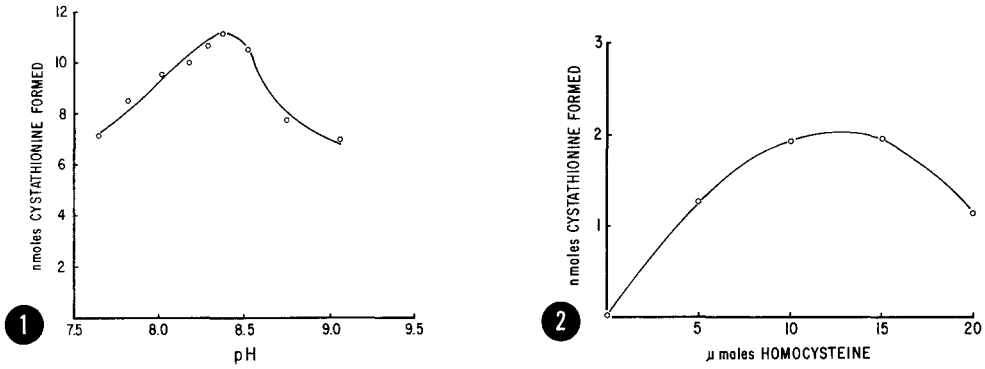
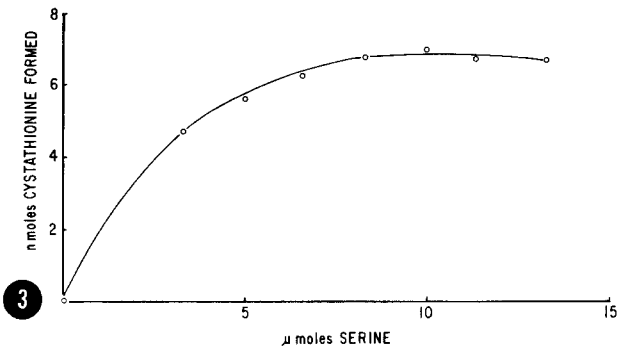


Fig. 1. Effect of pH on cystathionine synthase from cultured human skin fibroblasts. Assay procedure as in the text, except that solutions and buffer were at the pH indicated.
Fig. 2. Rate of reaction of cystathionine synthase from cultured human skin fibroblasts as a function of L-homocysteine concentration.

PHA-stimulated lymphocytes (6) and cultured skin fibroblasts (7,8) have been used, but overlap between heterozygotes and controls, or between heterozygotes and homozygous affected individuals, has cast doubt upon the reliability of the procedure. We now report an improved assay system, developed to measure synthase activity in cultured cells, which allows us to distinquish obligate heterozygotes from clinically typical homozygotes for synthase deficiency and from controls.

Methods

Skin biopsies were obtained from the inner forearm of 5 patients, 6 obligate and 2 potential heterozygotes, and 17 normal controls. Fibroblast cultures were established and maintained according to standard tissue culture procedures. Care was taken to assure that all cultures were harvested at the same stage of confluence. After reaching confluence, the cultures were fed consecutively for three days and harvested on the fourth day. The cells were then washed with saline, lysed by freeze-thawing, and stored frozen at -70° until the time of assay. The assay procedure is based on the direct measurement, on an automatic amino acid analyzer, of cystathionine formed in the reaction. Conditions were established for maximal enzymatic activity. The following, in μmoles, were incubated for 4 h at 37° in a total volume of 0.4



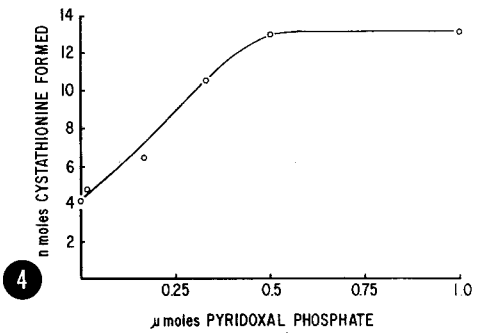


Fig. 3. Rate of reaction of cystathionine synthase from cultured human skin fibroblasts as a function of L-serine concentration.
Fig. 4. Rate of reaction of cystathionine synthase from cultured human skin fibroblasts as a function of PLP concentration.

ml: Tris-HCl buffer (pH 8.4), 60; PLP, 0.5; EDTA, 1; L-serine, 10; L-homocysteine made from the thiolactone (Calbiochem), 15; and cell lysate (usually 250 μl). The remainder of the procedure has been described by Gaull et al. (9). Protein concentrations were determined according to Lowry et al. (10). Enzymatic activity is expressed as nmoles cystathionine formed/mg protein/hour.

Results

The method developed in the present work involved several modifications of the conditions utilized in previous assays; these conditions were systematically established as optimum. The production of L-cystathionine by the cell extract was linear with protein concentration up to 1.3 mg/tube and with time of incubation up to 5 h. Enzymatic activity was greatest at pH 8.4 (Fig. 1). Activity was completely dependent upon the addition of L-homocysteine, the rate of reaction being maximal in the presence of 15 μmoles (Fig. 2). A serine concentration of 10 μmoles/tube produced the maximum rate of reaction (Fig. 3), but it was noted that when no serine was added, very small amounts of product (<0.6 nmole) were still detectable. This "blank" value, presumably the result of endogenous serine, was subtracted from the other points on the curve. The omission of PLP from the reaction mixture did not greatly alter the activity from that obtained with the standard amount of 0.015 μmoles (Fig. 4). However, when the PLP was increased to 0.5 μmoles a significant increase in prod-

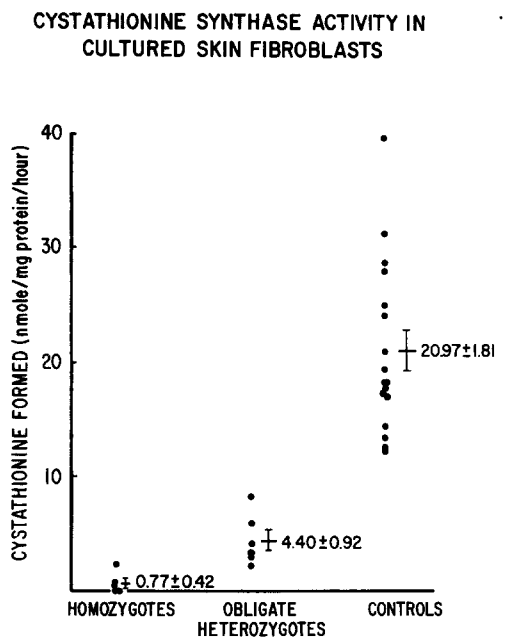


Fig. 5. Distribution of cystathionine synthase activity from cultured human skin fibroblasts derived from control subjects and from homozygotes and heterozygotes for cystathionine synthase deficiency. The value for each group gives the mean ± SEM.

uct formation was seen. Synthase activity was completely destroyed by boiling.

Enzymatic activity in cultured skin fibroblasts was highest in controls (mean ± SEM = 20.97 ± 1.81), intermediate in obligate heterozygotes (4.40 ± 0.92), and lowest in patients (0.77 ± 0.42) (Fig. 5). Of the five homozygotes studied, activity was detectable in three, two of whom demonstrated values of 0.74 and 0.83. The third, with enzymatic activity of 2.29 will be discussed below. Cultured skin fibroblasts from two potential heterozygotes were also investigated. The first had a synthase activity of 21.60 and was classified as normal, while the second showed an activity of 6.76, suggesting a heterozygous condition.

Discussion

The presence of cystathionine synthase activity in cultured skin fibroblasts was demonstrated in 1968 by Uhlendorf and Mudd (3). However, their assay system utilized conditions that had been developed for crude extracts of

rat liver (1), and, in addition, achieved sensitivity by use of a serine concentration well below the K_m for the enzyme. Subsequent studies on cultured cells (5-8,11) have used only slight modifications of this procedure. Rather than depending for sensitivity on a radioactive assay with rate-limiting substrate, the assay presented here is based upon the actual measurement of cystathionine formed under optimal conditions for cultured cells.

With the present procedure, differentiation of the heterozygous state of synthase deficiency from both the homozygous state and from normal controls is readily obtained with cells grown in tissue culture. This previously had been attempted with cultured skin fibroblasts (7,8), but overlap between the three genotypes was found, making heterozygote detection uncertain. Goldstein et al. (6) reported the detection of heterozygotes using PHA-stimulated lymphocytes; although the mean enzymatic activity for obligate heterozygotes was below that of the control group, the values for three of the heterozygotes overlapped the controls. Normal variation and/or the genetic heterogeneity strongly suggested in synthase deficiency (12), as well as the inclusion of heterozygotes in the control group, might account for the overlap seen by other workers. However, it is also possible that this overlap is the result of variation in activity caused by use of a non-optimal serine concentration on the steeply rising portion of the substrate curve. In the present study obligate heterozygotes demonstrated mean enzymatic activity intermediate between that of the homozygous affected group and the controls. The differences between the three means were significant. In addition, the only overlapping value was that of a clinically and biochemically atypical patient whose unusually high activity fell at the low extreme of the heterozygote range. His hepatic synthase activity is also unusually high for a homozygote (15% of mean control hepatic activity) (12), and thus, his classification as a minimally affected homozygote, rather than a severely affected heterozygote remains uncertain. On the basis of these results we conclude that this system of culturing skin fibroblasts and assaying extracts of the cells for enzymatic activity is a reliable method

for detecting the heterozygous state of cystathionine synthase deficiency.

The present results confirm and extend previous observations (6,7,12,14, 15) that mean synthase activities in heterozygotes are generally below the 50% mean control value reported in most inborn errors of metabolism and expected with a simple gene-dose relationship. The mean synthase activity in our heterozygote fibroblasts is 21% of that seen in the control group. To account for this low activity, we postulate that the synthase may be a dimeric protein which consists of two polypeptide subunits, each determined by one of a series of alleles, each allele coding for a molecularly different polypeptide chain. In the heterozygote, two different alleles are present, and two different subunits are synthesized, one normal and one aberrant. These two different subunits could combine to form a dimer in one of three ways: normal-normal, normal-mutant, and mutant-mutant, resulting in the formation of three molecularly different dimeric proteins. Thus, 25% of the total enzyme protein in the heterozygote would consist of dimers composed of two normal polypeptide chains and would have normal activity (as in the normal individual), 25% would consist of dimers composed of two mutant polypeptide chains with no (or very little) activity (as in the homozygous affected individual), and 50% would consist of "hybrid" dimers, composed of one mutant and one normal chain, unique to the heterozygote. If the hybrid dimer possessed no synthase activity the total activity in the heterozygote would be 25% of mean control activity, as we have observed. If the hybrid were completely active, the heterozygote could demonstrate 75% of mean control activity. Depending on the effect of the mutant polypeptide chain in combination with a normal one, and thus the activity of the hybrid dimer, the total activity observable in the heterozygote could vary from 25-75%. Perhaps the 50% value so often "observed" in heterozygotes for enzymatic deficiencies actually represents a mean of individuals with enzymatic activities ranging from 25-75% of normal. Of course, the possibility of more than two subunits making up the enzyme protein, or of contributions from multiple gene loci (7,13) would result in even more complex patterns

of hybrid molecule formation and even greater variation in enzyme activity in the heterozygous individual.

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