Am J Hum Genet 34:73-83, 1982

Immunochemical Studies on Cultured Fibroblasts from Patients with Homocystinuria due to Cystathionine β-Synthase Deficiency

FLEMMING SKOVBY,[1] JAN KRAUS, CARRIE REDLICH, AND LEON E. ROSENBERG

SUMMARY

Fibroblast extracts from 20 individuals with homocystinuria due to cystathionine β-synthase deficiency were analyzed for the presence of immunoreactive synthase antigen as cross-reacting material (CRM). CRM was quantitated by competitive and direct immunotitration using rabbit antiserum against homogeneous human liver synthase. The lower limit of sensitivity for detection of CRM was 1.5% of the amount of synthase antigen in control extracts. Each of 14 mutant extracts with detectable synthase activity had detectable CRM ranging from 5% to 100% of the amount found in control extracts. No statistically significant correlation was observed between the percent residual activity and the percent CRM. Of six mutant extracts without measurable catalytic activity, three had no detectable CRM, while three had 13%, 17%, and 26% CRM, respectively. These results extend our information about the biochemical heterogeneity previously found in synthase deficiency, and emphasize that such deficiency is caused by a wide array of mutations affecting the structural locus for cystathionine β-synthase.

INTRODUCTION

Cystathionine β-synthase (CS; L-serine hydro-lyase [adding homocysteine]) (E.C. 4.2.1.22) deficiency is an autosomal recessively inherited disorder of sulfur amino acid metabolism and the major cause of homocystinuria in man. Its clinical variability and biochemical heterogeneity are well documented [1]. Nearly all patients have dislocated optic lenses, and other clinical manifestations such as mental

Received April 6, 1981.

This work was supported by grant AM-12579 from the National Institutes of Health. F. S. was the recipient of National Research Service Award GM-07439 from the National Institutes of Health.

[1] All authors: Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.

 0002-9297/82/3401-0006$02.00

retardation and thrombotic vascular disease occur in a much smaller fraction. Moreover, some affected individuals, but not all, respond to treatment with pyridoxine supplements with a marked fall in their characteristically elevated plasma and urinary concentrations of methionine and homocystine. At the cellular level, this heterogeneity was first evidenced by the observation that liver and fibroblast extracts from some patients lack detectable CS activity, whereas extracts from other patients retain variable amounts of enzyme activity [2].

For several years, our laboratory has attempted to define the mechanisms responsible for this biochemical heterogeneity and relate them to in vivo pyridoxine responsiveness. Fowler et al. [3] examined cell lines grown in pyridoxal-supplemented medium (1,000 ng/ml) from 14 CS-deficient patients for residual activity, for affinity of mutant CS for its coenzyme, pyridoxal 5'-phosphate (PLP), and for thermostability. They concluded that at least three general classes of CS-deficient mutant cells exist: those with no residual activity; those with reduced activity and normal affinity for PLP; and those with reduced activity and reduced affinity for PLP. Lipson et al. [4] extended this work in cells from controls and selected mutants with residual CS activity by growing fibroblasts in a pyridoxal-free medium, thereby depleting them of PLP and converting virtually all of their CS to apoenzyme. When such cells were then propagated in media containing increasing concentrations of pyridoxal, maximal saturation of apoCS with PLP in control fibroblasts was observed at a medium pyridoxal concentration of 10 ng/ml or less, whereas 25–50 ng/ml was required in fibroblasts from CS-deficient patients responsive to pyridoxine in vivo, and at 100 ng/ml, CS holoenzyme formation was still increasing in cells from in vivo nonresponsive patients. In vitro estimates of the K_m of CS for PLP in extracts of PLP-depleted cells were similarly much higher in the latter group, and, importantly, exceeded the capacity of cells to accumulate PLP intracellularly. Taken together, these studies imply that a larger number of different mutations cause CS deficiency—some by obliterating catalytic activity entirely, others by altering the apoprotein in such a way that its affinity for coenzyme and/or its stability are reduced variably.

CS from human liver has been purified to homogeneity recently in our laboratory by Kraus et al. [5], and a monospecific antiserum against the pure enzyme has been raised in rabbits. With this antiserum, it is possible to extend studies of the molecular basis of CS deficiency beyond measurements of catalytic activity. As an initial step, we report here on the quantitation of CRM in fibroblast extracts from 20 patients with homocystinuria due to CS deficiency.

MATERIALS AND METHODS

Materials

L-[U-^{14}C]serine was purchased from New England Nuclear, Boston, Mass.; PLP from Sigma, St. Louis, Mo.; L-cystathionine and L-homocysteine thiolactone from Calbiochem, La Jolla, Calif.; and L-serine from Schwarz/Mann, Orangeburg, N.Y. Anti-CS antiserum was obtained by immunization of a white New Zealand rabbit with homogeneous human liver CS. The immune serum was used without purification. Goat antiserum, raised against

rabbit γ-globulin and purchased from Calbiochem, was prepared in such a way that 125 lyophilized U were dissolved in 4 ml of 0.01 M potassium phosphate buffer, pH 7.2.

Cell Culture

Skin fibroblast lines from 20 unrelated individuals with CS-deficient homocystinuria and from two controls were used in these studies. All cell lines had been propagated for eight to 20 passages. Cells were cultured in 850-cm^2 polystyrene roller bottles (Corning, American Scientific, Bedford, Mass.) at 37°C in a 5% CO_2/95% air atmosphere using Eagle's minimal essential medium containing pyridoxal hydrochloride (1,000 ng/ml) and kanamycin (100 μg/ml), supplemented with 1% (v/v) nonessential amino acids and 10% (v/v) fetal calf serum. Cells were grown to confluence, harvested with 0.1% trypsin solution, washed twice with phosphate-buffered saline, pH 7.4, centrifuged at 800 *g* for 10 min at 4°C, and then stored as cell pellets at −70°C.

Cell Extracts

Fibroblast pellets were sonicated in 0.03 M potassium phosphate buffer, pH 6.0, containing 1 mM PLP. The supernatants obtained after centrifugation at 10,000 *g* for 10 min at 4°C were used for immunotitration experiments. The protein concentration of these extracts was determined by the method of Lowry et al. [6] using crystalline bovine serum albumin as a standard.

Immunotitration

The quantitative assay for CRM in the mutant extracts was based on the inhibition by mutant extract of immunoprecipitation of control extract (competitive immunotitration). In parallel titration experiments, 50 μl vol of control fibroblast extract (protein concentration 2.0–5.0 mg/ml) were mixed either with equal volumes of mutant extract or with bovine serum albumin containing the same amount of protein (2.0–10.0 mg/ml) and PLP as found in the mutant extract. Increasing amounts of rabbit anti-CS antiserum, diluted in a 10 μl vol, were added, and these mixtures were incubated for 22 hrs at 4°C. Ten μl of goat antiserum to rabbit γ-globulin were added to each mixture, and the tubes were allowed to stand for 2 hrs at 4°C to permit formation of CS-anti-CS-antirabbit γ-globulin complexes. After centrifugation at 10,000 *g* for 10 min at 4°C, 80 μl of the supernatants were assayed for CS activity. In each pair of titration curves, the amount of antiserum required to inhibit each initial activity by 50% was denoted the Ab50. The percent of CRM in a mutant was defined as

$$\frac{\mathrm{Ab50}_{c+m} - \mathrm{Ab50}_{c}}{\mathrm{Ab50}_{c}} \times \frac{P_c}{P_m} \times 100 ,$$

where *c* refers to the control fibroblast extract, *c+m* to the mixture of control and mutant extracts, and *P* to the protein concentration of the respective fibroblast extracts.

Selected mutants with high residual catalytic activity were also immunotitrated directly using the procedure described above, their titration curves being compared with those obtained with control fibroblast extracts.

Assay of CS Catalytic Activity

CS catalytic activity was assayed as described by Fowler et al. [3], except that the amounts of [^{14}C]cystathionine formed were determined by counting in a Beckman (LS) 7500 liquid scintillation spectrometer. One U of CS activity is defined as that which produces 1 nmol of L-cystathionine per hr. This assay is capable of detecting as little as 0.25% of the CS activity found in control cells [3].

RESULTS

Properties of Anti-CS Antiserum

An average of 0.06 μl of the rabbit anti-CS antiserum utilized in this study produced a 50% inhibition of 1 U of CS activity. Using the specific activity of the pure liver enzyme (340,000 U/mg) as a conversion factor, it can be approximated that each unit of enzyme activity corresponds to 3 ng of fibroblast CS. As shown in figure 1, there was a linear relationship between the amount of antiserum required to precipitate 50% of initial enzyme activity (Ab50) and the amount of enzyme in the assay over at least the fivefold range of enzyme activity within which all immunotitrations presented here were performed. This linearity justifies the use of Ab50 values in calculation of CRM as defined under MATERIALS AND METHODS.

The insoluble antigen-antibody immunocomplexes retained full catalytic activity even when further complexed with goat antiserum against rabbit γ-globulin, suggesting that the antigenic determinants on the CS molecule are separate from the catalytic site(s).

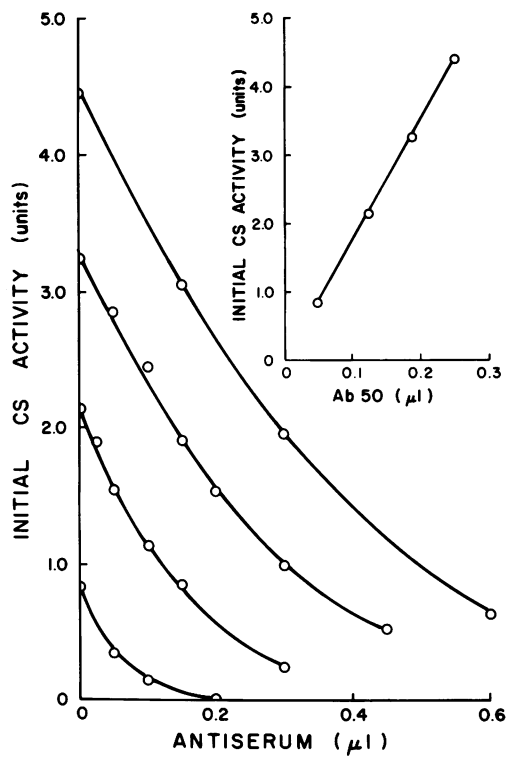

FIG. 1.—Immunotitration of control fibroblast extracts (no. 86) containing increasing amounts of CS activity. The Ab50 in each titration is determined graphically as the amount of antiserum required to inhibit the initial enzyme activity by 50%. *Inset* shows the Ab50 as a function of the amount of enzyme activity initially present.

Sensitivity of Immunotitration Method

To define the lower limit of sensitivity of the immunotitration assay, we determined the smallest amount of additional control fibroblast protein that, when mixed with standard amounts of that same control extract, could be detected as a separate titration curve of CS activity (i.e., could displace the control curve to the right). In numerous experiments (fig. 2), as little as 5% of additional control extract produced a detectable shift in the titration curve. Immunotitrations of control extracts containing less than 5% of additional protein could not consistently be separated from the baseline control curve. The data in figure 2 show, moreover, that the change in the Ab50 produced by addition of 5% and 10% increments of control extract, respectively, agreed well with the additional amounts of control extract added (i.e., 5% additional protein increased the Ab50 by 5.7%; 10% of additional protein increased the Ab50 by 11.5%). This finding indicates that, when equal amounts of protein from control and mutant extracts are mixed together, as little as 5% CRM can be detected. However, by increasing the amount of mutant fibroblast protein to four times that of control in a competitive immunotitration, the overall sensitivity for detection of CRM was increased to ~1.5%.

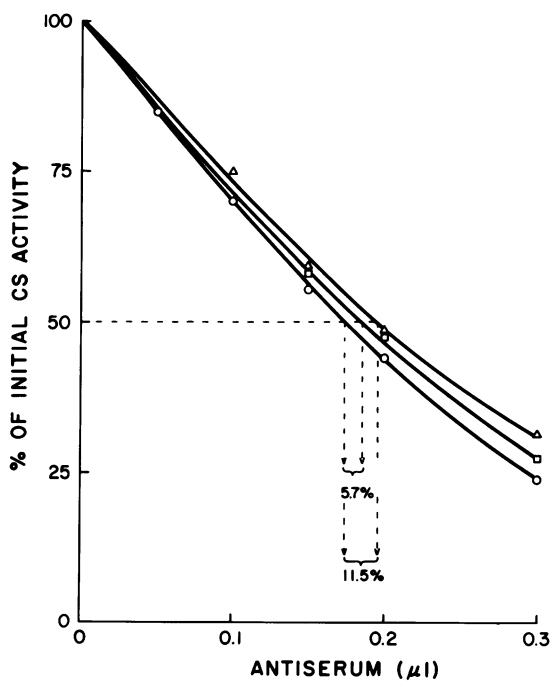

FIG. 2.—Sensitivity of immunotitration of CS activity in a representative control fibroblast extract (no. 86). The *two top curves* represent titrations of fibroblast extract containing 5% (□) and 10% (Δ) more extract protein than that present in the *baseline titration curve* (O). The differences between the Ab50 values obtained from the 5% and the 10% curves are 5.7% and 11.5%, respectively.

Competitive Immunotitration of CS-deficient Cell Lines

Results of at least two determinations of CRM by competitive immunotitration in each of the 20 CS-deficient fibroblast lines are summarized in table 1, along with their respective catalytic activities (expressed as percent of the mean catalytic activity of the control cell lines). Initially, equal amounts of control and mutant extract protein were mixed prior to competitive immunotitration. For those mutant extracts observed to contain close to or < 5% CRM when thus assayed, repeat competitive immunotitration experiments were conducted using mutant:control protein ratios of 3:1 or 4:1.

The catalytic activities in the mutants ranged from undetectable to 9% of control, while the amounts of CRM ranged from undetectable to 82%. Each of the 14 mutant lines with residual CS activity were CRM-positive (CRM^+). Figure 3 shows the immunotitration curve obtained with one such fibroblast line (no. 341). Although, as shown in table 1, all activity-positive mutants had relatively more CRM than catalytic activity, there was no statistically significant correlation between the two parameters ($P \simeq .2$).

Six mutant cell lines had no detectable activity. In three of these (nos. 342, 344, and 599), no CRM was detectable. As illustrated in figure 4, the extract from such a

TABLE 1

CRM AND RESIDUAL CYSTATHIONINE β-SYNTHASE ACTIVITY IN MUTANT FIBROBLAST EXTRACTS

Cell line no.*	CS activity (%)†	CRM (%)‡
676	5.1	82
341 (10)	0.5	45
375 (13)	8.5	39
343 (5)	4.3	35
42 (2)	6.8	25
338 (4)	9.0	17
34 (1)	1.5	16
860	3.8	12
382 (14)	3.0	12
380 (7)	0.5	12
729	2.4	10
458	0.3	7
168 (3)	1.2	5
578	0.6	5
339 (8)	ND†	26
366 (6)	ND	17
340 (9)	ND	13
599	ND	ND‡
344 (12)	ND	ND
342 (11)	ND	ND

* Denotes our laboratory accession number, as also used by Lipson et al. [4]. The numbers in parentheses relate to the numbering system employed by Fowler et al. [3].

† Values given represent the mean of multiple determinations, expressed as % of the mean specific activity (14.3 U/mg protein) of the control fibroblast lines used (nos. 83 and 86). ND = not detectable (less than 0.25% of control activity).

‡ Data shown are the mean of the results from at least two competitive immunotitrations on each mutant line. ND = not detectable (less than 1.5% CRM).

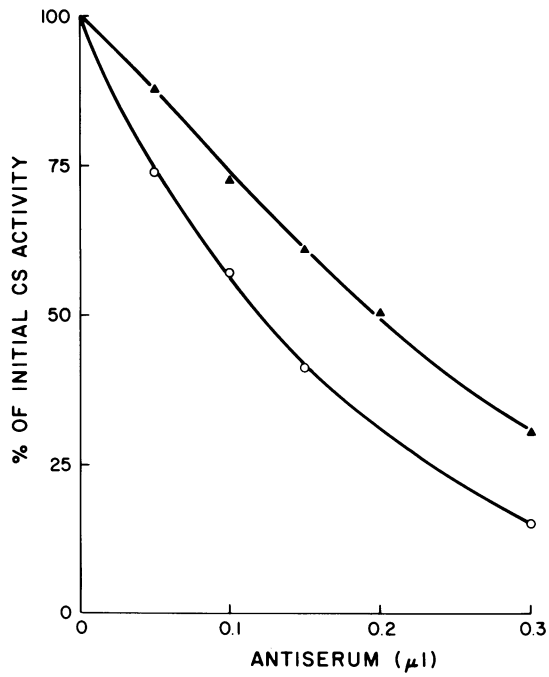

FIG. 3.—Competitive immunotitration of fibroblast extract from mutant cell line no. 341. *Top curve* (▲) shows the titration of control extract mixed with an equal volume of mutant fibroblast extract, while the *bottom curve* (O) is the immunotitration of the same control extract mixed with an equal volume of bovine serum albumin. The respective protein concentrations were: control extract, 4.0 mg/ml; mutant extract, 4.8 mg/ml; and bovine serum albumin, 4.8 mg/ml.

mutant (no. 599) failed to displace the control curve in a competitive immunotitration, even when four times as much protein extract was added to the control extract. The remaining three mutants without catalytic activity (nos. 339, 340, and 366), however, had clearly measurable CRM of 26%, 13%, and 17%, respectively.

Direct Immunotitration of CS-deficient Cell Lines

Table 2 shows the results obtained by direct immunotitration of extracts from four of the five mutants with the highest residual catalytic activities (nos. 338, 343, 375, and 676). As noted in table 2, the results are in excellent agreement with those CRM values obtained from independent competitive immunotitrations. Figure 5 illustrates two of the direct immunotitration curves from which these data were derived. To allow comparison with control data, the percent of initial CS activity in figure 5 is expressed on the ordinate as a function of the amount of anti-CS antiserum added per mg fibroblast extract protein on the abscissa, and the mean ± 1 standard error of the mean of the Ab50 values observed in the immunotitrations of control extracts is shown. The Ab50 values obtained with these mutants thus reflect directly the amount of mutant enzyme that immunoreacts with anti-CS

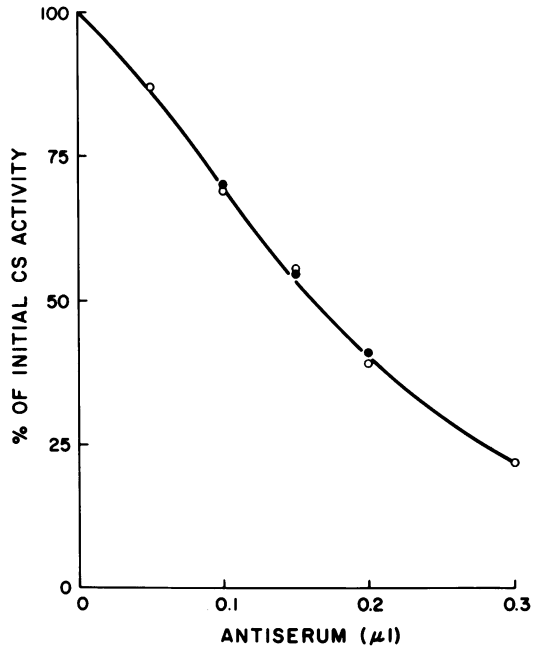

FIG. 4.—Competitive immunotitration of fibroblast extract from mutant cell line no. 599. Control fibroblast extract was mixed either with an equal volume of bovine serum albumin (O) or with an equal volume of mutant fibroblast extract (●). The respective protein concentrations were: control extract, 2.5 mg/ml; mutant extract, 10.0 mg/ml; and bovine serum albumin, 10.0 mg/ml.

antiserum. Cell line no. 676 is remarkable among all the mutants as being the only one with an amount of enzyme protein in the range of control values.

DISCUSSION

We have demonstrated that, in the presence of goat antirabbit γ-globulin, an antiserum raised in rabbits against homogeneous human liver CS precipitates CS protein from control fibroblast extracts. Because the immunoprecipitated CS molecules retain full catalytic activity, it is clear that the particular antibodies produced are directed against antigenic determinants distinct from those conferring catalytic activity. Using this antiserum, we have developed a sensitive immunotitration assay aimed at estimating the amounts of CS antigen in fibroblast extracts from a large group of patients with inherited CS deficiency. This assay is capable of detecting as CRM as little as 1.5% of the CS antigen present in control extracts.

Of the 20 mutant extracts studied, 14 had some residual CS catalytic activity. As expected, each of these lines had easily detectable CRM by direct and/or competitive immunotitration. With a single exception (line no. 676), each CRM^+ line contained distinctly less CRM than did control cells. Assuming that the affinity of such mutant CS molecules for the anti-CS antibodies present in the rabbit antiserum is comparable to the affinity exhibited by normal fibroblast CS, these data indicate

TABLE 2

COMPARISON OF RESULTS OBTAINED FROM COMPETITIVE AND DIRECT IMMUNOTITRATIONS OF MUTANT EXTRACTS WITH HIGH RESIDUAL ACTIVITY

	METHOD OF IMMUNOTITRATION	
CELL LINE NO.	Competitive (%)*	Direct (%)†
676.....	82	120
375.....	39	39
343.....	35	38
338.....	17	21

* Data are extracted from table 1.

† Values given are the mutant Ab50 values per mg fibroblast extract protein, expressed as % of the mean control Ab50 per mg protein. Experimental data from which such values were derived are shown in figure 5. The Ab50 value for no. 676 is within 1 SE of the mean control Ab50 (fig. 5).

that in all CRM^+ lines except no. 676, the number of molecules of CS protein is decreased. We can conclude, further, that such a decrease cannot be explained merely by such simple mechanisms as a reduced rate of synthesis or an accelerated rate of destruction of catalytically normal CS molecules because all of the CRM^+ lines (including no. 676) had distinctly more fractional CRM than fractional residual catalytic activity. Thus, it is apparent that these CRM^+ mutants reflect the presence of mutant CS molecules altered at their catalytic sites, as well as, generally, in their rates of synthesis and/or degradation. Since fibroblast extract from mutant no. 676 contains a normal amount of CRM but only 5% of residual activity, it would appear that the mutation in this particular cell line alters the catalytic site(s) profoundly, with little or no effect on the rate of enzyme synthesis or degradation.

Additional analysis of these mutants with residual enzymatic activity did not reveal any correlation between residual activity and CRM. Furthermore, when comparing the latter to the data previously obtained in our laboratory on in vivo responsiveness to pyridoxine [3], and on affinity of apoCS for PLP [4], no consistent relationship was noted.

The six mutant lines without residual activity fall into two groups. Three had clearly measurable, although much reduced amounts of CRM. The presence of immunoreactive material devoid of catalytic activity can, again, best be explained by structural changes in the CS molecule that alter the catalytic site(s) even more drastically than in those CRM^+ mutants that retain some activity. The remaining three cell lines lack both detectable CRM and residual activity. Numerous mechanisms could account for the findings of this group: a putative regulatory gene mutation; deletion of some or all of the structural gene for CS; frameshift or nonsense mutation of the structural locus; errors in processing or transport of the CS messenger RNA; and failure of the antibodies in the antiserum raised against normal enzyme to recognize existing mutant CS molecules.

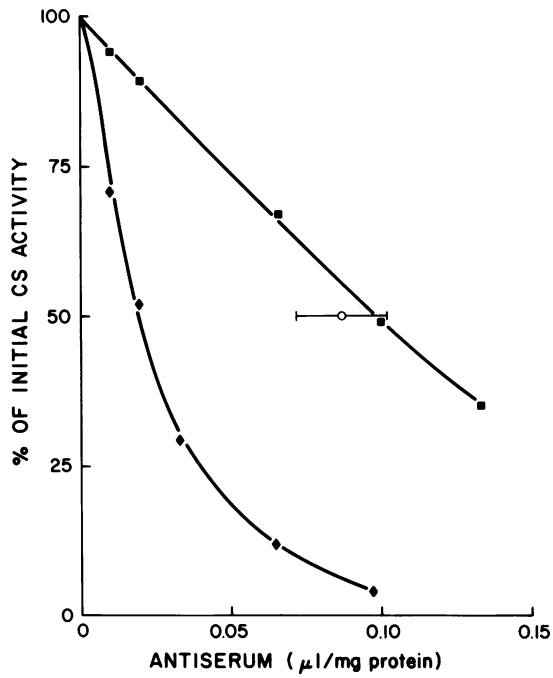

FIG. 5.—Direct immunotitration of fibroblast extracts from cell lines no. 338 (◆) and no. 676 (■). To enable comparison between mutant and control data, the abscissa is expressed in μl antiserum added per mg fibroblast extract protein, and the mean Ab50 ± 1 SEM of control fibroblast extract protein is shown (|—○—|). While the Ab50 obtained in the titration of extract from mutant cell line no. 676 is in the range of control data, that from cell line no. 338 is only 21% of the mean control Ab50 (see table 2).

Immunologic heterogeneity similar to that observed in the CS mutants devoid of catalytic activity has been described in Sandhoff disease (complete deficiency of HEX A and B). No evidence of CRM could be found in the liver from one patient with this disorder [7], while another contained both the HEX A and the HEX B antigens, and a third patient's liver contained only the HEX A antigen [8]. These results, however, do not conform to the published data on several other inherited disorders characterized by specific enzymatic deficiency. For example, erythrocytes from each of three patients with complete galactose-1-phosphate uridylyltransferase deficiency contained normal amounts of CRM [9], whereas hemolysates from 14 patients with complete hypoxanthine phosphoribosyltransferase (HPRT) deficiency lacked any detectable CRM [10, 11]. Absence of CRM has also been reported in patients with total deficiency of muscle phosphorylase [12], sucrase-isomaltase [13], and purine nucleoside phosphorylase [14]. While examination of immunoprecipitated CS molecules from radiolabeled fibroblasts may define the biochemical differences among some CRM^+ mutants, explanations for the heterogeneity observed among the CS mutants without catalytic activity, as well as an understanding of the differences between these mutants, on the one hand, and uridylyltransferase

or HPRT mutants on the other, must await studies capable of probing the molecular structure of the respective genes and messenger RNA molecules.

REFERENCES

1. MUDD SH, LEVY HL: Cystathionine β-synthase deficiency, in *The Metabolic Basis of Inherited Disease*, 4th ed, edited by STANBURY JB, WYNGAARDEN JB, FREDRICKSON DS, New York, McGraw-Hill, 1978, pp 464–488
2. UHLENDORF BW, MUDD SH: Cystathionine synthase in tissue culture derived from human skin: enzyme defect in homocystinuria. *Science* 160:1007–1008, 1968
3. FOWLER B, KRAUS J, PACKMAN S, ROSENBERG LE: Homocystinuria: evidence for three distinct classes of cystathionine β-synthase mutants in cultured fibroblasts. *J Clin Invest* 61:645–653, 1978
4. LIPSON MH, KRAUS J, ROSENBERG LE: Affinity of cystathionine β-synthase for pyridoxal 5′-phosphate in cultured cells. A mechanism for pyridoxine-responsive homocystinuria. *J Clin Invest* 66:188–193, 1980
5. KRAUS J, PACKMAN S, FOWLER B, ROSENBERG LE: Purification and properties of cystathionine β-synthase from human liver. Evidence for identical subunits. *J Biol Chem* 253: 6523–6528, 1978
6. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275, 1951
7. CARROLL M, ROBINSON D: Immunological properties of *N*-acetyl-β-D-glucosaminidase of normal human liver and of GM_2-gangliosidosis liver. *Biochem J* 131:91–96, 1973
8. SRIVASTAVA SK, BEUTLER E: Studies on human β-D-*N*-acetylhexosaminidases. III. Biochemical genetics of Tay-Sachs and Sandhoff's diseases. *J Biol Chem* 249:2054–2057, 1974
9. TEDESCO TA, MELLMAN WJ: Galactosemia: evidence for a structural gene mutation. *Science* 172:727–728, 1971
10. UPCHURCH KS, LEYVA A, ARNOLD WJ, HOLMES EW, KELLEY WN: Hypoxanthine phosphoribosyltransferase deficiency: association of reduced catalytic activity with reduced levels of immunologically detectable enzyme protein. *Proc Natl Acad Sci USA* 72: 4142–4146, 1975
11. GHANGAS GS, MILMAN G: Radioimmune determinations of hypoxanthine phosphoribosyltransferase crossreacting material in erythrocytes of Lesch-Nyhan patients. *Proc Natl Acad Sci USA* 72:4147–4150, 1975
12. DREYFUS JC, ALEXANDRE Y: Immunologic studies on glycogen storage diseases type III and V. Demonstration of the presence of an immunoreactive protein in one case of muscle phosphorylase deficiency. *Biochem Biophys Res Commun* 44:1364–1370, 1971
13. GRAY GM, CONKLIN KA, TOWNLEY RRW: Sucrase-isomaltase deficiency. Absence of an inactive enzyme variant. *N Engl J Med* 294:750–753, 1976
14. OSBORNE WRA, CHEN SH, GIBLETT ER, BIGGAR WD, AMMANN AA, SCOTT CR: Purine nucleoside phosphorylase deficiency. Evidence for molecular heterogeneity in two families with enzyme-deficient members. *J Clin Invest* 60:741–746, 1977