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CHARACTERIZATION OF HUMAN CYSTATHIONINE β-SYNTHASE

EVIDENCE FOR THE IDENTITY OF HUMAN L-SERINE DEHYDRATASE AND CYSTATHIONINE β-SYNTHASE

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SUMMARY

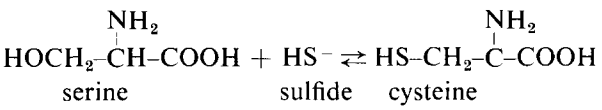
A protein has been studied in human fetal liver that is able to condense serine with several sulfur donors. The two activities of greatest interest are cystathionine β-synthase (EC 4.2.1.22) which catalyzes the reaction of sulfide with serine to form cysteine, and L-serine dehydratase (EC 4.2.1.13) which condenses homocysteine with serine to form cystathionine. These two activities were followed through several purification steps and were always found in the same fractions. The ratio of the activities remained constant at 1.7 ± 0.1 with homocysteine as the preferred sulfur donor. When the two activities were assayed simultaneously, sulfide did not interfere with cystathionine synthesis, but a sulfide-to-homocysteine ratio of at least 1.5 was needed for cysteine production. Both activities showed similar thermal denaturation profiles and sensitivity to hydroxalamine. Further evidence that these activities are associated with the same protein comes from the observation that a patient with homocystinuria is deficient in cystathionine β-synthase as well as L-serine dehydratase.

INTRODUCTION

Homocystinuria, a disease characterized by high urine and plasma levels of homocysteine [1–3] and low tissue levels of cystathionine [4, 5] is usually associated with a deficiency or absence of L-serine dehydratase which is an enzyme in the transsulfuration pathway from methionine to cysteine [6]. Some patients respond to treatment with pyridoxine by showing increased L-serine dehydratase activity and decreased levels of homocysteine in urine and plasma [7]. The pyridoxine treatment did not, however, increase either cystathionine or cysteine to normal levels. Furthermore, this treatment results in only a slight increase in L-serine dehydratase (from 1–2% to 3–4% of the control activity) [7]. Thus, the question arose as to whether some other pathway for cysteine synthesis was responsible for the effect of pyridoxine in the treatment of homocystinuria.

Since cystathionine β-synthase is a pyridoxal phosphate-containing enzyme [8] which could provide an alternate pathway of cysteine synthesis, we thought it would

be of interest to determine whether or not human tissues contain this enzyme and whether or not it is present in patients lacking L-serine dehydratase. Cystathionine β-synthase which catalyzes the following reaction



was found in human liver, brain and fibroblasts. This enzyme had not been previously described in man.

The purpose of this study was to determine whether cystathionine β-synthase activity was present or absent in patients lacking L-serine dehydratase and to determine if these activities reside in the same or different enzyme protein(s).

METHODS AND MATERIALS

Materials

L-[U-^{14}C]Serine was obtained from Schwartz BioResearch, DL-homocysteine-thiolactone–HCl from Sigma, Minimum Essential Medium from Grand Island Biological Company, and fetal calf serum from Irving Scientific Sales. Cytochrome *c* (horse heart) was obtained from Mann Research Laboratories; lactate dehydrogenase (rabbit muscle) and catalase (beef liver) were purchased from Worthington Biochemical Corporation, and 2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole from New England Nuclear. Cellulose MN300 pre-coated, plastic thin-layer sheets were obtained from Brinkman. All other chemicals were reagent grade.

Cystathionine β-synthase assay

The reaction mix included 10 μl of 1 M Tris-HCl, pH 8.3, 10 μl of 5 mM pyridoxal phosphate, 0.5 μCi of L-[U-^{14}C]serine (156 Ci/mole), 10 μl of 0.2 M Na_2S, and a variable amount of enzyme in a volume of 100 μl. Na_2S was prepared fresh daily under N_2 atmosphere, and the reaction was terminated by the addition of 10 μl of 100% (w/v) trichloroacetic acid. The tubes were chilled on ice and centrifuged at 2600 $\times$ *g* for 5 min. The supernatant was stored frozen in sealed tubes prior to chromatography.

L-*Serine dehydratase assay*

The reaction was performed as above except the incubation was carried out in air, and DL-homocysteine was used in place of Na_2S. DL-Homocysteine was prepared fresh daily from DL-homocysteine thiolactone-HCl by reacting the thiolactone with 1 M KOH for five minutes at room temperature, then neutralizing with HCl in Tris–HCl buffer, pH 8.3, as described by Mudd et al. [9].

Thin-layer chromatography

The substrates and products from the reactions above were separated by thin-layer chromatography. 10 μl of the enzyme assay supernatant was spotted on 0.1-mm thick cellulose MN300-precoated plastic sheets. Carrier cysteine and serine were

added to the spots, and the samples were oxidized with 30% H_2O_2 before developing in a chromatography solvent that consisted of 74 ml methyl alcohol, 8 ml pyridine, 16 ml 1.25 M HCl. Chromatograms were developed for approximately 90 min, air-dried, and sprayed with ninhydrin to determine the location of the products and substrates. Cystathionine, cysteine and serine are easily separated by this technique. The chromatograms were covered with transparent tape and cut into pieces 2 cm × 0.5 cm and counted in toluene containing 4 g/l butyl-2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole.

Purification

Fetal tissue was obtained at hysterotomy and stored at −70 °C. Fetuses were phenotypically normal. Throughout the purification, enzyme preparations were kept at 0–4 °C unless otherwise specified. Human fetal liver (37.55 g) was minced and homogenized in 20 ml of 10 mM Tris–HCl, pH 7.4, containing 10 mM NaCl and 1.5 mM $MgCl_2$ and then sonicated until all the cells were broken. The homogenate was then centrifuged at 150 000 × *g* for 60 min. The pellet was discarded, and the supernatant was brought to 20% saturation with solid $(NH_4)_2SO_4$. The pellet was collected by centrifugation at 12 000 × *g* for 10 min. The pellet was discarded, and the supernatant was brought to 45% saturation with $(NH_4)_2SO_4$. The pellet was again collected by centrifugation at 12 000 × *g* for 10 min, resuspended in 5 ml of 5 mM Tris–HCl, pH 8.3, and dialyzed 30 h against 1 l of buffer with two buffer changes. This dialyzed fraction was placed in a 50 °C water bath for 10 min. Denatured protein was removed by centrifugation at 27 000 × *g* for 1 h. The supernatant fraction was placed on a column containing 45 ml of DEAE-cellulose which had been equilibrated with 5 mM Tris–HCl, pH 7.6. The column was washed with 60 ml of 5 mM Tris–HCl, pH 7.6, and a continuous gradient of 200 ml of 1 mM K_2HPO_4 and 200 ml of 25 mM K_2HPO_4 was used to elute the enzyme. Active fractions were concentrated using a PM 10 membrane, and further fractionated on a 57 cm × 2.4 cm Sephadex G-200 column. Active fractions were concentrated to approximately 2 ml using PM 10 and UM 10 membranes. 1 ml of this concentrated fraction was placed on a continuous 5–20% (w/w) sucrose gradient and centrifuged at 179 000 × *g* for 24 h. The refractive index of each 0.8-ml fraction was determined, and the fractions were dialyzed overnight against 2 l of 5 mM Tris–HCl, pH 8.3, before assaying.

Assay of marker enzymes in sucrose gradient

Cytochrome *c* was determined by its absorption at 406 nm. Lactate dehydrogenase and catalase were assayed as previously described [10].

Tissue culture and preparation of fibroblast extracts

Fibroblast cell lines were started from skin biopsies and grown at 37 °C in minimum essential medium, 10% fetal calf serum in the presence of 5% CO_2. At confluency, the medium was removed; the cells were washed with isotonic phosphate-buffered saline. The cells were then loosened with 0.5% trypsin; serum was added to stop the trypsin activity; the cells were washed with phosphate-buffered saline, suspended in phosphate-buffered saline with a rubber policeman and collected by centrifugation. The pellet was resuspended in 10 mM NaCl and 1.5 mM $MgCl_2$, and the cells were disrupted by sonication.

RESULTS

Purification experiments

An attempt was made to resolve cystathionine β-synthase from L-serine dehydratase by enzyme purification of human fetal liver. The details of the purification scheme are described in Methods and Materials. After $(NH_4)_2SO_4$ precipitation and heat treatment, the crude enzyme preparation was fractionated by DEAE-cellulose chromatography. Activity was eluted by a continuous phosphate gradient in a broad peak which followed the major protein peak. As shown in Fig. 1,both cystathionine

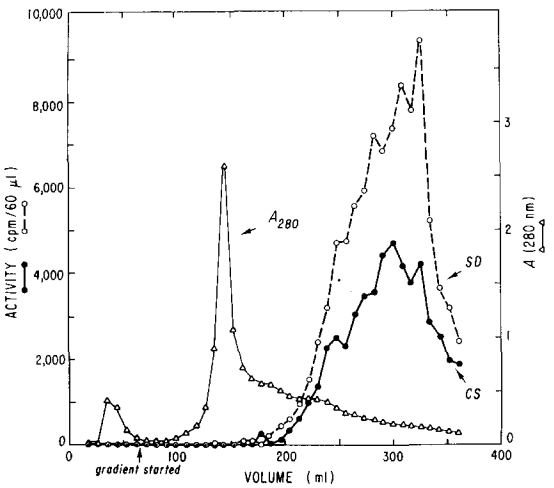


Fig. 1. Purification of L-serine dehydratase (SD) and cystathionine β-synthase (CS) from human fetal liver by DEAE-cellulose chromatography. Fractions 239–335 were combined and concentrated. The details of the assay are described under Methods and Materials.

β-synthase and L-serine dehydratase activities showed similar elution profiles. When the DEAE-cellulose fraction was concentrated and applied to Sephadex G-200, both activities appeared in the same fractions (Fig. 2). Thus, the two activities were not resolved by either a chromatography method based on charge (DEAE-cellulose) or by gel-filtration chromatography, a method of separation depending on differences in molecular size.

Next, the liver enzyme was sedimented on a continuous 5–20% (w/w) sucrose gradient. Both enzyme activities were found in the same peak as shown in Fig. 3A. Simultaneous sedimentation of horse heart cytochrome *c*, rabbit muscle lactate dehydrogenase, and beef liver catalase was performed on an identical sucrose gradient (Fig. 3B). Comparison of the sedimentation rates of cystathionine β-synthase-L-serine dehydratase to the marker enzymes gave a molecular weight of approximately 120 000. This observation is consistent with data obtained from the rat liver enzyme by Kimura and Nakagawa [11].

A summary of the purification is shown in Table I. Since the ratio of L-serine dehydratase activity to cystathionine β-synthase activity was constant at 1.7 ± 0.1, it is highly probably that these two activities are associated with the same protein.

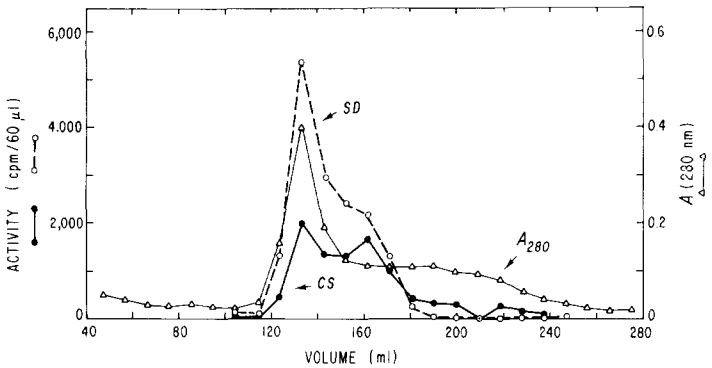


Fig. 2. Fractionation of L-serine dehydratase (SD) and cystathionine β-synthase (CS) on Sephadex G-200. The void volume, as determined by blue dextran, was 126 ml. Fractions eluting between 123.5 and 171 ml were combined and then concentrated. Details are described in Methods and Materials.

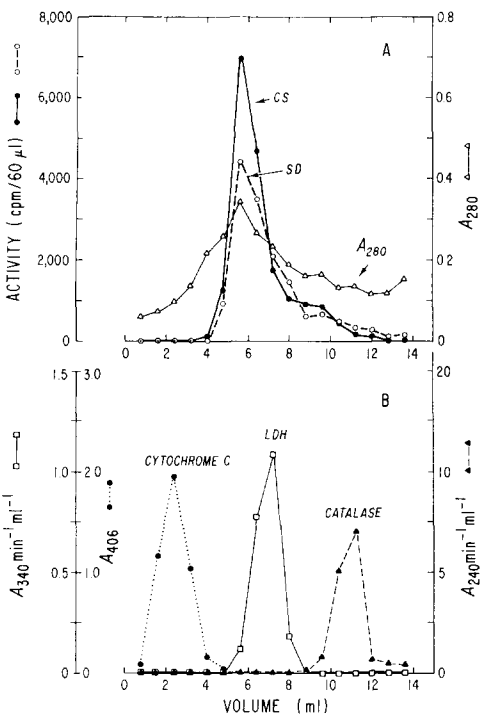


Fig. 3. A. Sedimentation of cystathionine β-synthase (CS) and L-serine dehydratase (SD) on a 5–20% (w/w) sucrose gradient. The gradient was collected from the top. B. Sedimentation of the marker enzymes, cytochrome *c* (horse heart), lactate dehydrogenase (LDH) (rabbit muscle) and catalase (beef liver). The sucrose gradients used in A and B were identical as determined by refractive index.

TABLE I

PURIFICATION OF CYSTATHIONINE β-SYNTHASE AND L-SERINE DEHYDRATASE FROM HUMAN FETAL LIVER

Enzyme activity is expressed as cpm/mg protein. Purification is described in Methods and Materials. CS, cystathionine β-synthase; SD, L-serine dehydratase.

Purification step	CS activity	SD activity	SD activity / CS activity
$(NH_4)_2SO_4$	17 500	28 600	1.6
Heat treatment	45 800	78 000	1.7
DEAE-cellulose	168 000	302 000	1.8
Sephadex G-200	219 000	358 000	1.6
Sucrose gradient	692 000	1317 000	1.9

The activity of the crude liver homogenate was not included because prior to the $(NH_4)_2SO_4$ fractionation cystathionine β-synthase activity was not linear with respect to protein concentration. The nature of the interferring reactions or compounds is not known. After the initial purification steps, both assays were linear with time and with the amount of enzyme added. During all other attempts at purification, just as the purification shown here, L-serine dehydratase and cystathionine β-synthase activity were found in the same fractions.

Comparison of the properties of L-*serine dehydratase and cystathionine β-synthase*

In order to gain further information regarding similarities between L-serine dehydratase and cystathionine β-synthase, certain characteristics of the two reactions were compared. For example, Fig. 4 shows that the heat inactivation curves for the two reactions were quite similar. In the experiment shown, enzyme and buffer were incubated at the indicated temperature for ten minutes. Then the reaction was initiated by the addition of substrate and carried out as described in Methods and Materials. For each reaction, incubation for 10 min at 55 °C caused approximately 50% inhibition.

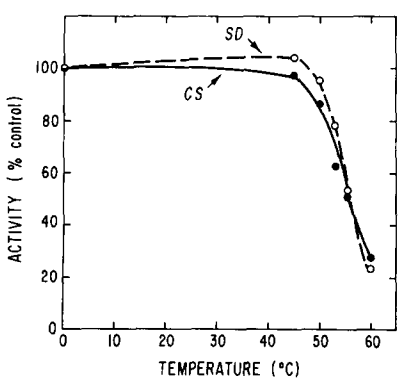


Fig. 4. Thermal inactivation of cystathionine β-synthase (CS) and L-serine dehydratase (SD). The enzyme and buffer were incubated 10 min at the indicated temperature prior to the initiation of the reaction by the addition of substrate.

Although L-serine dehydratase and cystathionine β-synthase activity are detectable in the absence of pyridoxal phosphate, both reactions were enhanced by the cofactor. Activity at 0.5 mM was approximately 14% of that obtained in the absence of pyridoxal phosphate (data not shown).

Fig. 5 shows that L-serine dehydratase and cystathionine β-synthase reactions were both inhibited by 90% at 1 mM hydroxylamine. Even though the hydroxylamine

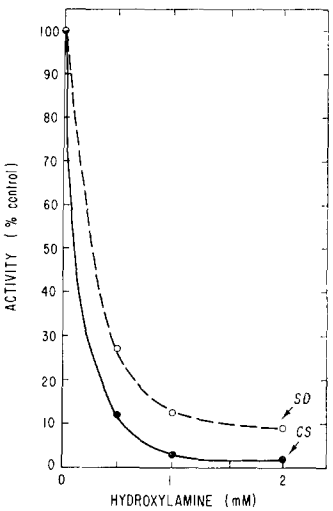


Fig. 5. Inhibition of cystathionine β-synthase (CS) and L-serine dehydratase (SD) activities by hydroxylamine. Hydroxylamine was added to the enzyme at 0 °C prior to substrate and pyridoxal phosphate addition.

inhibition was not overcome by the subsequent addition of 1 mM pyridoxal phosphate, this inhibition is probably due to an effect of hydroxylamine on the pyridoxal phosphate of this enzyme since both L-serine dehydratase and the cystathionine β-synthase [8] of other species are known to contain pyridoxal phosphate and since hydroxylamine is known to remove the cofactor from a number of enzymes.

Since the purification and characterization data suggested that cystathionine β-synthase and L-serine dehydratase activities reside on the same protein, the question arose as to whether the two reactions are independent of each other. Experiments were then done in which both homocysteine and sulfide were added to the reaction mixture in varying concentrations, and the two products, cysteine and cystathionine, were separated by the same thin-layer chromatography system routinely used to separate cysteine and cystathionine from serine. The activity of L-serine dehydratase at varying ratios of homocysteine to Na_2S is expressed as a percentage of the cystathionine activity obtained in the absence of sulfide. As shown in Fig. 6A, the addition of sulfide did not interfere with L-serine dehydratase activity. In fact, there appears to be an unexplained enhancement. On the other hand, homocysteine blocks cystathionine β-synthase activity unless the ratio of Na_2S to homocysteine is greater than 1.5 (Fig. 6B).

The effect of homocysteine in inhibiting the cystathionine β-synthase reaction

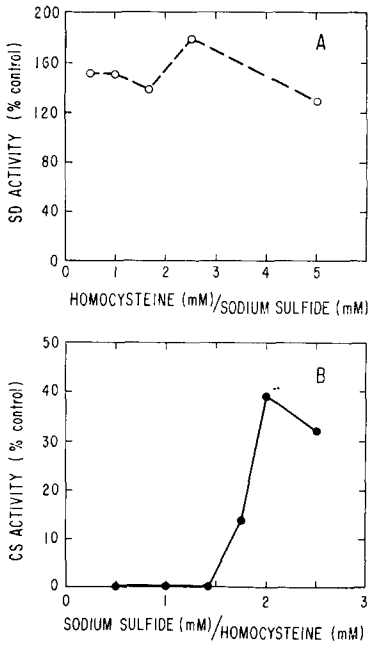


Fig. 6. The effect of Na_2S on L-serine dehydratase (SD) activity and the effect of homocysteine on cystathionine β-synthase (CS) activity. Reactions were carried out in which radioactive serine was incubated with both Na_2SO_4 and homocysteine, and the radioactivity of both products, cysteine and cystathionine was measured. Activity was expressed as a percentage with 100% indicating the SD activity obtained when serine and homocysteine were incubated without Na_2S (A), or the CS activity obtained when serine and Na_2S were reacted in the absence of homocysteine (B).

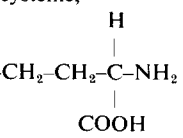
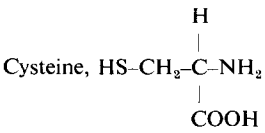
is not likely to be a pH effect since the homocysteine was prepared in buffer and its pH checked before use. If sulfide and homocysteine bind to the enzyme at separate sites, one would expect the two reactions to be independent of each other. However, these results indicate that the binding of homocysteine to the enzyme interferes with sulfide binding. These data could be interpreted to mean that homocysteine and sulfide bind to the same site with the enzyme preferring homocysteine. On the other hand, the two substrates could bind at different sites, and homocysteine could interfere with sulfide binding by inducing a conformational change in the protein. Further experimentation would be required to distinguish between these two possibilities. The fact that there is some interaction between the two substrates strengthens the argument that both reactions are catalyzed by the same protein.

Since the same enzyme apparently catalyzes the reaction of either sulfide or homocysteine with serine, it was of interest to determine whether this enzyme would catalyze the reaction of serine with other thio-containing compounds. As shown in Table II, the enzyme combines serine with 2-aminoethanethiol, mercaptoethanol, cysteine and thioglycolic acid. No activity was obtained with mercaptopropionic acid or *N*-acetyl cysteine. However, since the condensation product of the reaction of these compounds with serine was not available, activity could have been present and not detected if the product migrated in the same position as serine in the thin-layer chromatography system used. These data show a marked difference in activity between

TABLE II

COMPARISON OF SUBSTRATES

Assay was done as described in Methods and Materials and activity is expressed as a percentage of the activity obtained with homocysteine ± S.D. of the mean.

Substrate	Concentration (mM)	Activity (%)
Homocysteine, 	10	100
Sodium sulfide, Na_2S	20	70 ± 30
2-Mercaptoethanol, $HS-CH_2-CH_2OH$	140	60 ± 20
2-Aminoethanethiol, $HS-CH_2-CH_2-NH_2$	10	50 ± 20
Cysteine, 	10	6 ± 6
Thioglycolic acid, $HS-CH_2-COOH$	10	4 ± 3

homocysteine and cysteine. The reason for the difference in activity toward these two structurally similar compounds is not known. Perhaps there is steric hinderance to cysteine binding because the amino and carboxyl groups are on the carbon adjoining the thiol group.

Absence of cystathionine β-synthase in fibroblasts of a homocystinuric patient

An attempt was made to measure cystathionine β-synthase and L-serine dehydratase in crude extract of fibroblasts grown from a skin biopsy of a homocystinuric patient, R.M., who responded to pyridoxine treatment. Activity was simultaneously

TABLE III

ACTIVITY OF CYSTATHIONINE β-SYNTHASE AND L-SERINE DEHYDRATASE IN FIBROBLAST EXTRACTS OF A HOMOCYSTINURIC PATIENT AND CONTROLS

Activity is expressed as cpm/mg protein ± S.D. of the mean. R.M., fibroblasts of a homocystinuric patient who responds to pyridoxine treatment; M.O., Human adult fibroblasts; N.D., Not detectable. CS and SD, see Table I.

Subject	CS activity	SD activity
R.M.	N.D.	700 ± 200
M.K.	5600 ± 1200	10 400 ± 1300
A.F.	4900 ± 900	11 100 ± 1800
M.O.	2900 ± 200	3 600 ± 500

measured in fibroblasts obtained from a skin biopsy of a normal adult, M.O., and from fetal fibroblasts, M.K. and A.F., grown from fetal skin obtained at hysterotomy.

L-Serine dehydratase activity of R.M. fibroblasts was less than 10% of the fetal control activity and less than 20% of the adult control. Cystathionine β-synthase activity was not detected (Table III). Because the cystathione β-synthase assay is slightly less sensitive than the L-serine dehydratase assay, activity in the range of 5–10% of the control could be difficult to detect. As mentioned above, cystathionine β-synthase is difficult to measure in crude extracts of fetal liver; however, these problems do not occur in crude fibroblast extracts where activity is linear with protein concentration as shown in Fig. 7.

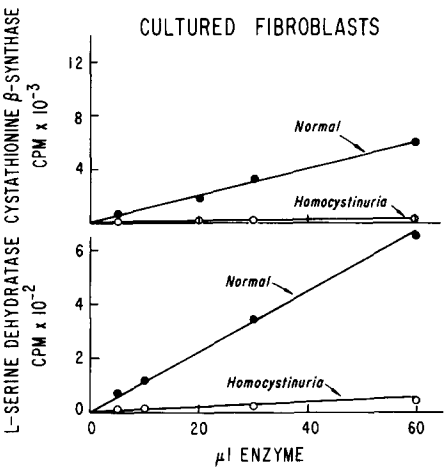


Fig. 7. Time course of cystathionine β-synthase and L-serine dehydratase reactions. Incubations were carried out at 35 °C as described in Methods and Materials.

DISCUSSION

Prior to this work, cystathionine β-synthase activity had not been reported in man, but cystathionine β-synthase had been reported in yeast by Schlossman et al. [12], *Aspergillus*, spinach, chicken liver and rat liver by Bruggeman et al. [13] and in chicken liver by Sentenac and Fromageot [14].

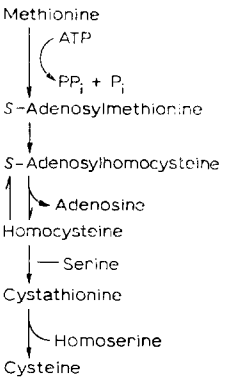
Braunstein et al. [15] compared chicken liver cystathionine β-synthase and rat liver L-serine dehydratase and suggested that they are the same enzyme. Interpretation of their data was complicated by the fact that they were comparing preparations by different procedures. Furthermore, we thought this question was worthy of further investigation because of complications in their cystathionine β-synthase assay. They measured the production of sulfide from cysteine by a colloidal PbS determination. The fact that this assay requires mercaptoethanol, homocysteine or cysteamine in addition to cysteine, and the fact that these compounds react with cysteine during the assay raises the question as to whether the sulfide measure was directly indicative of cystathionine β-synthase activity or of some more complicated reaction sequence. Because of this uncertainty, we chose to assay cystathionine β-synthase by deter-

mining the radioactivity in cysteine after the incubation of L-[U-^{14}C]serine with sulfide.

The data presented here support the idea of Braunstein and co-workers [15] that L-serine dehydratase and cystathionine β-synthase are the same enzyme. Specifically, the data show a parallel purification of cystathionine β-synthase and L-serine dehydratase from the same species, i.e. man. In addition, the two activities have similar sensitivity to heat and hydroxylamine. The experiments in which both sulfide and homocysteine were added to the same assay indicate that both reactions are catalyzed by the same enzyme and that the enzyme prefers homocysteine. The strongest evidence that L-serine dehydratase and cystathionine β-synthase are identical in humans is the finding that a homocystinuric patient lacking L-serine dehydratase also lacks cystathionine β-synthase.

During the course of this work, Pienaiazek et al. [16] reported a mutant of *Aspergillus nidulans* which simultaneously lacked L-serine dehydratase and cystathionine β-synthase. Therefore, the finding that "mutants" of both humans and *Aspergillus* which lack L-serine dehydratase also lack cystathionine β-synthase gives strong support to the argument that these are the same protein.

It has been assumed that the only pathway of cysteine biosynthesis from methionine in humans was;



The question now arises as to how the cystathionine β-synthase reaction fits into this metabolic scheme. The L-serine dehydratase reaction is likely to predominate under most circumstances since homocysteine is the preferred substrate and since intracellular sulfide concentrations are likely to be limiting. However, there may be instances where the cystathionine β-synthase reaction is important. For example, cystathionine β-synthase activity may be metabolically significant in fetal tissues. Sturman et al. [17] have shown that cystathionase, the final step in the transsulfuration pathway, is absent in human fetal brain and liver. Cystathionine β-synthase, which can make cysteine directly from serine and sulfide, may, therefore, be essential in fetal metabolism because it provides a pathway for the synthesis of cysteine that is independent of cystathionase.

On the other hand, the results shown in Fig. 6B suggest that high concentrations of sulfide are required in order to overcome the effects of homocysteine and stimulate the cystathionine β-synthase reaction. Curtis et al. [18] have described the toxicity of sulfide and its rapid oxidation to sulfate. Thus, the activity of cystathionine β-synthase may be a biologic protective mechanism for sulfide detoxication should

tissue concentrations of sulfide be increased. Finally, cystathionine β-synthase may be of importance in cysteine synthesis in cystathionuria patients, who are deficient in cystathionase.

In summary, it appears that cystathionine β-synthase and L-serine dehydratase activity are part of the same enzyme protein and that L-serine dehydratase has a greater physiological importance in the transsulfuration pathway in man under ordinary circumstances. However, in certain specific instances, such as fetal development and cystathionuria, cystathionine β-synthase may be especially important.

ACKNOWLEDGEMENTS

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