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STUDIES ON CYSTATHIONINE SYNTHASE OF RAT LIVER PROPERTIES OF THE HIGHLY PURIFIED ENZYME*

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SUMMARY

The enzyme cystathionine synthase has been purified over 400-fold from rat liver. A new spectrophotometric assay procedure for this enzyme has been employed based upon the observation that the ninhydrin reaction chromogen with cystathionine gives an absorption peak at 455 mμ in acid solution. The purified enzyme exhibited no dehydratase activity for any hydroxyamino acid tested.

The optimum activity of the enzyme is at pH 8.3. The enzyme is strongly inhibited by *p*-chloromercuribenzoate and Hg^{2+}. The inhibition by the former is relieved by the addition of reduced glutathionine. The enzyme is stable to heating to temperatures of up to 50° and can be lyophilized without loss of activity. The enzyme is colored yellow with an absorption peak at 430 mμ. Upon reduction with sodium borohydride this peak disappears and a shoulder develops at 330 mμ. Dialysis against cysteine causes a loss of enzyme activity that can be almost completely restored by the addition of pyridoxal-5-*P*. These results indicate strongly that the latter is a coenzyme for cystathionine synthase.

The addition of enzyme-free protein filtrates to the enzyme enhances its activity by as much as 50%.

INTRODUCTION

SELIM AND GREENBERG[1,2] and NAGABHUSHANAM AND GREENBERG[3] reported that cystathionine synthase activity (EC 4.2.1.21)*** was associated with an enzyme that deaminated L-serine and L-threonine. This was shown to be erroneous by BROWN AND MALLADY[4] and BROWN *et al.*[5]. Indirect evidence indicating that cystationine synthase and serine–threonine dehydratase (EC 4.2.1.13; 4.2.1.16)*** are distinct and separable enzymes has been published by several investigators[6,7]. Recently several

* An abstract of this work has appeared in *Federation Proc.*, 28 (1969) 668.

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*** *Report of the Commission on Enzymes of the International Union of Biochemistry 1961*, Pergamon, Oxford, 1961, p. 126, 127.

reports have appeared on the partial purification and certain properties of cystathionine synthase[8,9]. Recent work in our laboratory has resulted in the development of a new spectrophotometric method of assay for this enzyme, a purification procedure that gives more than 400-fold enrichment of the enzyme, and the determination of certain chemical and kinetic properties of the enzyme.

Our results agree with and extend the observations of NAKAGAWA AND KIMURA[9], but differ in certain respects from the more recent report of BROWN *et al.*[8].

The results of the investigation of this enzyme in our laboratory are reported in this communication.

EXPERIMENTAL PROCEDURE

Materials

L-Serine, DL-cystathionine, DL-allocystathionine, L-threonine, pyridoxal-*P* and ninhydrin were purchased from Calbiochem. DL-Homocysteine thiolactone hydrochloride was a product of Nutritional Biochemicals. DL-Allothreonine was obtained from Pierce Chemical Company and DL-homoserine from Sigma Chemical Company. Other chemicals employed in this work were all commercial preparations.

DEAE-cellulose (from Serva Laboratory) was washed with 1 M NaOH, 1 M HCl, ethanol, 1 M NaOH and finally with water[3]. Sepharose 6B was obtained from Pharmacia Fine Chemicals.

Analytical method for cystathionine

A new method for cystathionine estimation was developed, based on a relatively specific cystathionine–ninhydrin color chromogen absorbing at 455 mμ in a strongly acidic medium.*

The ninhydrin reagent was prepared as follows: 1 g of ninhydrin was dissolved in 100 ml of glacial acetic acid, then one-third volume of glacial phosphoric acid was added and the solution mixed well.

For the determination of cystathionine, 0.2 ml of cystathionine solution (0.1–3 μmoles) was mixed completely with 3.3 ml of the ninhydrin reagent and heated exactly for 5 min in a boiling-water bath. After heating, the solution was cooled in an ice bath for 2 min. The color developed was measured after 20 min at the absorption maximum of 455 mμ with a Gilford–Beckman automatic recording spectrophotometer. The absorption spectra of the ninhydrin reaction chromophore of chemically prepared cystathionine and of the enzymic reaction product are shown in Fig. 1**.

A calibration curve that shows the linear increase in absorption with concentrations is given in Fig. 2. L-Serine and DL-homocysteine used as substrates for

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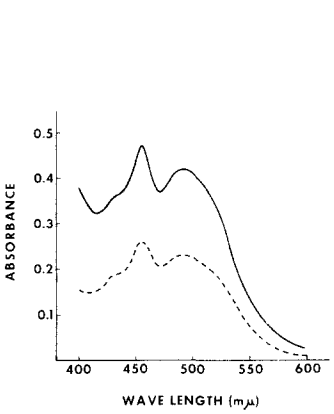
* The observation of the absorbance of cystathionine at 455 mμ was made in cooperation with Dr. MARTIN A. APPLE.

** Sulfur amino acids that absorb significantly at 455 mμ and thus could interfere with the assay method, if present, are cysteine and cystine. Cysteine is formed from cystathionine through the catalytic action of γ-cystathionase, which is present in liver. The addition of 0.5 mM Cu^{2+} to the incubation mixture completely inhibites the activity of γ-cystathionase and the formation of cysteine as reported by MATSUO AND GREENBERG[10]. This has repeatedly been confirmed by us. The presence of cysteine as a product of the reaction would easily be detected as the chromophore formed from it in the ninhydrin reaction has a deep red color in contrast to the orange color of the cystathionine chromophore.

cystathionine synthase gave about 1% of the absorbance of cystathionine. Tests on a mixture of both compounds showed the linear increase in color at 455 mμ seen in Fig. 2. This observation also shows that the color intensity due to a mixture of serine and homocysteine decreases linearly as these compounds are reacted to form cystathionine and can be neglected under the conditions employed in this work. From the stoichiometry of the reaction, namely that one mole each of serine and homocysteine produces one mole of cystathionine, the net amount of cystathionine formed in the



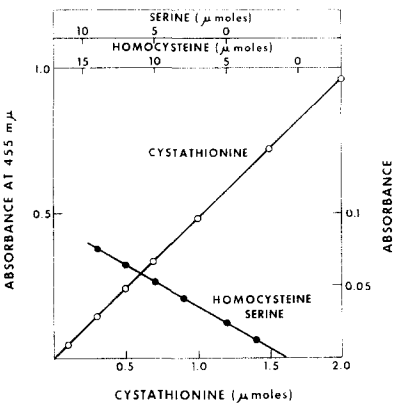


Fig. 1. Absorption spectra of colored ninhydrin reaction product of authentic cystathionine and of the enzymic reaction product in acid medium. Measurements performed in a Beckman–Gilford automatic recording spectrophotometer in cuvette of 1 cm light path. ———, 1.0 μmole (0.1 ml) of DL-*allo*-DL-cystathionine mixed with 0.1 ml of standard incubation medium without enzyme or substrate, mixed with 3.3 ml of ninhydrin reagent and color developed as described in text. — — —, 0.1 ml of deproteinized enzymic reaction mixture (0.1 ml of 50% trichloroacetic acid added per ml of incubate) diluted to 0.2 ml with distilled water and heated with 3.3 ml of ninhydrin reagent. The incubation was performed for 45 min at 37° with 73 μg of purified enzyme. Spectral readings corrected for blanks with enzyme and with substrates treated in the same manner. The molar extinction coefficient of cystathionine was calculated from the curve for authentic cystationine as follows: $\varepsilon_{max} = (0.47 \times 3.5 \times 10^6)/1000 = 1650$.

Fig. 2. Calibration curve for assay of cystathionine synthase by ninhydrin reaction with cystathionine. ○—○ (left hand scale), linear relation between absorbances and concentration of cystathionine in solution of 0.1 M Tris–HCl buffer (pH 8.3). ●—● (right hand scale), absorbance of mixtures of DL-homocysteine and L-serine at 455 mμ. Substrate mixtures dissolved in 0.1 M Tris–HCl buffer (pH 8.3). Absorbance measured as described in *Analytical methods for cystathionine*.

enzymic reaction can be calculated from the equation given below when 0.1 ml of deproteinized incubation mixture is treated as described in the procedure for the development and measurement of the ninhydrin color.

Cystathionine (μmoles) $= \{(S - B)/A\} \times 11$, where, S is the observed absorbance *minus* that of a blank without substrates, B is the absorbance of a blank without enzyme, and A is the absorbance of 1 μmole of standard cystathionine. Under the conditions of the enzyme assay described below the value of A is 0.47. If 0.2 ml of the incubation mixture is employed for the color measurement, the factor 11 is decreased to 5.5.

The amount of cystathionine formed in μmoles/h is given by multiplying the value calculated from the above equation by 60/45.

Enzyme assay

The reaction mixture contained 154 μmoles of DL-homocysteine (prepared just before use by heating the thiolactone hydrochloride for 3 min at 100° with 2 equiv. of NaOH). 99 μmoles of L-serine, 0.12 μmole of pyridoxal-*P* and enzyme preparation in a total volume of 1.0 ml of 0.1 M Tris buffer (pH 8.3). In the case of crude enzyme preparations the mixture contained also 0.5 μmole of $CuSO_4$ to inhibit cystathionase activity[10].

The incubations were carried out for 45 min in a Dubnoff metabolic shaking incubator at 37°, after preincubation for 10 min at the same temperature without substrates. At the end of the incubation period 0.1 ml of 50% trichloroacetic acid was added, the protein precipitate was removed by centrifugation, and 0.1 or 0.2 ml of the clear supernatant fluid was assayed for cystathionine synthase activity.

Assays for serine, threonine and allothreonine dehydratase activities were also run spectrophotometrically. The assay mixture contained 0.3 μmole of pyridoxal-*P*, 200 μmoles of DL-serine or L-threonine or 400 μmoles of DL-allothreonine, 4.5 μmoles of EDTA, 0.32 μmole of NADH and 1 μl of L-lactate dehydrogenase (rabbit muscle, crystallized in 2.2 M $(NH_4)_2SO_4$, Calbiochem) and enzyme preparation in a total volume of 3 ml of 0.1 M borate buffer (pH 8.3). Homoserine dehydratase (cystathionase) was assayed in 3 ml of 0.1 M potassium phosphate buffer (pH 7.5) with 0.3 μmole of pyridoxal-*P*, 480 μmoles of DL-homoserine, 0.15 μmole of 2,3-dimercaptopropanal, 0.32 μmole of NADH, 1 μl of lactate dehydrogenase and enzyme preparation. The decrease in absorbance at 340 mμ was followed in a Gilford–Beckman recording spectrophotometer at 25°.

Enzyme units

An enzyme unit is defined as the amount that yields one μmole of cystathionine per h under the conditions of the assay. The specific activity of the enzyme is expressed as the number of enzyme units/mg of protein.

Protein determination

Protein concentrations were determined by a biuret reaction with crude enzyme preparations[11,12] and by ultraviolet absorption[13] with purified enzyme preparations. Crystalline serum albumin was employed as a standard.

A linear relation between the rate of cystathionine formation and enzyme concentration or incubation time was observed (Fig. 3).

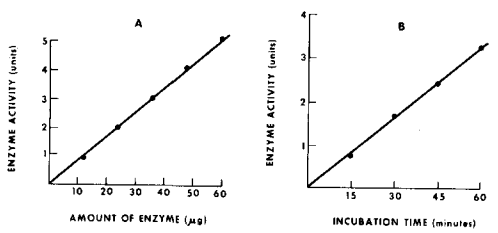


Fig. 3. Linearity of enzyme activity with protein concentration (A) and enzyme activity with incubation time (B).

RESULTS

Purification of enzyme

Preparation of crude homogenate. All operations were carried out in a cold room at 4–5°. Centrifugation was performed at 12 000 × *g* in a refrigerated centrifuge. Adult albino rats were killed by decapitation. The livers were removed immediately and washed with cold 1.15% KCl. The liver was then homogenized in a Waring Blendor for 5 min at full speed with 3 vol. of 1.15% KCl.

The crude extract was centrifuged 30 min and the sediment was discarded without washing.

pH 5 treatment. The crude extract was carefully adjusted to pH 5.0 with 0.2 M acetic acid, which resulted in the precipitation of a large amount of inert material. The precipitate was removed by centrifugation for 10 min and discarded, and the supernatant layer was quickly adjusted to pH 7.5 with 2 M NH_4OH. This step has been shown to inactivate the serine dehydratase[9].

$(NH_4)_2SO_4$ fractionation. The slightly turbid supernatant solution was brought to 30% saturation with $(NH_4)_2SO_4$ by the slow addition of the required amount of solid salt[14] with constant mechanical stirring. The precipitate was centrifuged and discarded. The supernatant solution was adjusted to 40% saturation with solid $(NH_4)_2SO_4$ in the same manner. The precipitate was collected by centrifugation and dissolved in 0.1 M potassium phosphate buffer (pH 7.5). The solution was dialyzed overnight against the same buffer and centrifuged to remove insoluble material that had formed.

Calcium phosphate gel treatment. To the clear solution calcium phosphate gel[15] was added in the proportion of 1 mg of gel per mg protein, the buffer concentration was adjusted to approx. 0.03 M with respect to phosphate, and the mixture was stirred for 30 min, followed by centrifugation.

Second $(NH_4)_2SO_4$ precipitation. The clear supernatant liquid from the preceding step was brought to 30% saturation of $(NH_4)_2SO_4$ as given above. The precipitate was centrifuged and discarded. The supernatant solution was adjusted to 37.5% saturation with solid $(NH_4)_2SO_4$ in the same manner. The precipitate was collected by centrifugation and dissolved in 0.08 M potassium phosphate buffer (pH 6.5). The solution was dialysed against the same buffer overnight.

DEAE-cellulose column chromatography. A column (2.3 cm × 15 cm) of DEAE-cellulose, treated as described under EXPERIMENTAL PROCEDURE, was prepared and equilibrated with 0.08 M potassium phosphate buffer (pH 6.5). The enzyme preparation from the previous step was centrifuged to remove insoluble materials and applied onto the column. After absorption of the protein, the column was washed thoroughly with 500 ml of the 0.08 M potassium phosphate buffer (pH 6.5). A large amount of protein was eluted in this effluent. The enzyme was fractionated by linear gradient elution. The mixing chamber contained 250 ml of 0.08 M potassium phosphate buffer (pH 6.5) and the reservoir contained an equal volume of 0.08 M potassium phosphate buffer (pH 6.5) with 0.2 M KCl. The eluate was collected in 5.8-ml fractions at a rate of 8.1 ml/h.

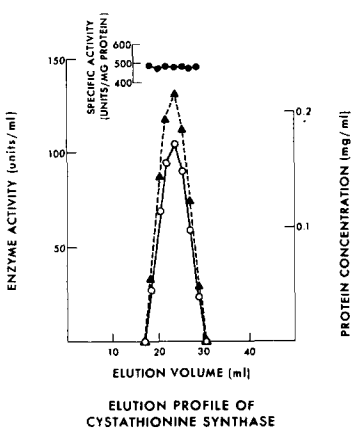
Sepharose 6B column chromatography. The active fractions from the previous step were combined and precipitated with solid $(NH_4)_2SO_4$ (40% saturation). The precipitate was dissolved in a small volume of 0.1 M potassium phosphate buffer

TABLE I

SUMMARY OF PURIFICATION OF CYSTATHIONINE SYNTHASE

Enzyme preparation purified in Department of Biochemistry, Nagoya University. Present procedure differs in use of Sepharose 6B in place of Bio-Gel. Specific activity achieved is over 3-fold higher than by earlier method.

Procedure	*Vol. (ml)*	*Protein (mg)*	*Total activity (units × 10⁻³)*	*Specific activity (units/mg protein)*	*Yield (%)*
Homogenate	1090	56800	51.1	0.90	100
Centrifuged supernatant	910	30100	50.0	1.66	98
pH 5 treatment	885	16400	46.7	2.85	91
First $(NH_4)_2SO_4$ fraction	66	2920	34.6	11.8	68
Calcium phosphate gel treatment	367	1600	26.5	16.6	52
Second $(NH_4)_2SO_4$ fraction	17.4	520	20.2	38.8	40
DEAE-cellulose eluate 40% $(NH_4)_2SO_4$ precipitate	1.2	9.4	3.36	358	6.6
Sepharose 6B filtrate 40% $(NH_4)_2SO_4$ precipitate	0.6	3.7	1.80	487	3.5



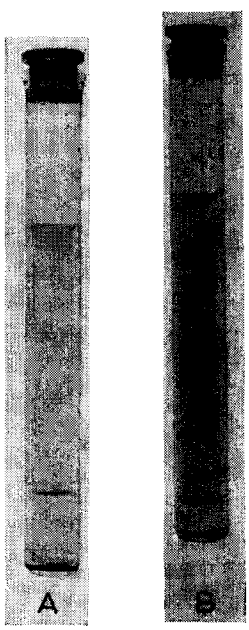


Fig. 4. An aliquot of the purified enzyme was rechromatographed on the Sepharose 6B column (1.1 cm × 44 cm). The eluate was collected in 1.8-ml fractions at a rate of 9.0 ml/h. Buffer, 0.1 M potassium phosphate buffer (pH 7.5). ○—○, enzyme activity; ▲— —▲, protein concentration; ●—●, specific activity.

Fig. 5. Disc gel electrophoresis was performed in 4% polyacrylamide gel containing Tris–glycine buffer solutions at pH 8.5. The enzyme was added to a saturated sucrose solution and layered on top of the gel. A constant current of 2.5 mA was applied. Gels were stained with 0.25% Amido Black in 7% acetic acid. Top is cathode, bottom the anode. A, contained 16 μg enzyme protein run for 40 min. B, contained 32 μg of the same enzyme run for 40 min.

(pH 7.5). The clear solution was applied onto the Sepharose 6B column (2.1 cm × 140 cm), which was previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) and chromatographed with the same buffer. The eluate was collected in 5.8-ml fractions at a flow rate of 6.8 ml/h. Each fraction was analyzed for protein by the method of ultraviolet absorption[13].

The purification and recovery of the enzyme in each step of the purification procedure is contained in Table I.

The elution profile of the enzyme from the Sepharose 6B column is shown in Fig. 4. Acrylamide gel electrophoresis run on the enzyme at this stage of purification showed the presence of only a single band of protein (Fig. 5).

Stoichiometry of the reaction

This was established in the following manner: L-serine and DL-homocysteine were incubated in the presence of pyridoxal-*P* with an aliquot of purified enzyme. Serine and cystathionine were separated on a Dowex 50-X8 cation exchange resin column. DL-Homocysteine was not determined because of its instability. The elution patterns of these amino acids are shown in Fig. 6. The peaks corresponding to serine and cystathionine were confirmed by paper chromatography in a system of *tert.*-butanol–formic acid–water (70:15:15, by vol.). The result shown in Table II indicates a 1:1 correspondence between the decrease in serine and the formation of cystathio-

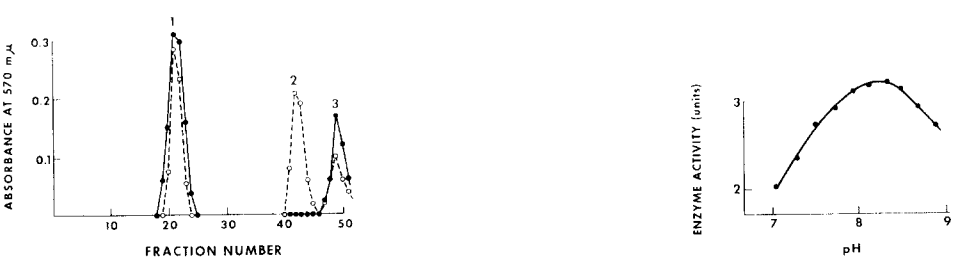


Fig. 6. Chromatographic determination of enzymic cystathionine formation. Reaction mixture consisting of DL-homocysteine (154 μmoles); L-serine (100 μmoles); pyridoxal-*P* (0.12 μmole) and purified enzyme (100 μg) in total volume of 1 ml of 0.1 M Tris–HCl buffer (pH 8.3) incubated for 3 h at 37°. Reaction stopped by addition of 0.1 ml 8 M HCl, aliquot of 0.3 ml of deproteinized supernatant applied onto Dowex 50-X8, (200–400 mesh, Na^+ form) column (1.2 cm × 15 cm) previously equilibrated with 0.2 M sodium citrate (pH 3.2)[16]. The same buffer was used to elute serine at a flow rate of 6.5 ml/6 min per fraction. At Fraction 36, the buffer was changed to 0.2 M sodium citrate (pH 4.2) and the flow rate was reduced to 2.9 ml/6 min. Each fraction was analyzed by the ninhydrin reaction[16] with a Gilford 300 spectrophotometer. ○— —○, products of enzyme reaction; ●—●, nonenzymic control. Peak 1, serine; Peak 2, cystathionine; Peak 3, unidentified compound (probably residual unoxidized homocysteine).

Fig. 7. pH–activity curve of cystathionine synthase. Incubation mixture is the same as described in *Enzyme assay* with 50 μg of purified enzyme in Tris buffer of varying pH.

nine, and, furthermore, a good agreement in cystathionine estimation between the Dowex 50-X8 column procedure and the direct spectrophotometric analytical method used for the present enzyme assay.

pH–activity

The pH–activity curve obtained is shown in Fig. 7. Cystathionine synthase has its optimal activity in Tris–HCl buffer at pH 8.3.

TABLE II

STOICHIOMETRY OF REACTION

The incubation was performed as described in Fig. 4, except that the amount of protein was 100 μg for Expt. I and 60 μg for Expt. II. Incubation was at 37° for 3 h. Ninhydrin values were calculated for their respective authentic compounds. Figures in parentheses show the amounts of cystathionine determined after incubation by the analytical methods given in the text.

Expt.	*Before reaction*	*After reaction*	
	L-*Serine added (μmoles)*	L-*Serine (μmoles)*	*Cystathionine formed (μmoles)*
I Nonenzymic control	100	106	
Enzyme reaction net change	100	73	29 (28)
		−33	+29 (+28)
II Nonenzymic control	107	110	
Enzyme reaction net change	107	95	13 (16)
		−15	+13 (+15)

Test of activity of hydroxyamino acid

The lack of enzyme activity of the present preparation of cystathionine synthase on hydroxyamino acids was demonstrated by experiments with L-serine, L-threonine, DL-allothreonine and DL-homoserine. The tests were performed as described in EXPERIMENTAL PROCEDURE for assay of hydroxyamino acid deamination by coupling the reaction with the lactate dehydrogenase–NADH system. The change in absorbance at 340 mμ was followed for at least 30 min at 25° with each of the above hydroxyamino acids, employing 75 μg of enzyme as against 40 μg used to test cystathionine synthetase activity. No reaction was observed with any of the hydroxyamino acids. This has previously been reported by BROWN *et al.*[5] and by NAKAGAWA AND KIMURA[9].

Effect of metal ions

Test of the effect of various divalent metal ions on the cystathionine synthase reactions with 45 μg of purified enzyme showed inhibition only by the following: Fe^{2+} (1 mM), 21%; Cd^{2+} (10 mM), 35%; and Hg^{2+} (10 μM), 53%; (0.1 mM), 68%; and (1 mM), 84%.

Carbonyl and metal chelating reagents

In agreement with reports of others[5,9], only hydroxylamine was markedly inhibitory, producing a 63% inhibition at 1 mM. L-Cysteine was slightly inhibitory, 20% at 0.1 mM. Semicarbazide (1 mM) and KCN (1 mM) had no effect.

Of metal chelating agents, only *o*-phenanthroline produced a small degree of inhibition (20% at 1 mM); 8-hydroxyquinoline (1 mM), nitrilotriacetic acid (5 mM), 2,2′-bipyridine (10 mM) and EDTA (1 mM) showed no inhibition. It appears unlikely that a metal ion is involved in cystathionine synthase activity.

Effect of sulfhydryl reagents

Among the several sulfhydryl reagents listed in Table III, only *p*-chloromercuribenzoate was found to be a powerful inhibitor of cystathionine synthase activity.

TABLE III

EFFECT OF SULFHYDRYL REAGENTS ON CYSTATHIONINE SYNTHASE ACTIVITY

Incubations were performed as described under *Enzyme assay* with 60 μg of enzyme (specific activity 85) and with sulfhydryl reagents as indicated.

Compound added	*Concn. (mM)*	*Activity (units)*	*Inhibition (%)*
None		5.1	
p-Chloromercuribenzoate	1	0.9	82
	0.1	3.3	35
Iodoacetate	1	5.3	
	0.1	5.2	
N-Ethylmaleimide	1	5.5	
	0.1	5.3	

As mentioned above, Hg^{2+} also is strongly inhibitory. Neither *N*-ethylmaleimide nor iodoacetate inhibited the enzyme activity at all.

The inhibition caused by *p*-chloromercuribenzoate (1 mM) was relieved by the addition of 5 mM GSH or dithiothreitol (Table IV). Tests with other thiol compounds were not run.

The effect of some compounds related to homocysteine metabolism on the cystathionine synthase activity was investigated. L-Cysteine, DL-homocysteine, α-ketobutyrate and pyruvate had no effect on the enzyme activity at the concentration tested (1 mM). $(NH_4)_2SO_4$ (10 mM) inhibited the enzyme activity by 50%.

TABLE IV

EFFECT OF SULFHYDRYL COMPOUNDS ON CYSTATHIONINE SYNTHASE ACTIVITY INHIBITED BY *p*-CHLOROMERCURIBENZOATE

Incubation performed as described under *Enzyme assay* with 50 μg of enzyme (specific activity 80) and *p*-chloromercuribenzoate (1 mM) or sulfhydryl-reducing agents (5 mM). Enzyme pre-incubated with mercurials for 10 min and inhibited enzyme similarly with sulfhydryl compounds.

Compound added	*Activity (units)*	*Inhibition (%)*
None	4.0	0
p-Chloromercuribenzoate	0.6	85
p-Chloromercuribenzoate + GSH	4.1	0
p-Chloromercuribenzoate + dithiothreitol	3.1	22

Effect of heat

Cystathionine synthase resists heat inactivation up to about 50° for 5 min. The enzyme loses activity at 62°, approximately one-third by heating for 2 min, and completely by a 10-min heating. No activation due to heat was observed at temperatures at which the enzyme is stable.

Coenzyme of cystathionine synthase

Considerable evidence has been obtained that pyridoxal-*P* is the coenzyme for this enzyme. The purified enzyme is yellow in color and has an absorption peak at 430 mμ at pH 5.5–9.5 characteristic of pyridoxal-*P* enzymes (Fig. 8). The absorption

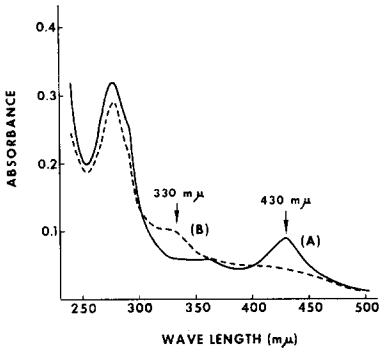


Fig. 8. Absorption spectra of cystathionine synthase before (A) and after (B) reduction with sodium borohydride. Lyophilized enzyme was dissolved in 0.01 M potassium phosphate buffer (pH 7.5) and dialyzed against 500 vol. of the same buffer for 12 h. After centrifugation to remove any insoluble material, an aliquot of enzyme solution (0.34 mg/ml) was then dialyzed for 5 min against 500 vol. of 0.01 M potassium phosphate buffer (pH 7.5) containing 0.005 M sodium borohydride. At the end of the 5-min period, the cellophane bag was transferred to a container with 500 vol. of 0.01 M potassium phosphate buffer (pH 7.5) and dialysis was continued for 4 h with one change of the buffer. Spectra were recorded in a cuvette of 1 cm light path with the Cary automatic spectrophotometer.

peak is moved to 410 mμ at pH 4.5 in sodium acetate buffer and to 420 mμ in 0.1 M NaOH. In acid solution (0.1 M HCl) the peak is shifted to 360 mμ. The absorbance also is lowered in alkaline and acid solutions. At all pH values the absorption goes through an isobestic point at 385 mμ. Reducing the enzyme with sodium borohydride eliminates the peak at 430 mμ in the yellow region and produces a shoulder at 330 mμ (Fig. 8). The reduced enzyme was completely inactive when tested in the absence of pyridoxal-*P*. 40 μM pyridoxal-*P*, produced a slight activation of the reduced enzyme to a specific activity of 12.4 (Table V).

TABLE V

ENZYMIC ACTIVITY OF CYSTATHIONINE SYNTHASE BEFORE AND AFTER REDUCTION WITH SODIUM BOROHYDRIDE

Incubations were performed as described under *Enzyme assay*. Enzyme was reduced as described in Fig. 6.

	Specific activity (units/mg protein)	
	With added pyridoxal-P	*Without added pyridoxal-P*
Before reduction	110.0	97.9
After reduction	12.4	0

The pyridoxal-P was demonstrated to be attached to the ε-amino group of a lysine residue by hydrolyzing a sample of borohydride-reduced enzyme and comparing the isolated reduction product of pyridoxal-*P* with authentic ε-pyridoxyllysine by paper chromatography[18].

The borohydride-treated enzyme was hydrolyzed in 6 M HCl in a sealed tube for 20 h. After hydrolysis, the HCl was removed *in vacuo* and the residue was dissolved in a small volume of water. Aliquots were spotted on Whatman No. 1 paper and developed (descending method) in the dark at room temperature in the following solvent systems: water–methanol–ethanol–benzene–pyridine–dioxane (25:25:10:10:10:10, by vol.)[18] and *n*-butanol–acetic acid–water (4:1:1, by vol.). Each chromatogram showed a fluorescent spot which traveled identically with synthetic ε-pyridoxyllysine. The spot was eluted and rechromatographed on Whatman No. 1 paper. The spot corresponding to the authentic ε-pyridoxyllysine, run at the same time, was quenched by ammonia fumes and gave a positive ninhydrin reaction.

The coenzyme was dissociated from cystathionine synthase by dialysis against cysteine at a slightly alkaline pH (ref. 19). The lyophilized enzyme was dissolved in 2 ml of phosphate buffer (pH 7.5) (1.2 mg/ml, specific activity 106) and dialyzed against 500 vol. of the same buffer containing 0.05 M cysteine for 39 h at 5° with one change of buffer solution. The enzyme was then assayed in the usual manner. The activity of the cysteine-dialyzed enzyme solution was reduced to 28.2 units/mg protein, a decrease of about 75% in activity. Addition of 0.1 mM pyridoxal-*P* to the assay medium increased the enzyme activity to 98 units/mg protein, 96% of the original activity.

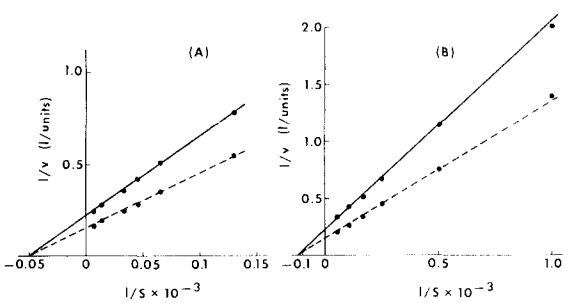


Fig. 9. Double reciprocal plots of DL-homocysteine (A) and L-serine (B) in the presence (●— —●) and absence (●—●) of 40–70% $(NH_4)_2SO_4$ fraction. The supernatant from the precipitation of the enzyme at 25–40% $(NH_4)_2SO_4$ saturation was further fractionated with solid $(NH_4)_2SO_4$ up to 70% saturation. The precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.5) and dialyzed against the same buffer overnight. Approx. 40 μg of purified enzyme and 0.8 mg of 40–70% $(NH_4)_2SO_4$ fraction were contained in usual incubation mixture with 0.5 mM $CuSO_4$ (●— —●). The same amount of purified enzyme was used with 0.5 mM $CuSO_4$ in the absence of 40–70% $(NH_4)_2SO_4$ fraction as a control experiment (●—●).

In another experiment performed to observe the change in absorbance at 430 mμ on dialysis with cysteine, the cysteine treatment resulted in a 40% decrease in absorbance; incubation with 17 μM pyridoxal-*P* for 10 min at 37° increased the 430-mμ absorbance to 85% of the untreated enzyme.

Michaelis constants

The reaction rates for the formation of cystathionine were determined at constant homocysteine and varying serine concentration and *vice versa*. The results obtained are plotted in Fig. 9. K_m values obtained from Lineweaver–Burk plots were 0.02 M for DL-homocysteine and 8.3 mM for L-serine, respectively. The figure for

homocysteine agrees well with that of NAKAGAWA AND KIMURA[9], but is about 6-fold greater for L-serine than found by these authors.

Activation of cystathionine synthase

Cystathionine synthase activity is mainly concentrated in a fraction between 25 and 40% saturation of $(NH_4)_2SO_4$ and only slight activity was found in fractions over 40% $(NH_4)_2SO_4$ saturation. When the 40–70% $(NH_4)_2SO_4$ fraction was added to the purified cystathionine synthase preparation, considerable activation of the enzymatic activity was observed. The activation by the addition of an increasing amount of the 40–70% fraction proceeded hyperbolically (Fig. 10A); the activator behaves somewhat like a substrate for cystathionine synthase. Boiled extract of the 40–70% $(NH_4)_2SO_4$ fraction and water extract of the ashed 40–70% $(NH_4)_2SO_4$ fraction showed no activating effect on the enzyme activity. These facts strongly suggest that the activator is a protein-like substance.

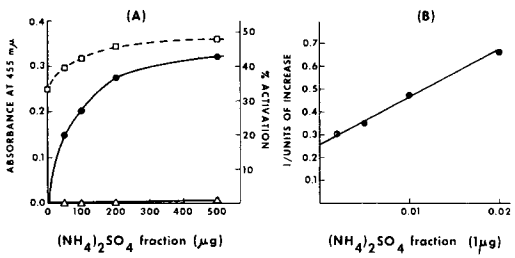


Fig. 10. Concentration–activation curve of cystathionine synthase activity by protein from the 40–70% $(NH_4)_2SO_4$ fraction (A) and double reciprocal plot of the same (B). Incubation procedure was the same as described in *Enzyme assay* with 7.8 units (approx. 55 μg) of purified enzyme and 0.5 mM $CuSO_4$. (A) □– –□, absorbance at 455 mμ of ninhydrin chromogen of deproteinized incubation mixture (0.1 ml) with purified enzyme; ●—●, absorbance at 455 mμ of ninhydrin chromogen of deproteinized incubation mixture (0.1 ml) without purified enzyme. (B) Plot of reciprocal of increase in enzyme units calculated from absorbance at 455 mμ in A *vs.* reciprocal of activating fraction.

Velocity–substrate concentration curves of serine and homocysteine for cystathionine synthase activity upon addition of the activator were typically hyperbolic. K_m values for serine and homocysteine obtained from Lineweaver–Burk plots with added 40–70% fraction were exactly the same (Fig. 9, broken lines) as those from the control experiments (Fig. 9, solid lines). In the presence of both substrates the calculated v_{max} from a double reciprocal plot with added 40–70% fraction reaches a limiting value of approx. 60% higher than that without the fraction (Fig. 10B). Test of the 40–70% fraction showed only a specific activity of 2.2. The maximum amount of the 40–70% fraction added in the experiment was 500 μg or about 1 enzyme unit.

DISCUSSION

It has now abundantly been established that cystathionine synthase and serine dehydratase are distinct and separate proteins. In agreement with the above, it has here been shown that our cystathionine synthase preparation had no deaminative

activity on any of the hydroxyamino acids tested. The erroneous contrary conclusions of SELIM AND GREENBERG[1,2] and NAGABHUSHANAM AND GREENBERG[3] appear to be adequately explained by the observation of BROWN *et al.*[8] that α-keto acids form adducts with homocysteine.

Our cystathionine synthase preparation showed normal Michaelis kinetics with homocysteine (Fig. 9) as found by NAKAGAWA AND KIMURA[9], but BROWN *et al.*[8] reports a sigmoid curve with this substrate.

There is also disagreement between BROWN *et al.*[8] and NAKAGAWA AND KIMURA[9] and ourselves on the possibility that cystathionine synthase is a pyrixodal-*P*-dependent enzyme. We observed that dialysis against cysteine resulted in a 75% decrease in enzyme activity. In addition, cystathionine synthase has an absorption spectrum characteristic of pyridoxal-*P* enzymes. This could be restored to 95% of its initial activity by addition of pyridoxal-*P*. Also, contrary to BROWN *et al.*[8], *p*-chloromercuribenzoate and Hg^{2+} were both potent inhibitors of our enzyme preparation.

Cystathionine synthase is quite a stable enzyme. It withstands inactivation by heating at 50° and by lyophilization. A most interesting observation made in our work is that a protein fraction found in the filtrate from the enzyme on 40–70% saturation with $(NH_4)_2SO_4$ enhanced the enzyme activity by as much as 50%. The saturation curve of the activator (Fig. 10) is similar to that of a normal substrate. The nature of the activator, aside from the fact that it appears to be a protein, and its mode of action is not as yet known but is under further investigation.

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