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Increase in cystathionine content in rat liver mitochondria after D,L-propargylglycine administration

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Summary. Intraperitoneal administration of D,L-propargylglycine to rats resulted in an increase in the cystathionine content of whole liver and liver mitochondria. Cystathionine in mitochondria was identified by amino acid analysis, thin layer chromatography, high-voltage paper electrophoresis and liquid chromatography-mass spectrometry. The cystathionine content of whole liver was 5.37 ± 1.59 μmol per g of fresh liver at 14 h after the administration of 50 mg of D,L-propargylglycine per kg of body weight, while 0.07 ± 0.02 μmol of cystathionine per g of fresh liver was detected in the control rats. The cystathionine content of liver mitochondria from both groups of rats was 9.40 ± 1.20 and 0.19 ± 0.04 nmol of cystathionine per mg of protein, respectively. The mitochondrial cystathionine increased dose-dependently with the increase of D,L-propargylglycine administered. The increase was proportional to the time after the administration up to 12 h, and then decreased. The increase of cystathionine in the liver mitochondria was linearly proportional to that in the whole liver. These results suggest that cystathionine in liver mitochondria is in an equilibrium with that in the cytosol.

Keywords: Amino acids–Cystathionine–Mitochondria–Propargylglycine

Introduction

Cystathionine is an intermediate in the transsulfuration pathway of methionine metabolism and is metabolized mainly to cysteine by cystathionine γ-lyase (EC 4.4.1.1) in mammals (Greenberg, 1975). It is well known that cystathionine γ-lyase deficiency leads to cystathioninuria (Finkelstein, 1975).

D,L-Propargylglycine is an *in vitro* and *in vivo* inhibitor of cystathionine γ-lyase (Abeles and Walsh, 1973). Experimental cystathioninuria has been induced by D,L-propargylglycine administration (Uren et al., 1978; Kodama

et al., 1982). Accumulation of cystathionine in several tissues has been observed in D,L-propargylglycine-treated rats (Kodama et al., 1983; Awata et al., 1984; Mizobuchi et al., 1990). Subcellular distribution of the accumulated cystathionine, however, has not been investigated.

Liver mitochondria is believed to play a central role in the metabolism of amino acid nitrogen in mammals (Rawn, 1989). Involvement of mitochondria in sulfur compound metabolism has also been indicated (Cohen et al., 1972; Koj et al., 1975; Sörbo, 1975; Ubuka et al., 1977a, 1977b). It has been reported that sulfate may be formed through the 3-mercaptopyruvate pathway from L-cysteine in rat liver mitochondria (Ubuka et al., 1990; Ubuka et al., 1992). In the present paper we describe the increase in cystathionine content in rat liver mitochondria after administration of D,L-propargylglycine, suggesting a possible role of liver mitochondria in cystathionine metabolism.

Materials and methods

Materials

Male Wistar rats weighing 200–300 g were used in this study and maintained on a laboratory diet, MF, of Oriental Yeast Co., Ltd., Tokyo, Japan. D,L-Propargylglycine was obtained from Sigma Chemical Co., St. Louis, MO, USA. Sucrose, D-mannitol, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were purchased from Wako Pure Chemical Ind., Osaka, Japan. Dowex 50W (×8, 200–400 mesh) were from Bio-Rad Laboratories, Richmond, CA, USA. AVICEL, microcrystalline cellulose was purchased from Funakoshi Co., Ltd., Tokyo, Japan.

Administration of D,L-propargylglycine and preparation of tissue extracts

Aqueous solutions containing various amounts of D,L-propargylglycine were injected intraperitoneally to rats. At the appropriate time after the injection, the rats were decapitated and exsanguinated. Following procedures were performed at 0 °C. Livers were taken out and washed in 0.9% NaCl solution. Sulfosalicylate extract of the whole liver was prepared by homogenizing 1 g of liver with 1 volume of water and 2 volumes of 6% sulfosalicylic acid and centrifugation at 10,000 × g for 20 min. Mitochondrial fraction was prepared according to Griffith and Meister (1985). In the present study, twice washed mitochondria were used except in experiments to examine the effect of washing of mitochondria on cystathionine content. The mitochondrial pellet obtained was suspended in 3 volumes of the isolation medium composed of 200 mM D-mannitol, 70 mM sucrose, 2 mM K^+ Hepes (pH 7.4). Sulfosalicylate extract of mitochondrial fraction was prepared by sonication of 2 ml of this mitochondrial suspension together with 0.2 ml of 33% sulfosalicylic acid at 20 kHz for 10 min and centrifugation at 10,000 × g for 20 min.

Preparation of cystathionine fraction from liver mitochondria

Liver mitochondria were obtained from rats administered 50 mg of D,L-propargylglycine per kg of body weight at 14 h after the administration and from untreated rats for the control. Mitochondrial pellet, 9 ml, was suspended in 27 ml of the isolation medium. The resulting suspension was sonicated together with 12 ml of 20% trichloroacetic acid at 20 kHz for 10 min and centrifuged at 10,000 × g. Thirtyseven millilitres of the supernatant was applied to a column of Dowex 50W (×8, 200–400 mesh, H^+-form, 2.7 cm × 17 cm). The column was washed with 500 ml of H_2O and then eluted with 1 l of 2 M NH_3. The eluate was dried under reduced pressure at 40 °C. The resulting residue was dissolved in 1 ml of water. The solution, 0.5 ml, was fractionated by using amino acid separating

system of a Hitachi KLA-5 amino acid analyzer as described below, and the fraction containing cystathionine was collected. The fraction obtained (2.5 ml) was applied to a column of Dowex 50W (×8, 200–400 mesh, H^+-form, 1.5 cm × 5.7 cm). The column was washed with 50 ml of H_2O and then eluted with 100 ml of 2M NH_3. The eluate was dried as above. The resulting residue was dissolved in 0.5 ml of H_2O and analyzed by amino acid analysis, liquid chromatography-mass spectrometry, thin layer chromatography, and high-voltage paper electrophoresis as described below.

Amino acid analysis

Acidic and neutral amino acids were analyzed with a Hitachi KLA-5 amino acid analyzer using a column of custom ion-exchange resin No 2613, 0.9 cm × 55 cm. Chromatography was performed with 115 ml of 0.2 N sodium citrate-8% ethanol (pH 3.22) and 100 ml of 0.2 N sodium citrate-24% ethanol (pH 4.50), successively, at a flow rate of 1 ml per min at 55 °C. Cystathionine was eluted at 174 min between methionine and isoleucine under the present conditions.

Liquid chromatography-mass spectrometry

Twenty μl of the solution was applied to a Hitachi L-6200 high-performance liquid chromatograph equipped with a 5-μm Inertosil ODS-2 column (4.6 mm i.d. × 150 mm) from Gasukuro Kogyo, Ltd., Tokyo, Japan, and eluted with a mixture of 50 mM ammonium acetate and acetonitrile (85:15, by volume) at a flow rate of 0.9 ml per min. The eluate was introduced to a Hitachi M80B mass spectrometer-computer system through the interface for atmospheric pressure ionization and analyzed. The nebulizer and vaporizer temperatures were 270 and 390 °C, respectively.

Thin layer chromatography and high-voltage paper electrophoresis

Thin layer chromatography was performed on thin layer of AVICEL using solvent systems of isopropanol-formic acid-water (4:1:1, by volume), n-butanol-acetic acid-water (4:1:1, by volume) and pyridine-acetone-29% ammonia-water (45:30:10:10, by volume). Rf value of cystathionine was 0.40, 0.04 and 0.04, respectively.

High-voltage paper electrophoresis was carried out using 95% pyridine-acetic acid-water (0.5:10.0:89.5, by volume; pH 3.1) as a buffer solution (Ubuka, 1962) on Whatman No. 1 paper at 3000 V for 30 min. Cystathionine was migrated toward the cathode by the same distance as aspartic acid was migrated toward the anode.

One percent ninhydrin-2% pyridine in acetone was used for color development.

Protein determination

Protein determination of mitochondrial fraction was performed by biuret method in the presence of 0.25% sodium deoxycholate (Jacobs et al., 1956).

Statistics

Data were analyzed by the Student's t-test or the paired t-test. Differences were accepted as significant at $p < 0.05$. Correlation coefficients were calculated between cystathionine concentration in liver mitochondria and that in whole liver.

Results and discussion

Identification of cystathionine in liver mitochondria

The cystathionine fraction from liver mitochondria of the rats administered D,L-propargylglycine showed a single peak, a single spot and a single band by

amino acid analysis, thin layer chromatography and high-voltage paper electrophoresis, respectively, which coincided to those of authentic cystathionine.

Figure 1 shows mass chromatograms of the cystathionine fractions from liver mitochondria of rats administered D,L-propargylglycine, untreated rats and authentic cystathionine. The peak of the quasi molecular ion $[M+H]^+$ of cystathionine (m/z 223) was observed on mass chromatogram of the sample from the administered rats, but not from the untreated ones. The retention time of this peak, 1.6 min, was the same as that of authentic cystathionine. These results clearly show that D,L-propargylglycine administration resulted in the increase in cystathionine content in liver mitochondria.

Amino acid contents of liver mitochondria

Figures 2 and 3 show chromatograms of amino acid analyses of the whole liver and liver mitochondrial extracts prepared 14 h after intraperitoneal injection of D,L-propargylglycine (50 mg per kg of body weight) or 0.9% NaCl. Summary of 4 experiments is shown in Table 1. The cystathionine content in whole liver was 5.37 ± 1.59 μmol per g of fresh liver at 14 h after the administration of D,L-propargylglycine, while 0.07 ± 0.02 μmol of cystathionine per g of fresh liver was detected in the control liver. These values agreed well with previous reports (Kodama et al., 1983; Awata et al., 1984). The cystathionine content in liver mitochondria determined at the same time was 9.40 ± 1.20 and 0.19 ± 0.04 nmol of cystathionine per mg of protein, respectively. Thus, D,L-propargylglycine treatment resulted in the increase in

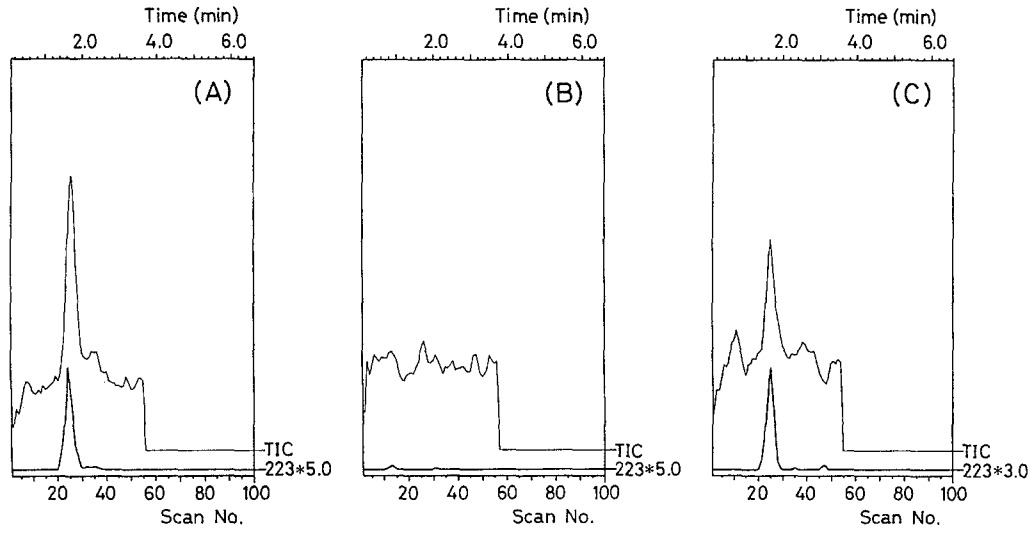

Fig. 1. Mass chromatograms of the cystathionine-containing fractions of liver mitochondria from rats administered D,L-propargylglycine (**A**), untreated rats (**B**) and authentic cystathionine (**C**). The quasi molecular ion of cystathionine, m/z 223, was detected. The mass spectrometer was scanned from m/z 150 to 250 at a rate of 4 s per scan. For details, see the section of Materials and methods

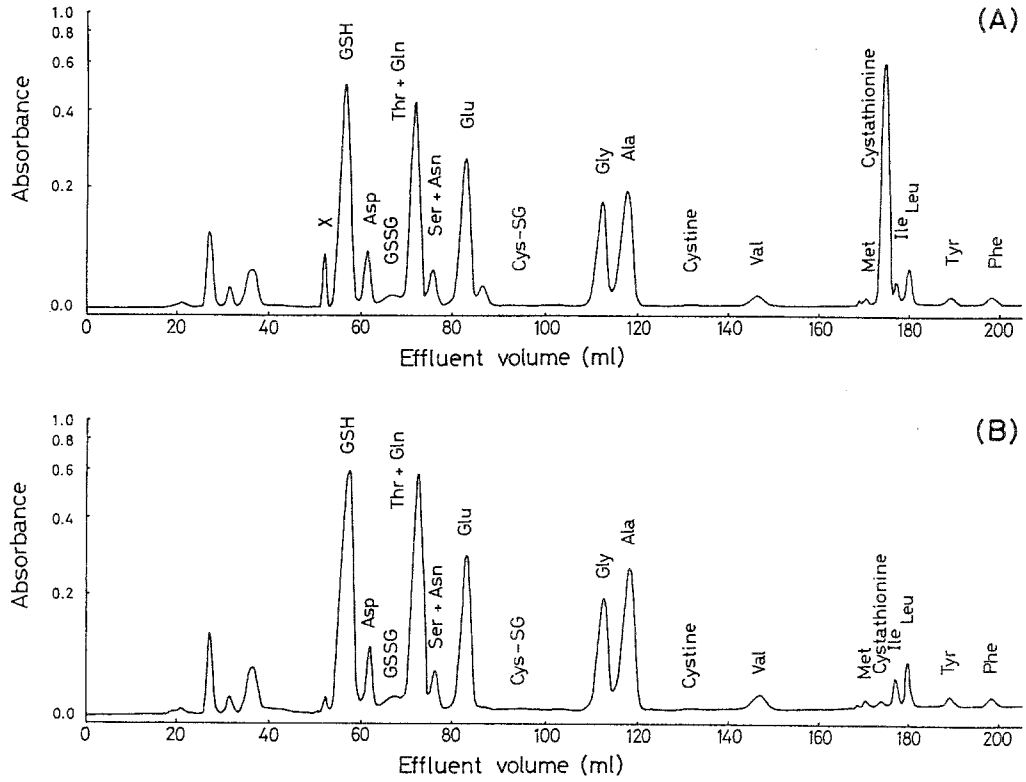

Fig. 2. Chromatograms of amino acid analysis of the whole liver at 14 hours after intraperitoneal injection of D,L-propargylglycine (50 mg per kg of body weight) (**A**) and 0.9% NaCl (**B**)

cystathionine both in whole liver and in liver mitochondria. A decrease in total glutathione in whole liver from L-propargylglycine-treated mice was reported (Shinozuka et al., 1982). In the present study, glutathione (GSH) and glutathione disulfide (GSSG) also showed a decreasing tendency. The decrease in the total GSH (GSH + 2GSSG) was significant in liver mitochondria ($p < 0.05$) as well as in whole liver ($p < 0.01$), confirming the previous conclusion that mitochondrial GSH is derived from cytosolic GSH (Griffith and Meister, 1985). GSH content in the control liver mitochondria shown in Table 1 agreed with those reported (Wahlländer et al., 1979; Ubuka et al., 1992), indicating mitochondria were prepared properly. Other known acidic and neutral amino acids showed no large changes, but an unknown peak, X, was observed before GSH on the chromatograms of liver and liver mitochondria after the administration of D,L-propargylglycine (Figs. 2A and 3A). The unknown compound giving this peak was suggested to be γ-glutamylpropargylglycylglycine (Ohta et al., in preparation).

Effect of washing of mitochondrial pellet on cystathionine content

The present procedure for the preparation of mitochondria from rat liver required 2 h. It is not clear whether cystathionine in mitochondria escaped

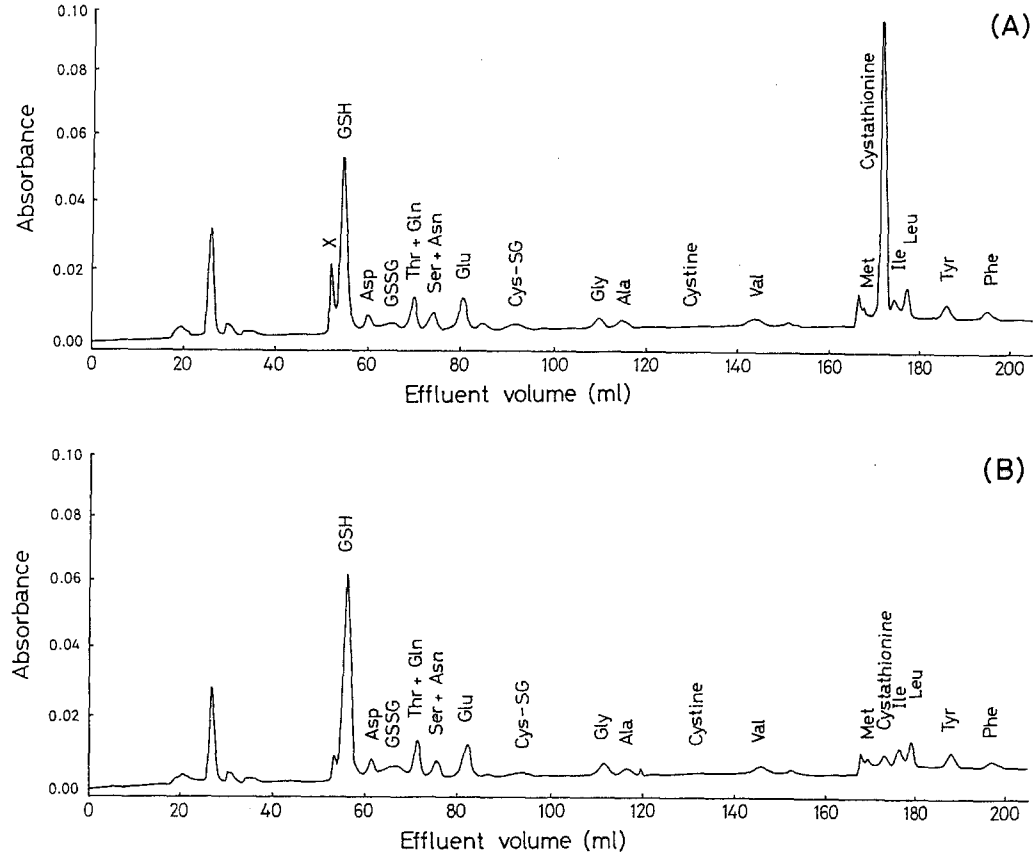

Fig. 3. Chromatograms of amino acid analysis of the liver mitochondria at 14 hours after intraperitoneal injection of D,L-propargylglycine (50 mg per kg of body weight) (**A**) and 0.9% NaCl (**B**)

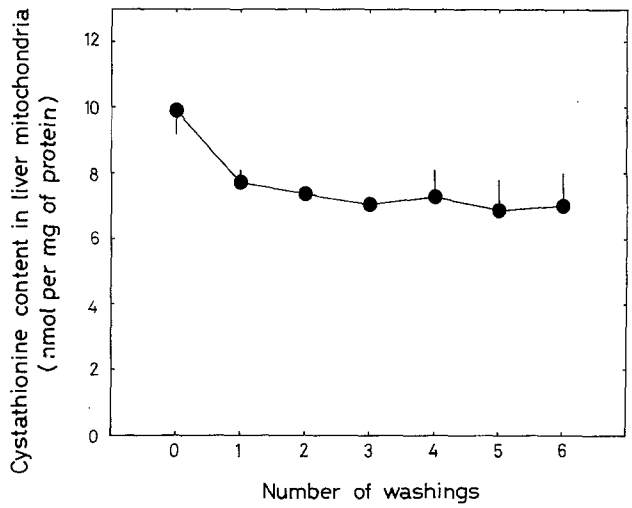

Fig. 4. Effect of washing of mitochondrial pellet on cystathionine content. Liver mitochondria were prepared at 14 hours after intraperitoneal injection of D,L-propargylglycine (50 mg per kg of body weight), according to Griffith and Meister (1985), except that the number of washings was varied from 0 to 6. The isolation medium composed of 200 mM D-mannitol, 70 mM sucrose, 2 mM K^+ Hepes (pH 7.4) was used for washing at 0 °C. Cystathionine was determined with a Hitachi KLA-5 amino acid analyzer as described under Materials and methods. Points represent the average values of 3 separate experiments and vertical bars indicate the standard deviations

Table 1. Free amino acid contents in liver mitochondria and whole liver[a]

Amino acid	Liver mitochondria (nmol per mg of protein)		Whole liver (μmol per g of liver)	
	Propargylglycine[b]	Control[c]	Propargylglycine[b]	Control[c]
GSH	6.59 ± 1.01	7.29 ± 0.26	5.15 ± 0.72[e]	7.65 ± 0.38
Asp	0.66 ± 0.15	0.56 ± 0.08	0.71 ± 0.22	0.76 ± 0.07
GSSG	0.54 ± 0.15[e]	0.78 ± 0.03	0.64 ± 0.35	0.90 ± 0.25
Thr+Gln	1.06 ± 0.41	0.63 ± 0.16	5.90 ± 1.26	4.90 ± 0.34
Ser+Asn	0.63 ± 0.14	0.49 ± 0.09	0.59 ± 0.05	0.55 ± 0.17
Glu	0.61 ± 0.25	0.59 ± 0.07	1.98 ± 0.63	2.24 ± 0.72
Cys-SG[d]	trace	trace	trace	trace
Gly	0.31 ± 0.15	0.21 ± 0.06	2.44 ± 0.12	2.22 ± 0.39
Ala	0.27 ± 0.11	0.32 ± 0.05	1.67 ± 0.48	2.53 ± 0.61
Cystine	trace	trace	trace	trace
Val	0.39 ± 0.12	0.20 ± 0.03	0.20 ± 0.05	0.25 ± 0.04
Met	0.20 ± 0.10	0.14 ± 0.05	0.05 ± 0.01[e]	0.08 ± 0.01
Cystathionine	9.40 ± 1.20[e]	0.19 ± 0.04	5.37 ± 1.59[e]	0.07 ± 0.02
Ile	0.49 ± 0.21	0.29 ± 0.08	0.17 ± 0.03	0.16 ± 0.03
Leu	0.58 ± 0.17	0.38 ± 0.08	0.25 ± 0.03	0.28 ± 0.05
Tyr	0.18 ± 0.07	0.12 ± 0.03	0.10 ± 0.03	0.11 ± 0.03
Phe	0.05 ± 0.03	0.06 ± 0.07	0.08 ± 0.02	0.11 ± 0.03

[a] Values are expressed as mean ± SD obtained from 4 separate experiments. [b] Fifty mg of D,L-propargylglycine per kg of body weight were administered to rats. Free amino acid contents in the tissues at 14 h after the administration were determined as described under Materials and methods. [c] Instead of D,L-propargylglycine solution, 0.9% NaCl was administered to rats. Free amino acid contents in the tissues at 14 h after the administration were determined as described under Materials and methods. [d] Cysteine-glutathione disulfide. [e] Significantly different ($p < 0.05$) from the value for the control by the Student's t-test.

from mitochondria during the procedure for preparation. Therefore, effect of repeated washing of mitochondria at 0 °C on the cystathionine content was examined. As shown in Fig. 4, cystathionine content in mitochondria showed no significant change (by the paired t-test) when the number of washings were varied from 1 to 6. Although there was a small decrease between no washing and 1 washing (significant by the paired t-test), the decrease could be explained by a small amount of residual cytosolic fraction contained in the no-washing mitochondria preparation. These results indicate that the present method for mitochondria preparation was suitable for the determination of mitochondrial cystathionine.

Change of cystathionine content in whole liver and liver mitochondria following administration of D,L-propargylglycine

Figure 5 shows the relationship of cystathionine content in whole liver and in liver mitochondria with the dose of D,L-propargylglycine. Cystathionine content both in whole liver and in liver mitochondria increased dose-

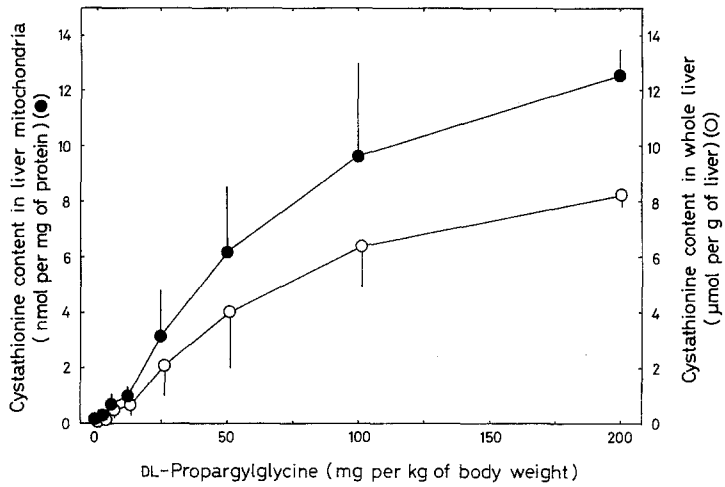

Fig. 5. Relationship of cystathionine contents in rat liver (○) and liver mitochondria (●) with the dose of D,L-propargylglycine. Tissue extracts were prepared at 14 hours after intraperitoneal injection of D,L-propargylglycine (0, 3.125, 6.25, 12.5, 25, 50, 100 or 200 mg per kg of body weight). Cystathionine was determined with a Hitachi KLA-5 amino acid analyzer as described under Materials and methods. Points represent the average values of 3 separate experiments and vertical bars indicate the standard deviations

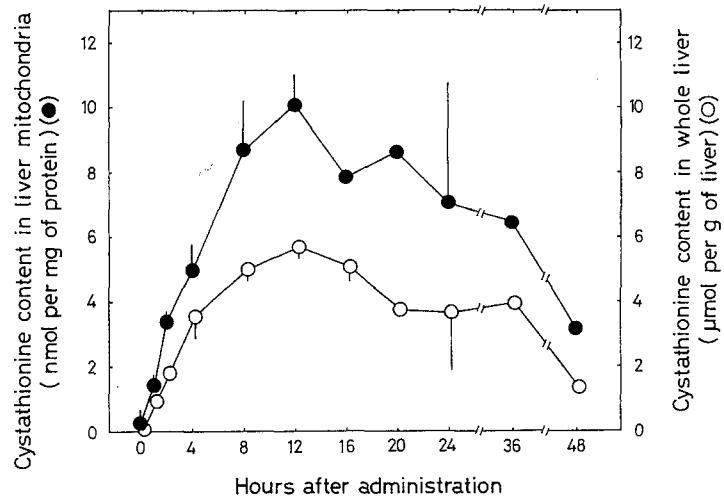

Fig. 6. Accumulation of cystathionine in rat liver (○) and liver mitochondria (●) after administration of 50 mg/kg of body weight of D,L-propargylglycine. Tissue extracts were prepared at the indicated time after intraperitoneal injection of D,L-propargylglycine (50 mg per kg of body weight). Cystathionine was determined with a Hitachi KLA-5 amino acid analyzer as described under Materials and methods. Points represent the average values of 3 to 5 separate experiments, except that the pints of 20, 36 and 48 h represent the value of a single experiment, and vertical bars indicate the standard deviations

dependently with the increase of D,L-propargylglycine. Figure 6 shows the cystathionine content in whole liver and in liver mitochondria at from 1 to 48 h after administration of 50 mg/kg of body weight of D,L-propargylglycine. Cystathionine content in both whole liver and liver mitochondria increased for 12 h after D,L-propargylglycine administration, and then gradually decreased. The increase in whole liver, shown in Figs. 5 and 6, agreed well with previous reports (Kodama et al., 1983; Awata et al., 1984).

Figures 5 and 6 show that the change of cystathionine content in liver mitochondria was similar to that in whole liver, suggesting cystathionine content in mitochondria is linearly proportional to that in whole liver. The data from 60 rats, summarized in Table 1, Figs. 5 and 6, were statistically analyzed. The coefficient of correlation between cystathionine content in liver mitochondria and in whole liver was 0.979, confirming a linear relationship between cystathionine content in liver mitochondria and in whole liver.

Cystathionine content could be converted to cystathionine concentrations assuming water contents of 0.8 μl per mg of protein in liver mitochondria (Wahlländer et al., 1979) and 0.74 ml per g of liver in whole liver (Bintz and Riedesel, 1967). Differences between calculated cystathionine concentrations in liver mitochondria and in whole liver were statistically analyzed by the paired t-test. Cystathionine concentration in mitochondria was significantly higher than that in whole liver at 200 mg D,L-propargylglycine per kg body weight in Fig. 5 and at 0, 2, 4, 8, 12, and 16 h after administration in Fig. 6 ($p < 0.02$). At the other points in Figs. 5 and 6 no significant difference was detected. The ratio of cystathionine concentration in mitochondria to that in whole liver observed in the present study was 1.84 ± 0.82 ($n = 60$; range, 1.08–6.70). This ratio was significantly higher than 1 by the Student's t-test. These results indicate that the cystathionine concentration in liver mitochondria was comparable with that in the cytosol. The mitochondrial water content may be assumed to be approximately 10% of the total cellular water (Wahlländer et al., 1979). Therefore, the mitochondrial cystathionine content could be estimated to be at least 10% of the total cellular cystathionine.

It is well known that cystathionine is formed from homocysteine and serine by cystathionine β-synthase (EC 4.2.1.22) in mammals (Greenberg, 1975). Kashiwamata (1971) and Rassin and Gaull (1975) reported that most of the cystathionine β-synthase activity in rat liver was found in the soluble fraction. Allsop and Watts (1975) reported that cystathionine β-synthase and cystathionine γ-lyase activities were not present in the mitochondrial fraction from rat liver. These previous results indicate that cystathionine in liver mitochondria from rats administered D,L-propargylglycine was derived from the cytosol. Although mechanism of transport of cystathionine across mitochondrial membranes has not been specified, the present results indicate that mitochondrial cystathionine is in an equilibrium with cytosolic cystathionine. Furthermore, this seems to indicate that liver mitochondria of untreated rats also may be supplied with cystathionine from the cytosol.

It is believed that the first 2 enzymes of the urea cycle, carbamoylphosphate synthase (ammonia) and ornithine carbamoyltransferase, are

located in liver mitochondria to metabolize ammonia (Rawn, 1989). Therefore, it seems that, in liver mitochondria, various amino acids may be actively transaminated to form oxo acids. As for sulfur amino acids, the transaminative metabolism of L-cysteine in rat liver mitochondria has been indicated (Ubuka et al., 1977a, 1992). The previous studies using bovine liver (Costa et al., 1986), kidney (Ricci et al., 1986), and brain (Costa et al., 1987) indicated that cystathionine is transaminated by glutamine transaminase to form cystathionine mono-oxo acid. Glutamine transaminase is present in rat liver mitochondria as well as existing as a soluble isozyme (Yoshida, 1967; Cooper and Meister, 1974). These findings suggest a possible role of mitochondrial cystathionine as substrate of mitochondrial glutamine transaminase.

Products of presumed cystathionine origin were found in the urine in cystathioninuric patients (Kodama et al., 1969, 1970, 1975) and normal humans (Matarese et al., 1987). Cystathionine mono-oxo acid has been recognized as the precurcer of these products (Kodama et al., 1969; Ricci et al., 1983; Okada et al., 1993). Liver mitochondria might take the part in the formation of these products of presumed cystathionine origin in both cystathioninuric and normal subjects.

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