Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7185–7189, September 1990
Biochemistry

High-affinity transport of glutathione is part of a multicomponent system essential for mitochondrial function

(glutathione deficiency/buthionine sulfoximine/glutathione esters/mitochondria)

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Contributed by Alton Meister, July 11, 1990

ABSTRACT **Glutathione, an essential cellular antioxidant required for mitochondrial function, is not synthesized by mitochondria but is imported from the cytosol. Rat liver mitochondria have a multicomponent system that underlies the remarkable ability of mitochondria to take up and retain glutathione. At external glutathione levels of <1 mM, glutathione is transported into the mitochondrial matrix by a high-affinity component (K_m, ≈60 μM; V_{max}, ≈0.5 nmol/min per mg of protein), which is saturated at levels of 1–2 mM and stimulated by ATP. Another component has lower affinity (K_m, ≈5.4 mM; V_{max}, ≈5.9 nmol/min per mg of protein) and is stimulated by ATP and ADP. Both components are inhibited by carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), glutamate, and ophthalmic acid. Increase of extramitochondrial glutathione promotes uptake and exchange; the intermembranous space seems to function as a recovery zone that promotes efficient recycling of matrix glutathione. The findings are in accord with *in vivo* data showing that (*i*) rapid exchange occurs between mitochondrial and cytosolic glutathione, (*ii*) lowering of cytosolic glutathione levels (produced by administration of buthionine sulfoximine) decreases export of glutathione from mitochondria to cytosol, and (*iii*) administration of glutathione esters increases glutathione levels in mitochondria more than those in the cytosol.**

A small but significant fraction of the oxygen utilized by mitochondria is converted to hydrogen peroxide (1). Much has been written about the toxicity of hydrogen peroxide, superoxide anion, and other reactive oxygen compounds and also about the antioxidant defenses that seem to protect cells against oxygen toxicity (see, for example, refs. 2–5). The suggested "primary defenses" (5) include the activities of such enzymes as superoxide dismutase, catalase, and glutathione (GSH) peroxidases and also smaller molecules such as ascorbate, α-tocopherol, GSH, β-carotene, and uric acid. Superoxide dismutase converts superoxide anion to hydrogen peroxide, which is destroyed in mitochondria (which lack catalase) by GSH peroxidase. GSH is involved in the reduction of dehydroascorbate to ascorbate and also in the maintenance of α-tocopherol in the reduced state. Thus, it appears that GSH plays a crucial role as a cellular antioxidant.

In the course of studies in this laboratory on the functions of GSH, we induced GSH deficiency *in vivo* in mice and rats by administration of buthionine sulfoximine (BSO), a selective and irreversible transition-state inhibitor of γ-glutamylcysteine synthetase (4, 6–9), the enzyme that catalyzes the first step of GSH synthesis. GSH deficiency leads to marked structural damage in several tissues, including skeletal muscle (10), lung (11), lens epithelia of newborns (12), and epithelia of the jejunum and colon (13). Cellular damage in each instance is characterized by severe mitochondrial degeneration and very low levels of mitochondrial GSH. Administration of GSH monoester (14–17) eliminated the BSO-induced GSH deficiency, prevented mitochondrial and other cellular damage, and led to substantially increased mitochondrial GSH levels (10–13).

These studies show that GSH deficiency, produced without application of additional stress (e.g., increased oxygen, drugs, radiation), leads to severe mitochondrial damage in a number of tissues. These findings, which demonstrate that GSH is required for normal mitochondrial function, establish the essentiality of GSH as a physiological antioxidant. Thus, GSH affords protection against the constant endogenous formation of hydrogen peroxide (or superoxide), which occurs during normal metabolism.

Although it has long been known that mitochondria contain GSH (see, for example, refs. 18–22), the origin of mitochondrial GSH was at first unclear (21–23). Later it was found that mitochondria do not contain the enzymes required for GSH synthesis, thus indicating that mitochondrial GSH is derived from the cytosol (24). This was supported by studies in which isolated rat liver mitochondria were suspended in solutions containing high (5–10 mM) levels of GSH and in which the total levels of GSH in the mitochondria were found to increase (25–27). That administration of GSH monoesters to mice leads to increased mitochondrial GSH levels in various tissues without comparable increase in the GSH level of the cytosol suggested that mitochondria are highly effective in transporting GSH from the cytosol (10–13).

In the present study we examined the question as to whether there is a GSH transporter in mitochondria; we have studied aspects of the kinetics of GSH uptake by isolated liver mitochondria. Studies were carried out on GSH transport into and out of the mitochondrial matrix at low and high external GSH levels and at short time intervals (e.g., 15 sec). The present findings show that mitochondrial GSH homeostasis is effected by a multicomponent transport system that includes a high-affinity component that functions at very low external GSH levels. Efflux of GSH from the matrix is affected by the external concentration of GSH and the respiratory state. The results of these studies on isolated liver mitochondria, which are in accord with previous *in vivo* studies on the incorporation of cysteine into mitochondrial and cytosolic GSH (24), indicate that there is rapid exchange of GSH among mitochondrial matrix, the intermembranous space, and the cytosol.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]GSH (44–147 Ci/mmol; 1 Ci = 37 GBq) (containing dithiothreitol) and [^{14}C]sucrose (350 mCi/mmol) were obtained from New England Nuclear. [^{35}S]GSH was mixed with carrier GSH, extracted with ethylacetate to remove dithiothreitol (28), adjusted to pH 7.0 by addition of

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Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; FCCP, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone.

2 M NaOH, and stored at −70°C under N_2; its purity (>98%) was checked by HPLC. GSSG was obtained from Boehringer Mannheim. Sodium L-glutamate, ATP, ADP, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), and other reagents were obtained from Sigma. Acivicin was supplied by L. J. Hanka (Upjohn). Ophthalmic acid (γ-L-glutamyl-α-L-aminobutyrylglycine) was obtained from Bachem. Ficoll (specific gravity, 1.068) was obtained from Pharmacia; after dialysis against glass-distilled water, it was dissolved in a solution containing 3 mM Tris·HCl, 220 mM mannitol, 70 mM sucrose, and 0.1 mM Na_2EDTA (adjusted to pH 7.2).

Methods. Mitrochondria were isolated from the livers of male rats (Sprague–Dawley; 300–500 g) after an overnight fast. The livers of ketamine/xylazine-anesthetized rats (11) were perfused with cold saline through the left ventricle, excised, minced on ice, and homogenized (10 strokes; Dounce apparatus) with 5 vol of solution A [3 mM Tris·HCl/220 mM mannitol/70 mM sucrose/0.1 mM Na_2EDTA/0.1% pig serum albumin (fatty acid-free), pH 7.2 at 4°C]. The homogenate was subjected to differential centrifugation (29), and the final mitochondrial pellet was suspended in 0.2 ml of solution A (30–40 mg of protein per ml) at 0°C. The mitochondria were used within 3 hr of preparation, during which time <15% of the GSH content was lost.

It must be emphasized that it is crucial to include EDTA in the isolation medium; in its absence we found that mitochondrial GSH is rapidly lost. Omission of EDTA may account for much lower reported (26, 30) control levels of GSH.

Assays. Initial rates of GSH uptake into the matrix were estimated in mixtures (volume, 235–244 μl) containing 5 mM Hepes buffer (pH 7.2), 220 mM mannitol, 70 mM sucrose, 0.1 mM Na_2EDTA, 0.1% pig serum albumin (fatty acid-free), 5 mM succinate, 1 mM potassium phosphate, and 0.05–10 mM (3.3 to 0.75 μCi per assay) [^{35}S]GSH. Uptake was initiated by adding (with mixing) 5 μl of the mitochondrial suspension (final concentration, 0.7–0.8 mg of protein per ml) and was terminated by centrifugation of the mitochondria through Ficoll at 10,000 × *g* for 3 min at 4°C. The mitochondrial pellet was washed twice with solution A at 4°C to remove GSH from the intermembranous space prior to determination of matrix GSH.

The efficacy of the washing procedure was verified in separate experiments in which [^{14}C]sucrose (0.2 μCi per sample; 1 Ci = 37 GBq) was added to the mitochondria. No ^{14}C was detected in the mitochondrial pellet after two washings. When mitochondria were washed for a third time, the values for the GSH levels decreased by only 4% from those found after two washes; the latter values were 20% of those found for mitochondria that were washed only once. A fourth washing led to a further loss of GSH of about 10%.

Mitochondrial pellets were suspended in 25 μl of 4.3% (wt/vol) 5-sulfosalicylic acid and frozen at −70°C. After thawing and centrifugation for 1 min at 4°C, the ^{35}S was measured in the supernatant (plus wash). After washing the residual pellet with 100 μl of 4.3% sulfosalicylic acid, the protein-bound GSH was determined by dissolving the washed pellet in 50 μl of 1.0 M NaOH (12–16 hr at 23–25°C); 30 μl of the alkaline solution was neutralized with 1 M HCl, and its radioactivity was determined. Careful study of this procedure showed that the protein-bound GSH fraction is not decreased by washing and indicated that the matrix protein-bound GSH is consistently 12–16% of the total GSH.

GSH efflux from the matrix was determined with mitochondria that were preloaded by incubation (as described above) with 10 mM [^{35}S]GSH for 2 min at 4°C. Mitochondria treated in this manner contain 10–12 nmol of GSH per mg of protein. Efflux was terminated by centrifugation through Ficoll as described above.

Protein was determined as described (31) with bovine serum albumin as the standard. Statistical analyses were performed by using a basic program (by J. C. K. Lai) for the one-way analysis of variance (ANOVA) and Tukey test for multiple comparisons. Computer curve-fitting and linear regression analyses were performed by using SIGMA PLOT (Jandel, Corte Madera, CA).

RESULTS

Uptake of GSH into the Matrix. The initial uptake of GSH into mitochondria [which contain 6–7 nmol of GSH per mg of protein (11)] in state 4 respiration was rapid and increased with increase of the external GSH concentration. Maximal uptake rates were observed within the first 20–30 sec, and equilibrium was attained within 1–2 min at external GSH levels of 0.05–10 mM at 23°C (Fig. 1 gives representative data). Studies with external GSH levels of 20–30 mM gave variable results, and equilibrium was reached at 5 min or later; such levels of GSH, which are not physiological, have been reported to cause mitochondrial damage (32, 33). Uptake of GSH at 4°C was slower than at 23°C, but the equilibrium values were higher because GSH efflux (see below) was very slow at 4°C.

The observed initial rates of GSH uptake into the matrix reflected function of a multicomponent system (Fig. 2). At low external GSH levels (0.05–1 mM), a high-affinity component was observed, with an apparent K_m value of about 60 μM and an apparent V_{max} of 0.53 nmol/min per mg of protein; this component was saturated at an external GSH level of 1–2 mM. At an external level of GSH of 1–8 mM, a low-affinity component was observed, with an apparent K_m of 5.4 mM and V_{max} of 5.9 nmol/min per mg of protein. When the external levels of GSH were about 8–10 mM or higher, uptake into the matrix seemed to occur by diffusion. [^{35}S]GSH transported into the matrix appeared to be compartmented under the conditions used; 84–88% of the ^{35}S taken up was associated with free GSH and the remainder with protein-bound GSH.

GSH Exchange-Efflux; Reuptake. The observed efflux of [^{35}S]GSH from the matrix of [^{35}S]GSH-preloaded mitochondria was apparently biphasic when examined with an external GSH level of 0.15 mM (Fig. 3). When the external level of GSH was increased, the initial rates of GSH loss from the matrix were substantially enhanced. With an external GSH level of 8 mM, about 60% of the [^{35}S]GSH initially present in the matrix disappeared from the matrix in 15 sec. It is notable, however, that after 120 sec, all of the initially effluxed [^{35}S]GSH was recovered in the matrix. If one assumes that the preloading procedure labels matrix GSH homogeneously, the initial efflux and subsequent uptake of [^{35}S]GSH suggest that there is rapid efflux of [^{35}S]GSH from the matrix and that

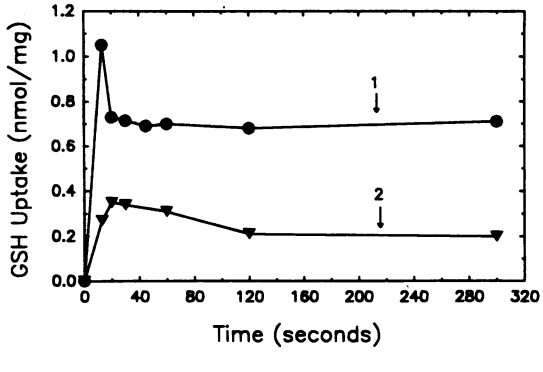

FIG. 1. Time course of GSH uptake into mitochondrial matrix at high and low external GSH concentrations. Curves: 1, 10 mM GSH; 2, 1 mM GSH.

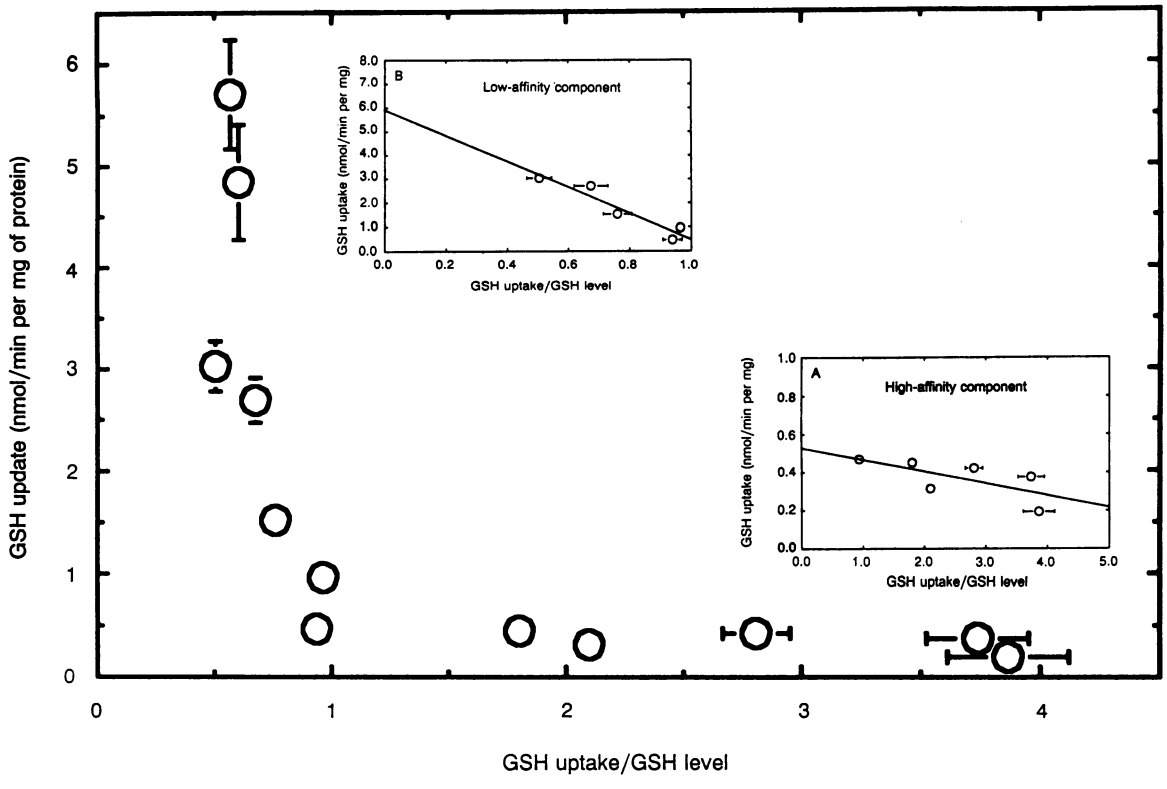

FIG. 2. GSH uptake into the matrix with various external concentrations of GSH. Uptake was determined after 15 sec. The data are shown as Eadie–Hofstee plots. (*Insets A* and *B*) Plots of the data for the high-affinity (*A*) and low-affinity (*B*) components. Error bars indicate ±SEM (n = 3–5).

the exported [^{35}S]GSH does not equilibrate with the unlabeled GSH in the medium. This lack of mixing with the extramitochondrial GSH would be expected if the exported [^{35}S]GSH were retained, under these conditions, in the intermembranous space. This phenomenon of efflux and reuptake of GSH was found with an external GSH level of 8 mM but not with one of 0.15 mM; it would appear that with an external GSH level of 8 mM, there is rapid flow of external GSH into the intermembranous space, which must facilitate very efficiently the reuptake of [^{35}S]GSH into the matrix. Thus, the findings indicate that there is exchange of GSH between the matrix and the intermembranous space, which is accelerated by increasing external GSH levels.

Initial Studies on Compounds That May Affect GSH Uptake. GSH uptake, at an external GSH level of 0.15 mM, was increased by ATP and decreased by glutamate, ophthalmic acid, FCCP, and ADP (Table 1). GSSG and acivicin [an inhibitor of γ-glutamyl transpeptidase (34, 35)] did not significantly affect initial GSH uptake. GSH uptake into the matrix at an external GSH level of 4 mM was increased by ATP and ADP and decreased by glutamate, ophthalmic acid, FCCP, and GSSG.

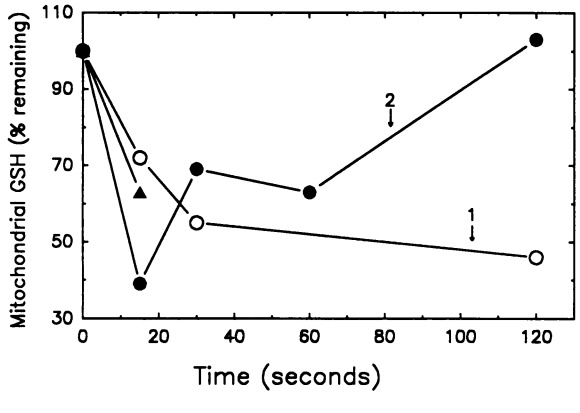

FIG. 3. GSH efflux–exchange from the matrix at various external levels of GSH. Curves: 1, 0.15 mM GSH; 2, 8 mM GSH. {When the experiment described in curve 2 was carried out in the presence of 1 mM ADP (state 3 respiration), about 45% of the [^{35}S]GSH was found in the matrix after 120 sec}. ▲, 4 mM GSH.

Table 1. Effects of various compounds on GSH uptake into mitochondria

Compound added	Uptake of GSH at two external GSH levels, pmol/min per mg of protein	
	(a) 0.15 mM	(b) 4 mM
None (control)	103	691
ADP	59	916
ATP	123	1,030
Glutamate	61	437
Ophthalmic acid	73	507
GSSG	87*	576
FCCP	73	541
Acivicin	106*	
Taurine		725*
Phenylalanine		642*

Uptake of GSH was determined at 23°C (15-sec values as described in the text) and are expressed as pmol/min per mg of protein; the values are means ± SD (<10%; n = 3–6). The levels of added compounds were ADP, 1 mM; ATP, 1 mM; L-glutamate, 0.5 (column a) and 2 (column b) mM; ophthalmic acid, 0.5 (column a) and 2 (column b) mM; glutathione disulfide (GSSG), 0.25 (column a) and 1 (column b) mM; FCCP, 40 μg/ml; acivicin, 0.5 mM; taurine, 2 mM; and phenylalanine, 2 mM.

*Not significantly different from the control (P > 0.05). The other values are significantly different from the appropriate controls (P < 0.05).

DISCUSSION

These studies elucidate the properties of mitochondria that underlie their remarkable affinity for GSH. In previous studies (25–27), uptake and accumulation of GSH by mitochondria were examined at external GSH levels of 5–10 mM, and the rates of GSH accumulation were derived from experiments carried out over a period of several minutes (26). In the present work, we estimated initial rates of GSH uptake by measuring uptake after 15 sec. Values obtained at later time points, which were lower than those found in the initial 15 sec, are probably less reflective of *in vivo* rates. The present studies indicate that the previous observations (25–27) reflect transport mediated by the low-affinity component observed here and by diffusion.

The components identified in the present studies correspond to carriers with apparent K_m values of about 60 μM and 5.4 mM. The data suggest that GSH uptake is an active, energy-dependent process; this is consistent with the observed activation by ATP and the inhibition found in the presence of FCCP. The apparent diffusional aspect of transport observed in liver mitochondria *in vitro* may occur in liver or lens *in vivo* because of the high levels of GSH in these tissues but may not be prominent in other tissues that have generally lower cytosolic GSH levels.

Although further studies are required to fully characterize the efflux of GSH from the mitochondrial matrix, it appears that efflux in the presence of low external GSH levels occurs in two phases. The initial, more rapid phase is not observed with mitochondria prepared in the absence of EDTA because, without EDTA in the isolation medium, a substantial amount of GSH is lost during preparation. Of interest, mitochondria that have been preloaded with [^{35}S]GSH discharge GSH more rapidly when external levels of GSH are increased, indicating the occurrence of exchange involving reversible transfer of GSH between matrix and the intermembranous space (Fig. 3).

The present findings strongly suggest the existence of carriers on the inner mitochondrial membrane that are accessible to GSH in the matrix and in the intermembranous space. That increasing external GSH levels accelerate the exchange of GSH across the inner mitochondrial membrane *in vitro* is in accord with several observations made *in vivo*. For example, depletion of tissue GSH by administration of BSO leads to marked decrease of cytosolic GSH without comparable decline of mitochondrial GSH (10–13, 24). In addition, administration of glutathione esters to such glutathione-depleted animals leads to a prompt and substantial increase in the level of GSH in the mitochondria without comparable increase of cytosolic GSH (10–13). These *in vivo* findings are interpretable in terms of decreased efflux of matrix GSH in the presence of low cytosolic GSH levels and the existence of GSH transporters in the inner mitochondrial membrane.

Further characterization of the transport components is required. That glutamate inhibits GSH uptake at extramitochondrial GSH concentrations of 0.15 mM and 4 mM is of interest and suggests that the putative transport proteins interact with the glutamyl portion of the GSH molecule. The possibility that GSH may be transported by the previously characterized glutamate transporters (36–39) needs to be considered, but seems unlikely. Thus, both the glutamate/hydroxyl transporter and the glutamate/aspartate antiporter do not exhibit multiple components and have K_m values of 4–6 mM (36, 37). The glutamate/aspartate antiporter is electrogenic and favors exchange of matrical aspartate with extramitochondrial glutamate (36–39); it is markedly inhibited by FCCP at much lower levels than that used in the present studies. We are unaware of published data on peptide transport by mitochondrial glutamate transporters (see refs. 36–39 and references cited therein).

Recent evidence suggests that there is an anion channel that transports anions electrophoretically across the inner mitochondrial membrane (see ref. 40 and references cited therein), and it is conceivable that GSH could be transported through this channel. However, this channel is regulated in a closed configuration and is thus not open under physiological conditions. The anion channel is a uniporter and shows very broad specificity for anions (including chloride, nitrate, phosphate, bicarbonate, sulfate, malate, succinate, fumarate, and citrate) (40). Since the present studies on GSH uptake were performed in the presence of succinate and phosphate, it is unlikely that GSH could have significantly entered the anion channel because of the presence of competing anions.

It is not surprising that ophthalmic acid inhibited uptake of GSH both at a low and at a high external concentration of GSH since this compound is a close analog of GSH. GSSG did not significantly inhibit uptake of GSH at external GSH levels of 0.15 mM, although there was an apparent inhibition when the external GSH level was 4 mM. The possibility that GSSG may be reduced to GSH in the intermembranous space needs to be considered in relation to earlier observations on GSSG transport (26, 30).

In summary, the mitochondrial GSH transport system appears to be designed in such a manner as to efficiently conserve mitochondrial GSH at the expense of cytosolic GSH. Since mitochondria continually produce reactive oxygen species and contain no catalase, they would appear to be largely, if not entirely, dependent on the GSH–GSH peroxidase system. Although one cannot exclude participation of other antioxidants, it would appear that, under normal physiological conditions, GSH is the principal functional antioxidant. GSH may function not only as substrate for GSH peroxidases but also in the maintenance of other protective compounds such as ascorbate.

It should be emphasized that the present and previous investigations have dealt with rat liver mitochondrial preparations and that it will be of importance to determine whether GSH transporters are present in mitochondria isolated from other tissues and also from other species. Although GSH is apparently essential for mitochondrial function in a number of tissues, there may well be quantitative and qualitative differences in the components of the various transport systems involved. Relationships between mitochondrial GSH and calcium metabolism have been discovered (41); thus, additional significant interrelationships, including the effects of hormones (42), need to be investigated.

We dedicate this paper to the memory of Prof. B. Sörbo, a pioneer in the study of sulfur-containing amino acids. This research was supported in part by a grant from the National Institutes of Health (2 R37 DK-12034) to A.M. J.M. acknowledges stipendary support from the Throne–Holst Foundation, Swedish Medical Society, Trygg–Hansa Research Fund, AGA.AB Medical Corporation Research Fund, Draco Medical Research Fund, and Crown Princess Margareta's Research Fund for Visual Disorders.

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