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Topical Review

Role of Membrane Transport in Metabolism and Function of Glutathione in Mammals

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Introduction

Glutathione[1] occurs in almost all living organisms, and its multifunctional properties have been attracting interest of many biochemists, physiologists, pharmacologists as well as clinical scientists. GSH[1] is a tripeptide with a structure of γ-L-glutamyl-L-cysteinylglycine, which is characterized by the γ-glutamyl peptide and the reactive thiol group. GSH is synthesized in two steps catalyzed by γ-glutamylcysteine synthetase [EC 6.3.2.2] (reaction 1) and glutathione synthetase [EC 6.3.2.3] (reaction 2), respectively.

L-glutamate + L-cysteine + ATP ⇌ γ-L-glutamyl-L-cysteine + ADP + P_i (reaction 1)

γ-L-glutamyl-L-cysteine + glycine + ATP ⇌ GSH + ADP + P_i (reaction 2)

Reaction 1 is considered rate limiting and inhibited by GSH, suggesting a physiologically significant feedback control of GSH synthesis (Meister & Tate, 1976). It is generally believed that cells are equipped with these enzymes to synthesize their own GSH for intracellular utilization. Therefore, the availability of the precursor amino acids is a regulatory factor for GSH synthesis, and it is highly likely that in mammalian cells amino acid supply from the outside of cells provides a control point in the synthesis.

The degradation of GSH is first catalyzed by γ-glutamyltransferase [EC 2.3.2.2]. This enzyme catalyzes transfer of the γ-glutamyl moiety of GSH to an acceptor. Although certain amino acids are active acceptors, the enzyme can catalyze hydrolysis of GSH; water can serve as the acceptor (McIntyre & Curthoys, 1979). γ-Glutamyltransferase is largely localized at the outer surface of membranes of certain epithelial cells (Novogrodsky, Tate & Meister, 1976). The enzyme is thus separated from its substrate, and this suggests that there is a mechanism for the movement of intracellular GSH to the place where the enzyme is present. Efflux of GSH from cells was first observed in perfused isolated liver preparations (Bartoli & Sies, 1978), in human fibroblasts in culture (Bannai & Tsukeda, 1979), and in human lymphoid cells (Griffith, Novogrodsky & Meister, 1979). The efflux is considered an important step in GSH utilization and is a property of many, perhaps most cells. GSSG[1] has also been known to be released from various cells and tissues (Sies & Akerboom, 1984). However, this efflux is often measured in response to oxidizing conditions, because under physiologic conditions GSSG is accumulated in the cells only to a very limited extent because of the action of GSSG reductase [EC 1.6.4.2]. The intracellular level of glutathione in mammalian cells is in the millimolar range and GSH is usually predominant.

Thus, two different types of membrane transport may take part in metabolism and function of glutathione. One is the transport of the precursor amino acids of glutathione, and another is the transport of glutathione itself. It seems that the former has not drawn much attention, so in this paper both will be described. Some recent reviews (Meister &

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[1] "Glutathione" is used to indicate material containing both reduced and oxidized forms. GSH or GSSG is used in the reduced or oxidized form of glutathione, respectively.

Table. Transport systems for precursor amino acids of glutathione

System	Occurrence	Na^+ dependence	Preferential substrate		Additional comments
			Precursor amino acid of glutathione	General	
ASC	Ubiquitous	Yes	Cysteine Serine Glutamine[a]	Neutral amino acids with small side chains, especially those having –SH or –OH group	Subject to *trans*-stimulation
x_c^-	Characterized so far in fibroblasts and hepatic cells	No	Cystine Glutamate	Anionic amino acids; aspartate largely excluded	Subject to adaptive control; enhanced by electrophilic agents
X_{AG}^-	Probably ubiquitous	Yes	Glutamate	Anionic amino acids; glutamate and aspartate	Not detectable in human erythrocytes
Gly	Widespread	Yes	Glycine	Glycine and sarcosine	
A	Ubiquitous	Yes	Glycine Serine Cysteine[a] Glutamine	Neutral amino acids with small, unbranched side chains	Subject to adaptive control; highly enhanced upon amino acid starvation
L	Ubiquitous	No	Methionine	Neutral amino acids with large, branched and apolar side chains	Methionine sulfur is utilized for cysteine synthesis mainly in liver
C	Sheep erythrocytes	No	Cysteine	Neutral amino acids with small side chains	Particularly high affinity for cysteine
N	Hepatocytes	Yes	Glutamine	Amino acid amides and histidine	

[a] In hepatocytes this amino acid is not accepted by the system.

Anderson, 1983; Orrenius, Ormstad, Thor & Jewell, 1983; Meister, 1984) will be referred to for the general knowledge on the metabolism and function of glutathione.

Transport of Precursor Amino Acids of Glutathione

As shown above, the reaction catalyzed by γ-glutamylcysteine synthetase is thought to be rate limiting in GSH synthesis. This enzyme has been purified from several sources and catalytic properties have been examined. The enzyme from rat kidney exhibits apparent K_m values for glutamate and cysteine of 1.8 and 0.3 mM, respectively (Richman & Meister, 1975). Although the intracellular concentration of cysteine has not been extensively studied, the available data suggest that it is regulated at a lower level than the apparent K_m value of γ-glutamylcysteine synthetase for cysteine. Therefore it is probable that the rate of GSH synthesis and, consequently, the GSH level is significantly influenced by the availability of intracellular cysteine (Tateishi et al., 1974). Evidence to show the correlation between the cellular levels of cysteine and GSH has been provided in rat liver (Demaster et al., 1984).

One of the important factors that regulate the intracellular level of cysteine is its membrane transport. The basic concepts on the transport of amino acids across mammalian cell membranes have been developed primarily by Christensen and his coworkers (Christensen, 1984). Uptake and release of amino acids is mediated by a number of transport systems which manifest broad and overlapping substrate specificity.

Cysteine is transported mainly by System ASC in a variety of cells (Kilberg, Handlogten & Christensen, 1981; Franchi-Gazzola, Gazzola, Dall'Asta & Guidotti, 1982; Bannai, 1984*a*). This system is Na^+-dependent and especially reactive with neutral amino acids having side chain of short to intermediate length. Although the system is highly concentrative, it mediates both inward and outward flows of the amino acids. The system is subject to *trans*-stimulation (Gazzola, Dall'Asta & Guidotti, 1980); a System ASC amino acid (an amino acid substrate for System ASC) at one side of the membrane stimulates the passage of another System ASC amino acid present at the other side of the membrane. System ASC seems to be constitutive, and in many

cells it does not respond to regulatory factors known to alter amino acid transport by other systems. It should be noted that the intracellular level of cysteine and probably of GSH is directly influenced by the extracellular levels of not only cysteine but also other System ASC amino acids (Bannai & Ishii, 1982). The extracellular cysteine acts to raise the intracellular level of cysteine. In contrast, the extracellular System ASC amino acids other than cysteine inhibit competitively the influx of cysteine (*cis*-inhibition) and stimulate the efflux of cysteine (*trans*-stimulation). Thus, System ASC amino acids such as alanine, serine and glutamine present outside of cells function to lower the intracellular level of cysteine.

Another important factor that regulates the intracellular level of cysteine is transport of cystine. Usually plasma concentration of cystine is much higher than that of cysteine (Saetre & Rabenstein, 1978; Bannai, 1984*a*), and cystine is readily converted to cysteine in cells. In the case of cells in culture, the situation is serious because cysteine in the culture medium is easily autoxidized to cystine. Furthermore, most of the cultured cells have only a limited, if any, ability to synthesize cysteine via the cystathionine pathway (Eagle, Washington & Friedman, 1966). Therefore, unless the cultured cells take up cystine and reduce it to cysteine, the intra- and extracellular levels of cysteine would be falling. Actually mouse ascites lymphoma L1210 cells are deficient in cystine transport activity, and therefore, when cultured in vitro, the intracellular levels of cysteine and GSH rapidly decrease and the cells fail to survive (Ishii, Hishinuma, Bannai & Sugita, 1981).

Transport of cystine is mediated by a system different from the well-known neutral amino acid transport systems, Systems A, ASC, and L. According to Christensen (1984), amino acid transport is an ion transport; Systems A, ASC, and L ordinarily mediate the transport of the zwitterionic form (or dipolar ion) of amino acids. Cystine is not a dipolar ion, but within the physiologic pH range it occurs as a tetrapolar (neutral as a whole) ion in the main and as a tripolar (anionic as a whole) ion for the rest (Bannai & Kitamura, 1981; Makowske & Christensen, 1982). Cystine is transported as the anionic form in human fibroblasts (Bannai & Kitamura, 1980, 1981) and in hepatic cells (Makowske & Christensen, 1982; Takada & Bannai, 1984). The system that mediates the transport of anionic cystine is designated as System x_c^-. This system is Na^+-independent and, among amino acids routinely present in the intra- or extracellular fluid space, only cystine and glutamate are reactive substrates. Recently evidence to show that System x_c^- mediates an exchange of cystine and glutamate has been obtained (S. Bannai, *submitted*). The physiologically significant flows of amino acids via System x_c^- is thought to be an entry of cystine accompanied by an exodus of glutamate, because cells are usually rich in glutamate, but not in cystine. It is interesting to think of a likeness between substrates of System x_c^- and of γ-glutamylcysteine synthetase. Cystine, after entering cells in exchange for glutamate, is reduced to cysteine, which then reacts with glutamate to form γ-glutamylcysteine. Apparently intracellular glutamate has a function to draw in extracellular cystine and to maintain an adequate balance between cysteine and glutamate in the cells.

One of the important functions of GSH is to protect cells from an endogenous or exogenous electrophilic attack by trapping the electrophilic agent. Thus electrophilic compounds are believed to deplete intracellular GSH. This is the case when the compounds are present in excess. However, when cultured human fibroblasts are exposed to such electrophilic agent as diethylmaleate at relatively low concentrations, GSH levels, though initially decreased a little, do increase and become twice the normal levels (Bannai, 1984*b*). Under these conditions it has been found that the activity of System x_c^- is greatly induced. The increase in GSH levels is ascribable to the enhanced influx of cystine via System x_c^-, because the GSH levels do not increase if the influx of cystine through System x_c^- is competitively inhibited by glutamate or homocysteate (both are System x_c^- amino acids). The mechanism of the induction of System x_c^- by the electrophilic compounds is unclear at present, but the phenomenon may reveal a protective function of the cells against an electrophilic attack. That is, when the cells are exposed to the electrophilic attack, the activity of System x_c^- is induced and the influx of cystine is enhanced, resulting in a stimulation of GSH synthesis. GSH thus accumulated may serve for a detoxication of the electrophilic agent. The similar results are obtained in isolated rat hepatocytes (Bannai, Takada & Tateishi, 1984). It has been thought that hepatocytes hardly take up cystine (Orrenius et al., 1983). However, the activity of System x_c^- is detectable upon culturing hepatocytes in the presence of some hormones (Takada & Bannai, 1984), and it is strongly induced when the cells are treated with an electrophilic agent such as sulfobromophthalein. GSH levels of isolated hepatocytes rapidly decrease after addition of sulfobromophthalein, but the levels are soon restored. This restoration is consequent on the enhanced activity of System x_c^-.

Hepatocytes, unlike other cells, actively synthesize cysteine from methionine and serine via the cystathionine pathway and utilize it for GSH synthesis. Transport of methionine into hepatocytes is largely mediated by Na^+-independent System L (Kilberg et al., 1981). The transport activity for methionine or serine may influence the GSH synthesis, though there is no evidence for this view at present.

Transport of amino acids across the erythrocyte membrane is required for normal erythrocyte function and viability. Mature mammalian erythrocytes possess a variety of amino acid transport systems (Young, Jones & Ellory, 1980). These cells do not synthesize protein, and a principal reason for the cells to transport amino acids is to provide the substrates cysteine, glutamate and glycine for GSH synthesis. Deficiency of GSH may associate with the occurrence of hemolytic anemia. Adequate membrane transport of cysteine is necessary for the erythrocytes, because they are hardly permeable to cystine and have no detectable activity of cystathionine pathway. Human erythrocytes transport cysteine by a Na^+-dependent, stereospecific uptake mechanism (Young et al., 1980). This system has a uniquely high affinity for cysteine and is equivalent to the System ASC in other cell types. At physiologic concentrations of cysteine in plasma, the system accounts for major part of the cysteine uptake into the cell. Human erythrocytes have also a low-affinity, Na^+-independent uptake component for cysteine flux, which is similar to System L. An interesting feature of erythrocyte amino acid transport is that the distribution of amino acid transporters in the erythrocytes of different species is highly variable. Thus, sheep erythrocytes lack Systems ASC and L. Instead, they possess an additional system (System C) responsible for the transport of cysteine (Young, Ellory & Tucker, 1976). The substrate specificity of the human System ASC and sheep System C are similar, but System C does not require Na^+. The sheep is known to exhibit inherited GSH deficiency. One type of GSH deficiency (approximately 30% of normal levels), which occurs most commonly in Finnish Landrace sheep (the breed frequency approximately 20%), results from an impaired membrane transport for cysteine; System C is defective in the low-GSH sheep erythrocytes (Young et al., 1976). It is noted that the inherited transport lesion is not expressed in reticulocytes, liver, kidney and other tissues. They have normal GSH contents, suggesting that System C is erythrocyte-specific (Young & Tucker, 1983).

Glutamate and glycine are also precursors of GSH. However, evidence to show that the transport of glutamate or glycine influences GSH biosynthesis is limited. These amino acids are synthesized via several metabolic pathways; glutamate is easily formed from glutamine by deamination, and glycine is formed from serine through the action of serine hydroxymethyltransferase [EC 2.1.2.1]. In human fibroblasts, intracellular glutamate or glycine is not depleted even if the cells are cultured in media lacking in glutamate or glycine, respectively (Bannai & Kitamura, 1982). Depletion of glutamate has been observed in cells cultured in glutamine-free medium (glutamate concentration is usually low in the culture media routinely used), and depletion of glycine has been found in cells cultured in glycine-and-serine-free medium. Under these conditions intracellular levels of GSH decrease significantly. Glutamine and serine are, therefore, used as sources of glutamate and glycine, respectively, in the fibroblasts. Since glutamine and serine are mainly transported by System ASC in this and some other types of cell, System ASC appears to play a large part in the supply of all three precursors for GSH synthesis.

The liver GSH content decreases on starvation and returns to normal on refeeding. It has been shown that the availability of glutamate on refeeding is favorable for GSH synthesis (Tateishi et al., 1974). However, in isolated hepatocytes exclusion of glutamate from the medium does not affect the rate of GSH synthesis (Thor, Moldéus & Orrenius, 1979). Presumably, glutamine in the medium is the chief source for cellular glutamate. In hepatocytes, glutamine is transported by a unique, Na^+-dependent system, System N (Kilberg, Handlogten & Christensen, 1980), whereas glutamate is transported by Na^+-dependent transport systems for anionic amino acids (Gazzola et al., 1981; Kilberg, 1982). The plasma concentration of glutamine is usually higher than that of glutamate, suggesting a major role of System N in the supply of glutamate in hepatocytes.

The erythrocytes of most mammalian species are essentially impermeable to glutamate, but glutamine enters via a Na^+-dependent system similar to System ASC (Ellory, Preston, Osotimehin & Young, 1983). A more likely route for the precursor of GSH is therefore transport of glutamine, which will be deaminated to glutamate intracellularly. Recently assimilation of α-glutamylpeptides by human erythrocytes has been proposed (King & Kuchel, 1985). Experimentally, the authors used proton spin-echo NMR spectroscopy to follow peptide hydrolysis, because peptide spectra are different from those of the free amino acids. The results suggested that α-glutamylpeptides such as α-glutamylalanine entered the red cell and were rapidly hydrolyzed there. It has been postulated that this route is a

possible means of glutamate supply for GSH synthesis in human erythrocytes. Although the results are of interest, it is clear that the transport and hydrolysis of α-glutamylpeptides should be examined by other methods with the use of the radiolabeled peptides.

Dog erythrocytes possess a high affinity, Na^+-dependent transport system for glutamate and aspartate (System X^-_{AG}). The ability of these cells to transport glutamate permits the use of extracellular glutamate for intracellular GSH biosynthesis. A dog family with hereditary high concentrations of GSH and some amino acids, such as glutamate and aspartate, in the erythrocytes has been found (Maede, Kasai & Taniguchi, 1982; Inaba & Maede, 1984). These erythrocytes exhibit (Na^+, K^+)-ATPase activity and contain high K^+ and low Na^+ concentrations, whereas normal dog erythrocytes lack the enzyme and contain low K^+ and high Na^+ concentrations. It has been concluded that a Na^+-concentration gradient induced by (Na^+, K^+)-ATPase causes the increased transport activity of System X^-_{AG} and consequently glutamate and aspartate are accumulated. The increased level of GSH (about six times the normal concentrations) is accounted for by the fact that the feedback inhibition of γ-glutamylcysteine synthetase by GSH is relieved by the very high level of glutamate (about 90 times the normal levels).

Glycine transport is usually mediated by several carrier systems. System Gly is a Na^+-dependent, high affinity system, selective for glycine and sarcosine. This system is considered major route for glycine entry into cells of many types at physiologic concentrations of glycine in the extracellular fluid (Christensen & Handlogten, 1981). Other systems, such as System A, take some part in the glycine transport in liver cells (Christensen & Handlogten, 1981), and the band 3 anion-exchange system partly mediates the glycine flux in human erythrocytes (Ellory, Jones & Young, 1981). It is possible that the membrane transport of glycine regulates GSH synthesis, but evidence to show that is not known as yet.

Transport of Glutathione

The efflux of GSH has been observed in a variety of cells and organs (Meister & Anderson, 1983). Since the enzymes that catabolize GSH are localized on the external surface of certain cells, the efflux constitutes the initial step in the degradation of GSH. It is noted that the efflux occurs independently of the subsequent enzymatic degradation. Thus, cells hitherto known to have a rapid turnover rate of GSH usually exhibit rapid export of GSH.

Hepatic GSH is translocated from hepatocytes into plasma and bile. Lauterburg, Adams & Mitchell (1984) have made quantitative observations on glutathione metabolism in the liver of intact animals. The efflux of GSH into blood and the excretion of GSH into bile in normally fed rats have amounted to 12.4 and 3.4 nmol per min per g liver, respectively. Total efflux has almost entirely accounted for the turnover of liver GSH in the basal state. The efflux of GSH from liver contributes over 90% of total glutathione inflow in the circulation. Thus, although the finding does not exclude the possibility that other organs may contribute GSH to plasma, the liver is the major source of plasma GSH.

A system of GSH transport across hepatocyte sinusoidal plasma membrane vesicles has been described (Inoue, Kinne, Tran & Arias, 1984). The membrane vesicles exhibit Na^+-independent transport of GSH into an osmotically active intravesicular space. Two transport systems with high and low affinity have been postulated. GSH transport via the latter system is markedly inhibited by GSSG or some glutathione conjugates, suggesting that these glutathione derivatives interact preferentially with the low-affinity transport system for GSH. A controversial point in this study is about the orientation of the sinusoidal membrane vesicle preparations used. These authors have suggested that the vesicles are mostly oriented right side out. It has been known that hepatic GSH transport occurs unidirectionally from the cell to the extracellular space. Therefore, an efflux of GSH from the vesicles is intrinsic and should be measured. Properties of the transporter across the vesicle membrane are not necessarily symmetrical. A GSH transport system in hepatocyte canalicular membrane vesicles has also been described (Inoue, Kinne, Tran & Arias, 1983). GSH uptake occurs into an intravesicular space of the membrane. This transport is Na^+-independent and saturable with respect to GSH concentration. The rate of GSH uptake is enhanced by valinomycin-induced K^+-diffusion potential. Therefore, GSH uptake into the vesicles appears to be mediated by an artificially imposed membrane potential difference, because these vesicles are oriented exclusively right side out. Under physiologic conditions GSH is unidirectionally secreted from hepatocytes into bile. This directionality might be explained on the basis that the direction of the transport is controlled by an electrochemical potential difference. Isolated plasma membrane vesicles may provide a useful preparation for the study of membrane process, independently from cellular metabolic events and with good control of the composition of the solutions at both the intra- and extra-

cellular sides of the plasma membrane. However, vesicles might undergo changes in transport properties and regulatory functions during their preparation. Conclusions with isolated vesicles therefore need to be confirmed for their applicability in intact cells. Further studies seem to be required to determine whether the reported systems in hepatic sinusoidal and canalicular membrane vesicles function in vivo in a similar manner as described.

The primary organ for clearance of circulating GSH is the kidney (McIntyre & Curthoys, 1980; Orrenius et al., 1983). GSH filtered by glomeruli is degraded to its constituent amino acids by the action of γ-glutamyltransferase and peptidases localized on the luminal surface of the brush border membrane of proximal tubules. It has been known that the amount of GSH extracted by the kidney exceeds the amount which could be accounted for by glomerular filtration. This imbalance leads to the proposal that basolateral uptake of nonfiltered, intact GSH occurs in the kidney (Rankin & Curthoys, 1982). However, since γ-glutamyltransferase is also associated with the basolateral membrane portions of the renal proximal tubule, the disappearance of GSH from the basolateral circulation may be due to catabolism of GSH. For this reason uptake of intact GSH by the kidney has been studied under conditions where degradation of GSH is inhibited. A transport system which mediates influx of intact GSH across renal basolateral membrane has been described (Lash & Jones, 1984). GSH transport in basolateral membrane vesicles from rat kidney is Na^+-dependent and electrogenic. GSH is cotransported with Na^+ at the coupling ratio of at least $2Na^+$/GSH. The system seems to have a sufficient driving force to allow uptake of GSH into the cells against the usual concentration gradient of GSH. Analysis of vesicle content has confirmed that the measured uptake of GSH represents transport of intact GSH rather than of its degradation products. Apparently the system is a general transport system for γ-glutamyl compounds, because γ-glutamyl amino acids interact with the GSH transport, and uptake of γ-glutamylglutamate with properties similar to GSH uptake also occurs in these membranes. In vivo experiments provide evidence for uptake of γ-glutamyl compounds in the kidney (Anderson & Meister, 1983). However, under physiologic conditions there is extensive degradation of GSH by the action of basolateral enzymes, and net basolateral transport of intact GSH is hardly detected (Abbott, Bridges & Meister, 1984). Our present understanding is that there is a unidirectional efflux of GSH from a variety of cells and tissues and that, although only a few published results demonstrate the influx of GSH (Lash & Jones, 1984; Linder, Burlet & Sudaka, 1984), some cells might take up intact GSH.

Although the kidney is the primary organ for clearance of plasma glutathione, its function with regard to glutathione is not solely to use it. The kidney synthesizes and secrets GSH actively. Thus in the kidney GSH is secreted into the tubular lumen and degraded there. The amino acids thus formed is reabsorbed and used for GSH synthesis (an intra-renal circulation). A similar circulation is observed in the liver. Glutathione-degrading enzymes are localized on the luminal surface of bile canaliculi (Inoue et al., 1984*a*). Therefore GSH secreted into the bile is partially degraded and the constituent amino acids may be transported back into the liver (an intra-hepatic circulation).

What is the physiological significance of the extracellular metabolism of glutathione? The first suggestion is that the hepato-renal transport of GSH functions to supply cysteine steadily to other tissues. This proposal is based on a view that liver GSH serves as a reservoir of cysteine (Tateishi et al., 1977). The intracellular concentrations of GSH far exceed those of cysteine, which is rapidly utilized for protein synthesis or catabolized. The function of GSH as a storage and transport form of cysteine has a distinct advantage. The sulfhydryl group of GSH is more stable than that of cysteine, and, probably due to this difference, GSH even at relatively high concentrations in cells is far less toxic than cysteine. The second suggestion is that extracellular GSH functions as a reductant that protects membranes from oxidative stress. There is good evidence that GSH has an important function of destroying reactive oxygen intermediates and free radicals which are formed physiologically. The rapid oxidation of GSH secreted in bile to GSSG indicates that the bile canalicular and ductile membranes are exposed to strong oxidative stresses. In addition, the plasma GSH may function as a substrate of glutathione peroxidase present in plasma (Hill & Burk, 1983).

Concluding Remarks

This paper summarizes some recent studies on the membrane transport in relation to the metabolism and function of glutathione. Although we do not know much about the physiological importance of membrane transport with respect to the general amino acid metabolism, it now appears obvious that the membranal events are indispensable to the metabolism of glutathione. The molecular aspects of transport of amino acids or GSH by mammalian cells is an area just starting to develop. Isolation of the membrane components involved in the transport process, mechanism of energization, and regulation of the transport remain to be challenged. Bet-

ter understanding of these problems will serve to illustrate a key role of the membrane transport in not only the metabolism of glutathione but also many other aspects of amino acid metabolism.

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