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Glutathione-mediated transport across intestinal brush-border membranes

Maria Teresa Vincenzini, Fabio Favilli and Teresa Iantomasi

Institute of Biochemistry, University of Florence, Florence (Italy)

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Glutathione transport was studied in brush-border membrane vesicles of rabbit small intestine in which γ-glutamyl transpeptidase (EC 2.3.2.2) had been inactivated by a specific affinity-labeling reagent, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT_{125}). Transport of intact [*glycine*-2-^{3}H]GSH occurred into an osmotically active intravesicular space of AT_{125}-treated membranes. The 0.1 M NaSCN gradient (Na^+ inside > Na^+ outside) in the transport medium could be replaced with KSCN or NaCl without affecting transport activity. The initial rate of GSH transport followed Michaelis-Menten saturation kinetics (K_m = 17 μM). The results suggest that, in these membranes, there was an Na^+-independent mediated transport for intact GSH with marked specificity and affinity. In fact glycine, glutamic acid and cysteine did not decrease GSH uptake, as was also true for glycylglycine and glycylglycylglycine; only γ-glutamylcysteinylglycyl ester, a derivative of GSH, partially inhibited GSH transport.

Introduction

Glutathione, L-γ-glutamyl-L-cysteinylglycine, is present in most cells in high concentrations (0.1–10 mM). GSH functions in metabolism, in transport and in mechanisms that protect cells and membranes against free radicals and toxic compounds of endogenous and exogenous origin [1,2]. The GSH transport mechanism across cell membranes and especially the uptake of intact GSH into cells are not well known. However, GSH transfer among organs has been demonstrated and there is considerable evidence that many cells export GSH [1,2]. Recent studies [3–7] have revealed that many tissues have secretory transport systems for GSH and its derivatives. Only in renal and in intestinal basolateral membranes has an Na^+-dependent active transport for GSH been identified, which may translocate GSH from plasma into cells [8,9]. At the intestinal level the data are scarce and contradictory [10,11]. At present, it would appear that the main mechanism of GSH uptake into cells involves extracellular degradation of GSH by membrane-bound γ-glutamyl transpeptidase and dipeptidase activities, followed by uptake of the resulting products and intracellular resynthesis of GSH in ATP-requiring steps [12]. Referrring to this problem, Meister [13] states that "one cannot exclude without appropriate experimental study the possibility that intact GSH is transported into some cells".

The present study was undertaken to investigate whether GSH can really be transported intact across small intestinal membranes and whether the GSH transport system resembles those of other di- and tripeptides. There is evidence that a number of small peptides are indeed transported as

Abbreviations: GSH, glutathione; AT_{125}, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: M.T. Vincenzini, Institute of Biochemistry, Viale Morgani 50, I-50134 Firenze, Italy.

such across the intestinal brush-border membrane, either by an Na^+-dependent or an Na^+-independent transport mechanism [14–19]. It was prompted in part by the suggested therapeutic roles of GSH and its derivatives as radioprotective agents and as compounds that can prevent and eliminate the toxicity resulting from the administration of chemotherapeutics and from the process of cellular ageing [9,20–22]. The transport of intact GSH was carried out in rabbit intestinal brush-border membrane vesicles whose γ-glutamyl transpeptidase (EC 2.3.2.2) activity was inactivated by a specific affinity-labeling agent, AT_{125} [23]. This experimental model enabled us to examine membrane GSH transport dissociated from the complications of cell metabolism and to define the role that Na^+ and K^+ play in the GSH uptake. Moreover, the use of AT_{125} allowed us to study the transport of intact GSH. In fact, γ-glutamyl transpeptidase is the only known enzyme which hydrolyzes the γ-glutamyl linkage of GSH and is located on the outer surface of the intestinal membrane [24].

Materials and Methods

Materials

[*Glycine*-2,^{3}H]GSH (spec. act. 20 Ci/mmol) and [U-^{14}C]glycine were obtained from New England Nuclear (Boston, MA), D-[U-^{14}C]glucose and L-[1-^{14}C]glucose from Amersham International (U.K.) At^{125} was a gift from the Upjohn Company (Kalamazoo, MI). Glutathione monoethyl ester was obtained by the procedure of Bergman and Zervas [25]. Nitrocellulose filters (0.45 μm pore size) came from Sartorius (Göttingen, F.R.G.). The dye-reagent concentrate for the determination of protein was obtained from Bio-Rad. All other chemicals used were reagent grade and were obtained from commercial sources.

Preparation of brush-border membrane vesicles

Brush-border vesicles were prepared from frozen rabbit small intestine (jejunal and ileal segments) by the calcium method, as first described by Schmitz et al. [26] and modified by Kessler et al. [27]. Following the last centrifugation, the membranes were suspended in a medium containing 300 mM mannitol, 0.1 mM $MgSO_4$ and 10 mM Tris-Hepes (pH 7). The protein concentration was about 15 mg/ml as determined by the method of Bredford [28]. The following enzymes were assayed as markers for the brush-border membrane: alkaline phosphatase, leucine aminopeptidase, γ-glutamyl transpeptidase and sucrase. Specific activities of these enzymes were enriched 16-, 10-, 8-, 11-fold, respectively, compared to that of intestinal crude homogenate. The activity of lactate dehydrogenase, a cytosolic enzyme, was reduced 70-fold and that of Na^+/K^+-ATPase, a marker enzyme of the basolateral membrane, was absent in the vesicles. Moreover, microscopic examination revealed predominantly unilamellar membrane vesicles.

Inactivation of γ-glutamyl transpeptidase by AT_{125}

The membrane vesicles were incubated at 30 °C with or without 0.5 mM AT_{125}. At designated times after incubation, aliquots of 2–10 μl were withdrawn and the remaining peptidase activity was assayed by the method of Persijn et al. [29]. Specific activity of the γ-glutamyl transpeptidase in untreated vesicles (without AT_{125}) was 13.6 unit/mg protein at 30 °C and pH 8.5 in the presence of 100 mM glycylglycine as substrate. The transpeptidase activity of untreated vesicles remained constant throughout the incubation period.

Transport measurements

The uptake of radiolabeled substrates into intestinal brush-border membrane vesicles was performed by a rapid filtration method as described by Hopfer et al. [30]. Deaerated solutions under nitrogen were used for transport studies. In a final volume of 30 μl the transport media contained 100 mM mannitol, 10 mM Hepes-Tris (pH 7), varying concentrations of the radioactive and unlabeled substrates, 0.1 M NaSCN or KSCN or NaCl or varying concentrations of mannitol to adjust medium osmolality.

Transport was started by adding untreated or AT_{125}-treated membrane vesicles (150–200 μg protein). When AT_{125}-treated vesicles were used in the transport media, the final concentration of this reagent was 0.16 mM. After suitable incubation times (from 5 s to 60 min), transport was terminated by the addition of 3 ml ice-cold 0.15 M

NaCl buffered with 1 mM Hepes-Tris (pH 7) 'stop solution'. The mixture was then quickly filtered through a nitrocellulose filter and washed with 3 + 3 ml 'stop solution.'. Vesicle-associated radioactivity on the filters was determined in a liquid scintillation counter. All values were corrected for radioactivity found on the filters in the absence of membrane vesicles. Washing membrane vesicles with a hypotonic solution eliminates most of the intravesicularly transported free-form ligands [31]. Thus, after equilibrium uptake, the material inside the vesicles on the filters was eluted with water and concentrated by lyophilization to a small volume. In this concentrated solution, GSH was measured by the glutathione reductase method using unlabeled GSH (10–200 ng/ml of assay mixture) as the standard [32]. This enzymic analysis revealed that about 90% of the radioactivity associated with AT_{125}-treated vesicles was due to intact GSH, as also reported by Inoue et al. [7] in renal brush-border membrane vesicles.

There were small variations in transport activity with different membrane preparations (the results agreed to within 10%); however all experiments were repeated at least three times, each experiment always being tripicated. Further experimental conditions are described in the figure legends.

Results

Inactivation of vesicle-bound γ-glutamyl transpeptidase by AT_{125}

To determine whether intestinal brush-border membranes posses a specific mediated transport for intact GSH, it is essential to prepare vesicles which virtually lack γ-glutamyl transpeptidase activity [6]. Fig. 1 shows the effect of AT_{125}, an affinity-labeling reagent for glutamine amidotransferases, on vesicles-bound transpeptidase activity as a function of time. After 20 min of incubation with 0.5 mM AT_{125} at 30 °C the transpeptidase activity was completely inactivated.

AT_{125} effect on membrane transport systems

Since AT_{125} alkylates various membrane proteins [33], changes in the transport of metabolites or membrane permeability of AT_{125}-treated vesicles may have occurred. To test this possibility, active transport and facilitated diffusion of D-glucose, L-glycine and diffusional flux of L-glucose were compared for the untreated and AT_{125}-treated vesicles.

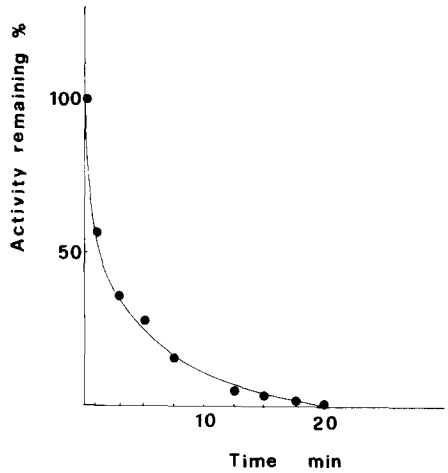

Fig. 1. Inactivation of vesicle-bound γ-glutamyl transpeptidase by AT_{125}. Membrane vesicles (15 mg protein/ml) were incubated with and without 0.5 mM AT_{125} at 30 °C. The reaction was started by adding to the reaction mixture aliquots of AT_{125}-treated or untreated (= control) vesicles at various times. The percentage of remaining activity was calculated with respect to the controls. See Materials and Methods for details.

Fig. 2 shows that the time-course of the Na^+-dependent and Na^+-independent glycine uptake into untreated vesicles was similar to that obtained with AT_{125}-treated vesicles. These results and those for D-glucose and L-glucose (data not shown) indicate that AT_{125} did not change either the transport systems or membrane permeability.

Time-course and sodium dependence of intact GSH uptake

GSH was taken up into the brush-border membrane vesicles in a time-dependent manner (Fig. 3). Since the membranes virtually lacked γ-glutamyl transpeptidase activity, intact GSH fully accounted for vesicle-associated radioactivity. There was no evidence of an overshoot in the presence of a Na^+ gradient (Na^+ inside > Na^+ outside) and replacement of NaSCN with KSCN or NaCl in the incubation medium had no marked effect on the time-course of GSH uptake. Only without an ion gradient (mannitol alone) was GSH uptake slightly lower at various incubation times. The equilibrium values were reached after 30 min

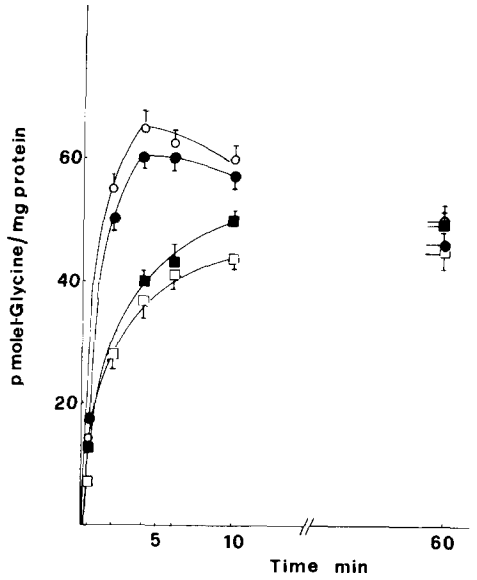

Fig. 2. Time-course of Na^+-dependent and -independent L-glycine uptake into AT_{125}-treated and untreated membrane vesicles. Transport was carried out at room temperature with AT_{125}-treated or untreated membrane vesicles (30 μl) in uptake media containing: 100 mM mannitol, 10 mM Hepes-Tris (pH 7), 50 μM [^{14}C]glycine and 100 mM NaSCN (○) (●) or 100 mM KSCN (□), (■). ○, □, membrane vesicles were preincubated with 0.5 mM AT_{125} at 30 °C for 45 min. ■, ●, membrane vesicles were preincubated without AT_{125} at 30 °C for 45 min.

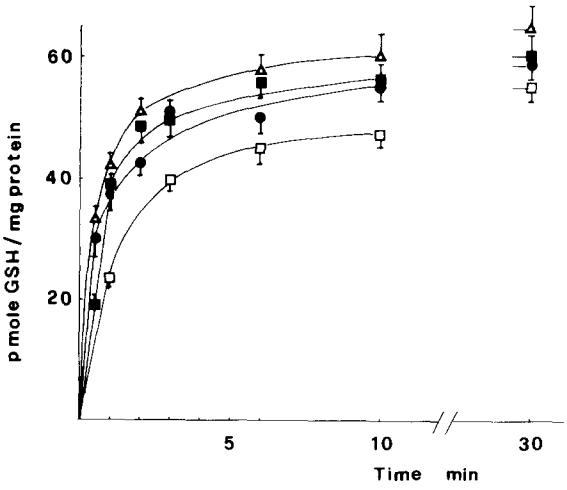

Fig. 3. Time-course of GSH uptake into AT_{125}-treated membrane vesicles. Transport was carried out at room temperature with membrane vesicles (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7), 5 μM [^{3}H]GSH, 45 μM unlabeled GSH, and 100 mM NaSCN (●), 100 mM NaCl (△) 100 mM KSCN (■) or 200 mM mannitol (□).

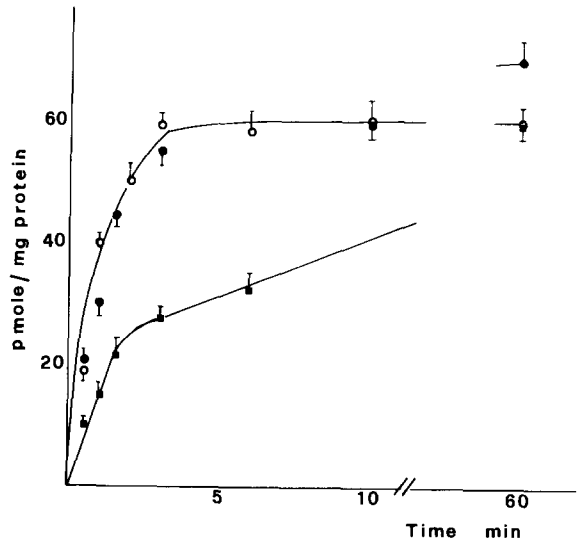

Fig. 4. Time-course of GSH, D-glucose, L-glucose into AT_{125}-treated membrane vesicles. Transport was carried out at room temperature with membrane vesicles (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7), 100 mM KSCN and 5 μM [^{3}H]GSH and 45 μM unlabeled GSH (○), 50 μM D-[^{14}C]glucose (●) or 50 μM L-[^{14}C]glucose (■).

of incubation and were approximately the same. The time-course of GSH uptake and of the Na^+-independent D-glucose uptake at the same concentration were identical (Fig. 4). Both reached the greatest uptake values during the first minutes of incubation, unlike L-glucose, which showed a typical diffusional behavior [27]. The half-equilibration time, $t_{1/2}$, i.e., the time required to reach half the equilibrium level, is about 30–40 s for GSH and D-glucose and 4–5 min for L-glucose.

GSH binding

Another problem in studying solute transport into vesicles is to distinguish between the binding to membranes and their transport. GSH can form mixed disulfides with many thiol compounds, including cysteinyl moieties of membrane proteins [34]. Moreover, GSH at pH 7 is negatively charged and can thus form ionic bonds with the membrane surface. To test whether vesicle-associated radioactivity really reflects transmembrane movement of GSH, the effect of medium osmolarity on the equilibrium GSH uptake was observed with and without an Na^+ gradient. The data shown in Fig. 5 indicate that with a decreasing ratio of osmolar-

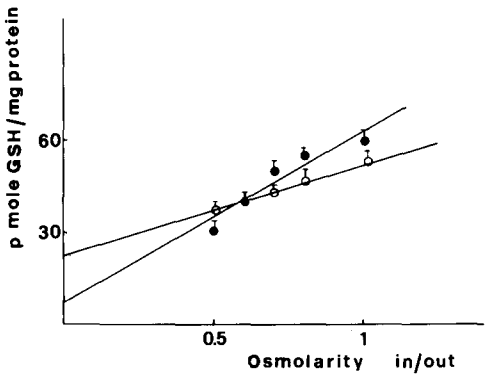

Fig. 5. Effect of medium osmolarity on the equilibrated GSH content of AT_{125}-treated membrane vesicles. Transport was carried out at room temperature with membrane vesicles (30 μl) in uptake media containing 10 mM Hepes-Tris (pH 7), 5 μM [^{3}H]GSH, 45 μM unlabeled GSH, varying concentrations of mannitol (300–700 mM) in the absence (○) and in the presence (●) of 100 mM NaSCN. GSH uptake values were determined after 60 min of incubation. On the abscissa are reported the ratios of osmolarity inside/osmolarity outside the vesicles. The y-intercept, which is greater than 0, shows that binding of GSH to the vesicles is 6% in the presence of Na^+ and 23% in its absence.

ity inside/osmolarity outside, the vesicles contained less GSH. Equilibrium values obtained from extrapolation to a zero intercept (infinite osmolarity outside the vesicles) show that the GSH binding is 6% of the total vesicle-associated GSH in the presence of the Na^+, while in its absence the binding is about 23%. Therefore the vesicle-associated radioactivity in the presence of Na^+ represents GSH transmembrane transport into an osmotically active space rather than binding to the membrane.

Concentration dependence of GSH uptake

To test whether the uptake into vesicles reflects a transport-mediated process, the rate of uptake was determined at varying GSH concentrations. The measurements were carried out in the presence of Na^+, which almost completely inhibited the GSH binding. The initial rates of GSH uptake (after 10 s of incubation) plotted as a function of GSH concentration seem to be best described as the sum of saturable and nonsaturable diffusion components (Fig. 6). The slope of this linear component of the graph was used to calculate the diffusion constant for GSH uptake into the vesicles. As shown in the inset, GSH uptake is linear over the first 20 s. Uptake values at the various GSH concentrations were then corrected for diffusion by subtracting each concentration's diffusion component to yield the net transport-mediated uptake shown in Fig. 7A. The reciprocal plot of the corrected data gave K_m of 17 μM with a V_{max} of 13 pmol/10 s per mg memrbane protein (Fig. 7B).

Specificity of GSH transport

Since labeled GSH contains the ^{3}H in the glycyl moiety, two experiments were performed to test whether the measured uptake might be due to a degradation product, glycine, which can arise extravesicularly from GSH through the activities of γ-glutamyl transpeptidase and dipeptidase that are abundant on the outer surface of intestinal brush-

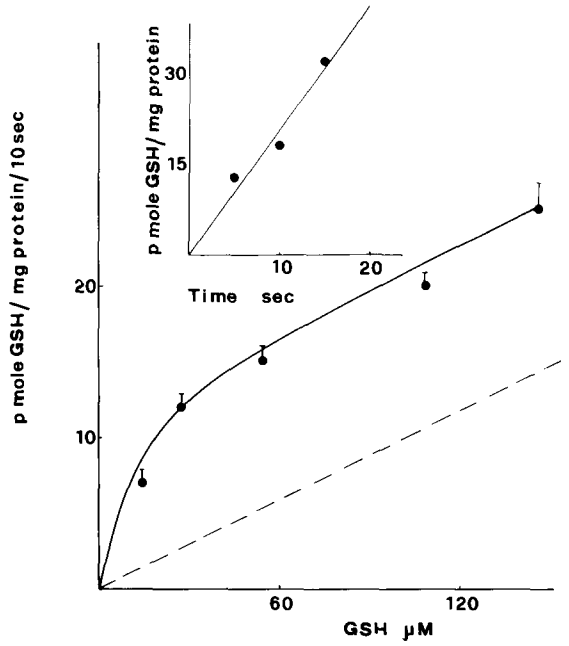

Fig. 6. Effect of varying GSH concentrations on the initial rate of GSH uptake. GSH uptake values were determined at room temperature after 10 s of incubation with AT_{125}-treated membrane vesicles (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7), 100 mM NaSCN and varying concentrations of [^{3}H]GSH (14–150 μM) (●). The dashed line is a graphical estimate of the nonsaturable component of GSH uptake. The linear relation between GSH uptake and initial incubation times (5–20 s) is shown in the upper plot. Experimental conditions are the same, the GSH concentration in uptake media was 50 μM.

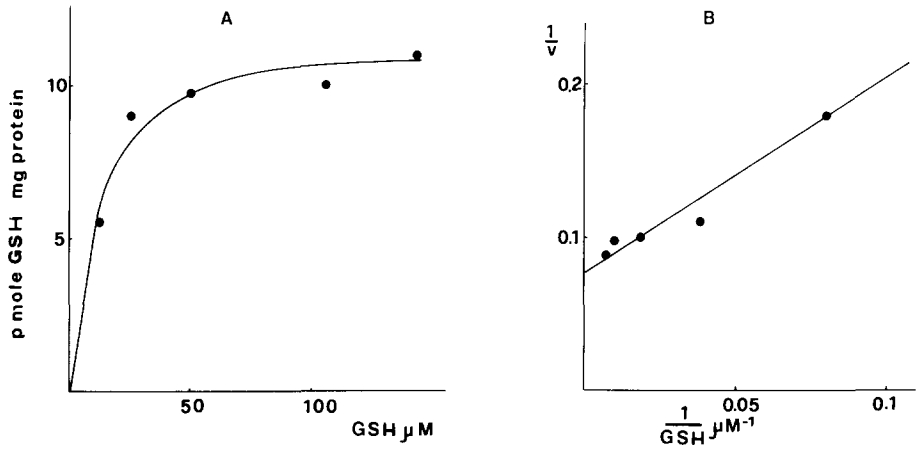

Fig. 7. (A) Initial rates of transport-mediated GSH uptake. The linear portion of the curve in Fig. 6 was used to estimate the non-saturable component of total GSH uptake. The data were obtained by subtracting the diffusion component for each concentration from the total GSH uptake. (B) Double-reciprocal plot of saturable uptake values of (A). v = pmol GSH/mg protein per 10 s.

border membranes [35]. The results of the first experiment (Fig. 2) showed an overshooting of glycine uptake into untreated and AT_{125}-treated vesicles in the presence of an Na^+ gradient, but not with a K^+ gradient. Therefore, glycine uptake appears to be primarily an Na^+-dependent active transport, while GSH uptake is an Na^+-independent mediated transport. In the second experiment, the rate of GSH transport was measured in the presence of unlabeled glycine or glutamic acid or cysteine at a concentration 10-times higher than that of the labeled GSH. The results indicated that these amino acids did not inhibit GSH uptake (Table I). Moreover, to test the structural specificity of the GSH transport system in membrane vesicles, GSH uptake was measured in the presence of peptides such as glycylglycine or glycylglycylglycine or a derivative of GSH, γ-glutamylcysteinylglycyl ester. This last compound reduced the rate of GSH uptake into the vesicles by about 40% at the first incubation times (Table I).

TABLE I

EFFECT OF STRUCTURALLY RELATED COMPOUNDS ON GSH UPTAKE

Transport was carried out at room temperature with AT_{125}-treated membrane vesicles (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7), 100 mM NaSCN, 5 μM [^{3}H]GSH, 45 μM unlabeled GSH and in the presence of 500 μM unlabeled compounds. The data represent means ± S.D. for triplicates.

Addition	Uptake (pmol GSH/mg protein)		
Incubation (min):	1	3	30
None	39 ±2.5	50 ±4	58 ±5
Glycine	37.5±2	49 ±5	60 ±5.5
Glutamic acid	36 ±5	48.5±3.5	58 ±5.5
Cysteine	38 ±4.5	50 ±6	58.5±6
Gly-Gly	39 ±3	47 ±6	55 ±6.5
Gly-Gly-Gly	36 ±5	46 ±6	55 ±7
GSH ethyl ester	20 ±3	31 ±2	49 ±3

Discussion

One of the chief problems in studying peptide transport is to distinguish transport of intact compounds from that of hydrolytic products (free amino acids). In fact, the complications due to hydrolysis of the peptides on the membrane surface have made such studies very difficult to interpret.

The transport process for intact GSH was observed in brush-border membrane vesicles of rabbit intestine after extensive inactivation of γ-glutamyl transpeptidase activity by AT_{125}. This affinity-labeling reagent for γ-glutamyl transpeptidase has also been used by others to study the transport of intact GSH across renal and liver canalicular membrane [4,5,7,8]. We demonstrated that at the intestinal level, too, this reagent in-

activated γ-glutamyl transpeptidase without changing the structural integrity of membranes, just as it does not alter the active and facilitated transport systems of D-glucose and L-glycine.

The GSH time-course was the same in the presence of an Na^+ or K^+ gradient; there was no overshoot in either case, so that the possibility of an Na^+-dependent active transport can be excluded. However, the greatest GSH uptake values are reached rapidly within the first minutes, as was the case for the D-glucose Na^+-independent transport; furthermore, we obtained saturation kinetics for varying substrate concentrations at the initial rate of GSH transport after the diffusional component is subtracted. These results strongly support the hypothesis that there is an Na^+-independent mediated transport for GSH across the intestinal membranes, a system analogous to that described for renal brush-border membrane, hepatocyte plasma membrane and liver canalicular membrane vesicles [7,6,4].

At the intestinal level, on the other hand, the data are contradictory. There are reports of an Na^+-independent transport of GSH into the rat intestinal everted sac [11] and of a Na^+-dependent active one into pig intestinal brush-border membrane vesicles [10]. In neither of these studies was the γ-glutamyl transpeptidase activity suppressed by AT_{125}; therefore, the presence of intact GSH transport cannot be clearly demonstrated. Moreover, in the intestinal everted sac preparation the cellular metabolic events that can interfere with GSH uptake were not eliminated.

We think it likely that the radioactivity recorded in the vesicles by these investigators may reflect the transport of labeled glycine, formed from GSH. Na^+-dependent and independent transport of glycine into intestinal brush-border membrane vesicles and into intestinal epithelia has indeed been demonstrated [36,37].

The major glycine transport pathway depends on the intestinal segment studies as well as on glycine concentration, as is the case for other amino acids [38]. In our experiments, the uptake of an extravesicular glycine concentration equal to that of GSH (50 μM) occurred primarily through an Na^+-dependent transport. An Na^+-dependent transport of intact GSH into rat intestinal basolateral membrane vesicles has been clearly demonstrated [9]. Lash et al. [9] also suggest that two different GSH uptake systems exist in the brush-border and in the basolateral membranes. These authors further demonstrate that enterocytes may utilize ingested GSH as well as plasma GSH for maintenance of intracellular concentrations and that exogenous GSH can be used to protect intestinal cells against oxidative chemical injury.

As for the effect of ions on GSH uptake, replacing Cl^- with a more permeant anion, SCN^-, does not significantly change its time-course. GSH uptake therefore does not seem to depend on a transmembrane diffusion potential, even though further studies are needed before one can exclude electrogenic transport. Moreover, the presence of Na^+ cancels almost completely the GSH binding, perhaps because cations can partially neutralize the negative charge of GSH and thus prevent ionic links on the membrane surface which facilitate GSH uptake into vesicles. In fact, under physiological conditions the luminal side of the intestinal brush-border membrane is more positively charged than the intracellular space [17]. On the other hand, there is considerable controversy over the exact role of Na^+ on the transport of intact peptides [17,39,40]. As is true for amino acids and peptides, in GSH uptake too there was a non-saturable component which partially masked the saturation kinetics.

The K_m value for the saturable mediated transport was very low (17 μM) and points to the presence of a high-affinity GSH transport system. This value is 10- or 20-times lower than that obtained in other membrane vesicles [4,6,7] and much lower than K_m values for facilitated transport systems of other peptides [39].

The affinity and specificity of this mediated transport for intact GSH are supported by a lack of inhibition of the amino acids studied, an observation which agrees with the previously established independence of the intestinal uptake of peptides from that of free amino acids [14]. Furthermore, other peptides, such as Gly-Gly and Gly-Gly-Gly, do not inhibit GSH uptake, either. These results are in line with the proposed involvement of a specific mediated transport of intact peptides in the small intestine [18]. Only the GSH derivative, glutathionemonoethyl ester, decreases GSH uptake.

Other researchers [4,6,7] have reported that the rate of GSH uptake into renal and liver membrane vesicles is inhibited only by GSH derivatives. Even though further studies are necessary, we suggest that glutathione monoethyl ester may be transported into vesicles by the same GSH transport system. In fact, after oral administration of this ester to mice, Meister [22] found increased cellular GSH levels, especially in the kidney and liver, and demonstrated that this derivative enters as such into cells and only subsequently is hydrolyzed in GSH.

The results of our study lead us to conclude that GSH was taken up intact by rabbit intestinal brush-border membrane vesicles by an Na^+-independent mediated transport with a marked specificity and affinity for GSH.

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