Effect of Nonprotein Thiols in the Intestinal Tissue on the Transport of Salicylate

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Rat intestinal salicylate transport showed concentration dependency, especially up to 22.5 mM in the mucosal medium, in an experiment using *in vitro* everted rat intestinal sac prepared from jejunum, ileum and colon. Thus, it is suggested that both saturatable and unsaturatable transport processes are involved in the intestinal salicylate transport. However, it was also estimated that intestinal salicylate absorption at high concentration in the clinical therapeutic context occurred predominantly through the unsaturatable (passive) transport process. Only the saturatable transport process seems to be influenced by nonprotein thiol levels in the intestinal tissue; there was a decrease of salicylate transport with decrease of nonprotein thiols. Further, since the saturatable transport process was restored even by the exogenous nonprotein thiols such as cysteamine, a process of nonspecific binding to the mucosal membrane rather than a specific carrier system may be involved in the saturatable transport of salicylate.

Keywords——salicylate; intestinal transport; rat; *in vitro* everted intestinal sac method; nonprotein thiol; diethyl maleate; diethyl ethoxymethylenemalonate; ethanol; cysteamine

There is still some controversy concerning the mechanism of the intestinal absorption of salicylate. Although it is generally believed that the intestinal absorption of salicylate is adequately explained by the pH-partition concept,[1] it has recently been reported that an apparent saturatable process was observed in part in the rat rectal absorption of salicylate[2] and it was suggested that absorption of salicylate in the rat small intestine might occur by active transport.[3] Those findings may indicate that intestinal salicylate absorption is influenced by physiological function(s) in the epithelial cells. Glutathione, a major endogeneous nonprotein thiol, is one of the physiological components required for gastric mucosal integrity[4] and is associated with the small intestinal active transport of L-amino acids.[5] Since salicylate is a hydrophilic compound at neutral pH and we have reported[6] that a decrease of nonprotein thiols in the intestinal tissue increased the intestinal mucosal permeability to cefmetazole, a hydrophilic compound, it seemed interesting to investigate the effect of nonprotein thiols in the intestinal tissue on the intestinal salicylate transport.

In the present report, we investigated the concentration dependency of the rat intestinal salicylate transport, employing an *in vitro* everted intestinal sac method. We also examined the effect of diethyl maleate (DEM), diethyl ethoxymethylenemalonate (DEEMM), ethanol, and/or cysteamine on the intestinal salicylate transport (DEM, DEEMM, and ethanol decrease the endogenous nonprotein thiols in the intestine,[6] and cysteamine is an exogenous nonprotein thiol).

Experimental

Materials——Sodium salicylate and ethanol were obtained from Nakarai Chemicals Co., Ltd. (Kyoto, Japan).

DEM and DEEMM were obtained from Sigma Co., Ltd. (Mo. U.S.A.). Cefmetazole was supplied by Sankyo Co., Ltd. (Tokyo, Japan). Commercial sulfamethoxazole and sulfadimethoxine were used after recrystallization from aqueous ethanol. Other reagents used were of analytical grade.

Animals——Wistar male rats, 200 to 225 g, were fasted for 16 h prior to experiments but water was given *ad lib.* Each animal study was started between 9 and 11 a.m.

Drug Transport Study——The transport study was carried out using the *in vitro* everted intestinal sac method described by Barr and Riegelman.[7] The everted sac of intestine containing 1 ml of Krebs–Ringer's solution (pH 7.0) was immersed in 10 ml of Krebs–Ringer's solution maintained at 37 °C and the mucosal fluid containing test compound and additive when necessary was continously gassed with 95% O_2 and 5% CO_2. At 40 min after starting the experiments, all serosal fluid was collected for the determination of the amount of drug transported, and then the intestinal tissue was homogenized for the assay of nonprotein thiols. Transport of drugs was generally represented in terms of clearance rate.

$$\text{clearance rate (ml/h/g)} = \frac{\text{(amount of drug in serosal medium)}}{[\text{(initial concentration of drug in mucosal medium)(experimental period)(tissue weight)}]}$$

Apparent transport rate of salicylate was also determined with the following equation.

$$\text{apparent transport rate (} \mu\text{mol/h/g)} = \frac{\text{(amount of salicylate in serosal medium)}}{[\text{(experimental period) (tissue weight)}]}$$

When additives were to be present in the experimental medium, DEM, DEEMM, or ethanol was added in the mucosal medium and cysteamine was added in the serosal medium. The wet weight of everted sac used in all experiments was 583 ± 38 mg for jejunum, 564 ± 26 mg for ilem and 927 ± 62 mg for colon.

Assays——Assay of salicylate,[2] cefmetazole,[8] sulfamethoxazole[9] and sulfadimethoxine[10] were carried out by high-pressure liquid chromatography as described previously. Assay of nonprotein thiols was carried out by the method described by Ellman,[11] and glutathione was used as a standard nonprotein thiol according to Szabo *et al.*[4]

Results and Discussion

Influence of Salicylate Concentration on Its Intestinal Transport

The clearance rate of salicylate transport from the mucosal side to the serosal side in the *in vitro* everted intestinal sacs prepared from segments of rat jejunum, ileum and colon was dependent on the salicylate concentration (especially up to 22.5 mM) in the mucosal medium; the clearance rate decreased with increase of salicylate concentration (Fig. 1A). The clearance
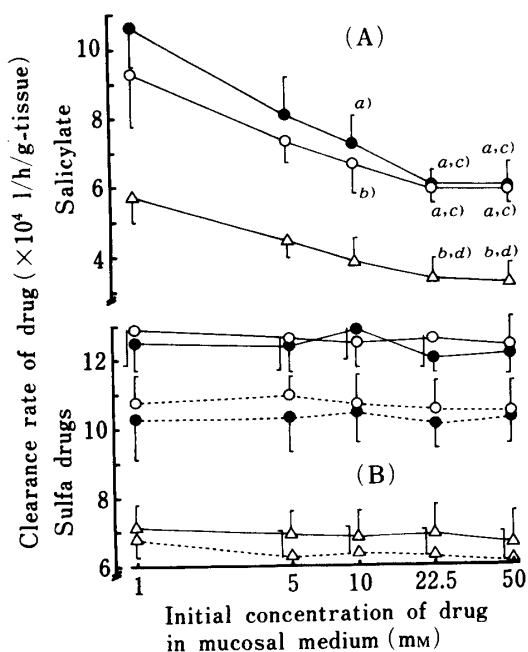

Fig. 1. Effect of Concentration of Salicylate (A), Sulfamethoxazole (B, ---) or Sulfadimethoxine (B, ——) on Clearance Rate of Drug Transport from the Mucosal Side to the Serosal Side in *in Vitro* Everted Rat Intestinal Sacs Prepared from Segments of Jejunum (○), Ileum (●) and Colon (△).

Each value represents the mean ± S.D. ($n > 3$). *a*) $p < 0.01$ *versus* 1 mM salicylate (Student's *t*-test), *b*) $p < 0.05$ *versus* 1 mM salicylate, *c*) $p < 0.05$ *versus* 5 mM salicylate, *d*) $p < 0.1$ *versus* 5 mM salicylate.

rate of salicylate per g-tissue weight was greater at the ileum and jejunum than at the colon (Fig. 1A). However, the clearance rates of sulfamethoxazole and sulfadimethoxine did not show such dependency on drug concentration in the mucosal medium within the concentration ranges used in this study (Fig. 1B).

The apparent transport rates of salicylate at various initial concentrations in the mucosal medium are shown in Fig. 2A to C, and apparent Lineweaver–Burk plots obtained from the data in Fig. 2A to C are shown in Fig. 2A' to C'. Since good linear relationships up to 10 mM salicylate were obtained in the Lineweaver–Burk plots, an apparent saturatable transport rates (estimated from the above straight lines, shown by solid lines in Fig. 2A' to C') and apparent unsaturatable transport rates (estimated from apparent total transport rates and apparent saturated transport rates) were calculated and are represented individually in Fig. 2A to C. The reason why the straight lines obtained for the apparent unsaturatable transport rate did not pass through the origin is considered to be overestimation of the rates of the
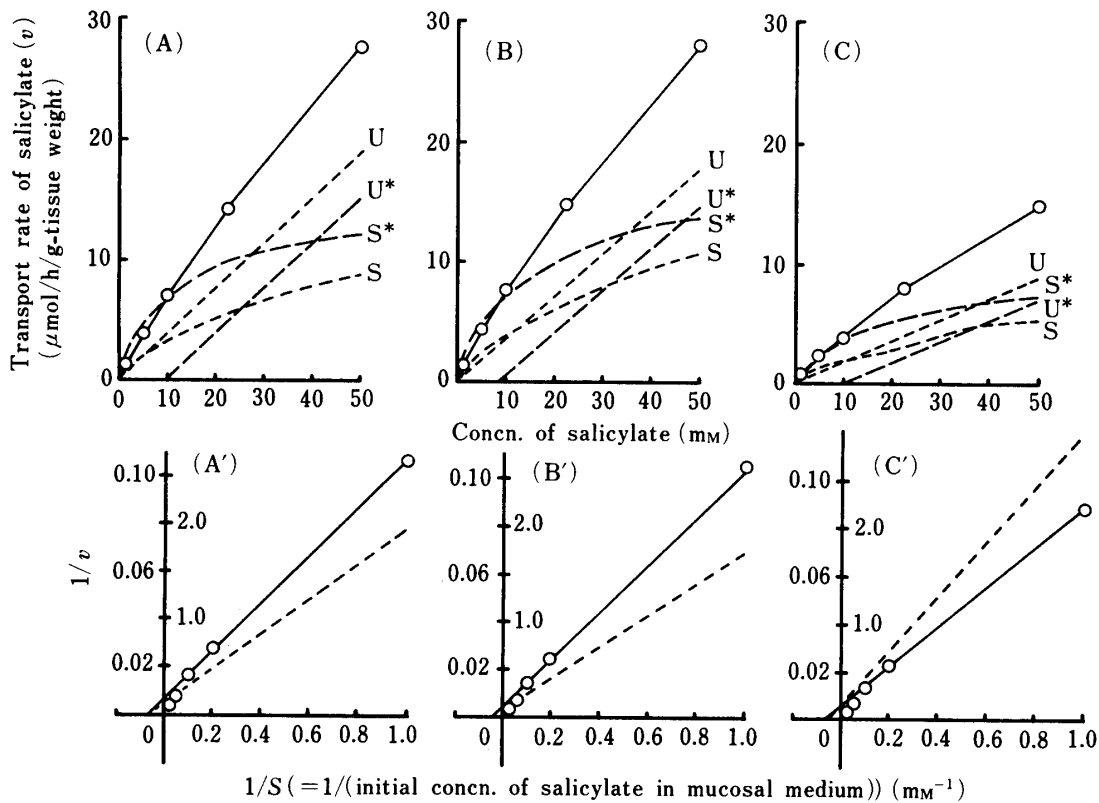

$1/S$ ($=1/($initial concn. of salicylate in mucosal medium)) (mM^{-1})

Fig. 2. Transport Rate (A, B, and C) and Lineweaver–Burk Plots (A', B', and C') of Salicylate in the Rat Jejunum (A and A'), Ileum (B and B') and Colon (C and C') in the *in Vitro* Everted Intestinal Sac Experiment

The transport rates shown by circles in this figure are the mean value at each salicylate concentration.

In A, B, and C, open circles represent the apparent total transport rate obtained from the data in Fig. 1A. Dotted lines, U* and S*, represent the rate of apparent unsaturatable transport and apparent saturatable transport, respectively (see the text). Dotted lines, U and S, represent the rate of estimated intrinsic unsaturatable transport and estimated intrinsic saturatable transport (determined according to the method described in the text), respectively. (for U*: A, $v = 0.342 \times S - 3.01$ ($r = 0.9988$); B, $v = 0.352 \times S - 3.13$ ($r = 0.9994$); C, $v = 0.176 \times S - 1.80$ ($r = 0.9999$)).

In A', B', and C', open circles represent the data indicated by open circles in A, B, C in this figure. Dotted lines represent the results obtained from the estimated intrinsic saturatable transport in A, B, and C in this figure. (for the open circles up to 10 mM salicylate: A', $1/v = 0.1014(1/S) + 0.0062$ ($r = 0.9994$); B', $1/v = 0.0881(1/S) + 0.0056$ ($r = 0.9990$); C', $1/v = 0.1600(1/S) + 0.01056$ ($r = 0.9999$)). For dotted lines: A', $1/v = 2.011(1/S) + 0.0914$ ($r = 0.9999$); B', $1/v = 1.582(1/S) + 0.082$ ($r = 0.9999$); C', $1/v = 3.045(1/S) + 0.145$ ($r = 0.9999$)).

TABLE I. Estimated Intrinsic Values of K_m, V_{max} and $k^{a)}$ for Intestinal Salicylate Transport
by the Saturatable Process (K_m and V_{max}) and Unsaturatable Process (k) and
the Ratios of Unsaturatable Transport at Various Salicylate
Concentrations are also Given

	Segments of intestine		
	Jejunum	Ileum	Colon
K_m (mM)	21.7 ± 5.2	20.1 ± 4.9	24.1 ± 6.9
V_{max} (μmol/h/g)	11.2 ± 1.9	11.9 ± 2.4	$5.9 \pm 1.3^{b)}$
$k \times 10^4$ (l/h/g)	3.51 ± 0.96	3.38 ± 0.61	$1.89 \pm 0.32^{b)}$
	Ratio of unsaturable process		
1 mM	0.42	0.37	0.45
5 mM	0.46	0.42	0.48
10 mM	0.50	0.46	0.52
22.5 mM	0.58	0.55	0.60
50 mM	0.69	0.67	0.70
100 mM	0.79	0.77	0.80
300 mM	0.91	0.90	0.91
1000 mM	0.97	0.97	0.97

a) k was obtained with the following equations. $(A/V_s)/(1\,h) = k'C$, $(A/1\,h) = (k' \times V_s)C = kC$. Here, A is the amount of salicylate transported by the unsaturatable process in serosal medium; V_s is the volume of serosal medium; k' is first-order kinetic constant of the unsaturatable transport process; C is salicylate concentration in the mucosal medium. k is apparent first order kinetic constant. The ratio of the unsaturatable transport process was determined by calculation with K_m, V_{max} and k. b) $p < 0.05$ versus jejunum and ileum. Each value of K_m, V_{max} and k represents the mean $\pm$ S.D. ($n = 7$).

saturatable transport process obtained from the apparent Lineweaver–Burk plots. Thus, lines parallel the above straight lines and passing through the origin may be estimated for an intrinsic unsaturatable transport rate of salicylate. From the above estimated intrinsic unsaturatable transport rates and the total transport rates of salicylate, intrinsic saturatable transport rates were estimated (Fig. 2A to C); Lineweaver–Burk plots for the estimated intrinsic saturatable transport rate are also shown in Fig. 2 A' to C'. The data shown with solid lines in Fig. 2A to C are the mean values of apparent transport rate of salicylate at various concentrations. However, to clarify the variation of the estimated intrinsic kinetic parameters of salicylate transport, K_m, V_{max} and k (first order kinetic constant for unsaturatable transport of salicylate; see Table I) were determined by random selection of the transport rate at various salicylate concentrations (7 times). The values of K_m for the estimated intrinsic saturatable transport of salicylate were similar in all the intestinal segments, the value of V_{max} at the small intestine being about twice that at the colon (Table I). The estimated ratios of unsaturatable transport of salicylate are given in Table I.

Since it has been reported that salicylate up to 125 mM (2% as sodium salt) does not damage colonic epithelial cells,[12] it is considered that the observed concentration-dependency of salicylate transport is due to an intrinsic transport mechanism.

As regards mechanisms involved in intestinal salicylate absorption, the following transport processes have been reported: 1) diffusion through the mucosal lipid layer,[1] 2) penetration through tight junction,[13,14] 3) active transport in the small intestine[3] and 4) a saturatable transport process in rectum.[2] In the present study, we showed that both an unsaturated transport process and a saturated transport process were involved in the intestinal salicylate transport (Fig. 2).

We have not investigated in detail the mechanism of the unsaturatable transport process of salicylate. Since small intestinal and colonic absorption of salicylate at high concentrations of about 0.3 M salicylate, where the unsaturatable transport process is dominantly involved, as
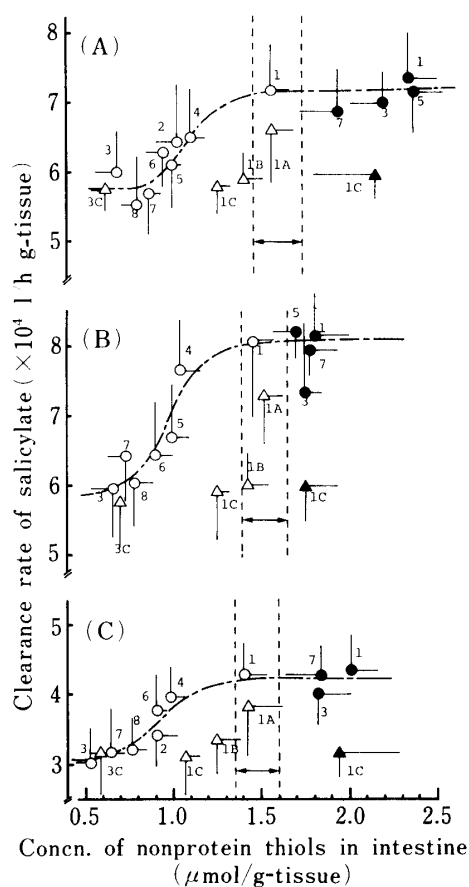
Fig. 3. Effect of Nonprotein Thiols on the Clearance Rate of Intestinal Salicylate Transport in Rat Jejunum (I), Ileum (II), and Colon (III) when DEM, DEEMM, Ethanol, or/and Cysteamine (● and ▲) were Added to the Medium in the *in Vitro* Everted Intestinal Sac Experiment

Arrows represent the normal concentration of nonprotein thiols in each intestinal tissue without any agent including salicylate in the medium. Initial concentration of salicylate in the mucosal medium was 5 mM (circles), 10 mM (triangles and A), 22.5 mM (triangles and B), or 50 mM (triangles and C). The concentration of additive in the mucosal medium was as follows; 1, no additive; 2, 0.5 mM DEM; 3, 1 mM DEM; 4, 1 mM DEEMM; 5, 5 mM DEEMM; 6, 5% ethanol; 7, 10% ethanol; 8, 20% ethanol. Cysteamine was added at a concentration of 1 mM to the serosal medium. Each value represent the mean ± S.D. ($n > 3$).

shown in Table I, was significantly facilitated by the coadministration of sodium ions[15,16] and colonic absorption of salicylate at such high concentration was also significantly suppressed by ouabain treatment,[17] penetration through tight junction may be the unsaturatable transport process of salicylate as Kunz *et al.*[13] and Yamashita *et al.*[14] have suggested. The high osmolarity in the epithelial intercellular fluid, induced by the efflux of sodium ions through the basolateral membrane of epithelial cells as a result of Na^+, K^+-adenosine triphosphatase activity after uptake of sodium ions into the epithelial cells following the coadministration of sodium ions, may lead to a solvent drag effect,[18] facilitating the penetration of salicylate through the tight junctions.

Influence of Nonprotein Thiols on the Intestinal Salicylate Transport

Since we have reported that a decrease of nonprotein thiols in the intestinal tissue increased the intestinal mucosal membrane permeability to cefmetazole, a poorly absorbed hydrophilic antibiotic,[6] we examined the effects of various agents which influence nonprotein thiol concentrations in the intestinal tissue on the intestinal salicylate transport.

The clearance rate of salicylate, when 5 mM salicylate was present in the mucosal medium of each intestinal segment, was decreased by the addition of DEM, DEEMM, or ethanol with a decrease of endogenous nonprotein thiols (Fig. 3). Addition of DEM in the mucosal medium did not affect the clearance rate of sulfa drugs (Fig. 4A and B) or salicylate at 50 mM (Fig. 3), but increased the colonic clearance rate of cefmetazole (Fig. 4C).

The decrease of clearance rate of intestinal salicylate transport when 5 mM salicylate was present in the mucosal medium seems to be related to the decrease of nonprotein thiols rather than the kind of additive (Fig. 3).

Addition of cysteamine, an exogenous nonprotein thiol, on the serosal side of the everted intestinal sac treated with DEM, DEEMM, or ethanol restored the clearance rate of salicylate
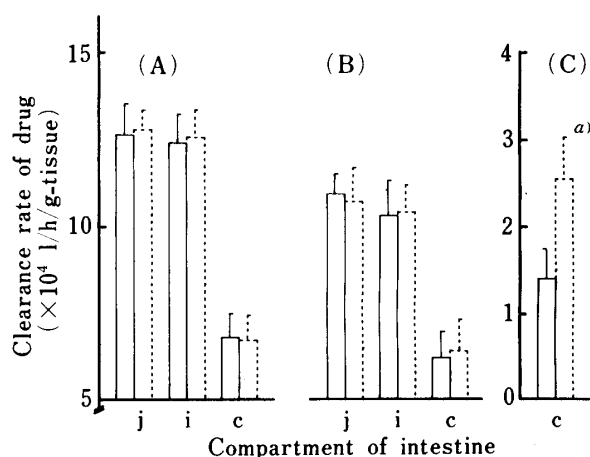
Fig. 4. Effect of DEM on the Clearance Rate of Transport of Sulfadimethoxine (A), Sulfamethoxazole (B) and Cefmetazole (C) from the Mucosal Side to the Serosal Side in *in Vitro* Rat Everted Intestinal Sacs Prepared from Segments of Jejunum (j), ileum (i) and Colon (c)

Sulfa drugs and cefmetazole at 5 mM initial concentration on the mucosal side were incubated without (□) or with (▦) 1 mM DEM on the mucosal side. Each value represents the mean ± S.D. ($n > 3$). a) $p < 0.05$ *versus* without DEM.

transport with an increase of nonprotein thiols in the intestinal tissue (Fig. 3). However, a significant increase of nonprotein thiols in the intestinal tissue over those in untreated tissue did not increase the clearance rate of salicylate transport over the clearance rate obtained in untreated tissue.

Further, 50 mM salicylate in the mucosal medium without any additive showed a tendency to decrease nonprotein thiol concentration in each intestinal tissue (Fig. 3) with a decrease of the clearance rate as mentioned earlier. However, the addition of cysteamine to the serosal medium did not increase the clearance rate of salicylate at 50 mM in spite of an increase of nonprotein thiols. In addition, a further decrease of nonprotein thiols did not decrease the clearance rate of salicylate at 50 mM as mentioned earlier.

Since it was estimated that about 70% salicylate is transported by the unsaturatable transport process when 50 mM salicylate was present (Table I), it is considered that the unsaturatable transport process of salicylate is not influenced by the nonprotein thiol concentration in the intestinal tissue. Thus, nonprotein thiols in the intestinal tissue may influence only the saturatable transport process of salicylate. Clearance rates of salicylate at 10 and 22.5 mM decreased, though there was no significant change of nonprotein thiols, in comparison with those at 5 mM salicylate (Fig. 3). This decrease of clearance rate with increase of salicylate concentration may be due to an increase of the ratio of the unsaturatable process of salicylate transport (Table I).

It is considered that the increase of nonprotein thiols in the intestinal tissue by cysteamine treatment is due to an accumulation of cysteamine. Thus, it is suggested that salicylate transport which was suppressed by the decrease of endogenous nonprotein thiols can be restored even by exogenous nonprotein thiols such as cysteamine; it has also been reported that the barrier function of the intestinal membrane against cefmetazole[6] and the gastric mucosal membrane integrity[4] were restored by exogenous nonprotein thiols. Furthermore, it has been reported that a decrease of nonprotein thiols in hepatocytes caused bleb formation of the plasma membrane,[19] and we recently found that a decrease of active transport of L-amino acid in rat small intestine owing to a decrease of endogenous nonprotein thiols was not restored by cysteamine treatment (unpublished data). We propose that a decrease of salicylate transport and an increase of cefmetazole transport, which are related to a decrease of nonprotein thiols, occur after partial loss of cell membrane integrity; *i.e.* nonprotein thiol loss in epithelial cells increases the membrane permeability to hydrophilic drugs but may suppress the presumed saturatable transport process of salicylate by disrupting the binding of salicylate to the intestinal mucosal membrane (as described below). Thus, the findings that nonprotein thiol loss caused a decrease of intestinal salicylate transport seems to indicate that the suppression of the saturatable transport process of salicylate by nonprotein thiol loss in the

intestinal tissue has a greater effect than a possible increase of mucosal membrane permeability to hydrophilic compounds.

It has been reported that uptake of only o-hydroxybenzoates (including salicylate) among benzoates into rat intestinal brush border membrane vesicles was suppressed by an amino-group blocker[20] and also that salicylate can bind to plasma membrane of epithelial cells.[21,22] Further, it was observed in the present study that exogenous nonprotein thiol (cysteamine) caused the restoration of salicylate transport, which was suppressed by nonprotein thiol loss, in spite of failing to restore active transport of L-amino acids. Thus, it is considered that the estimated saturatable process of intestinal salicylate transport involves nonspecific binding to the mucosal membrane, probably to amino groups of structural protein of the membrane, rather than a specific carrier system.

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