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The Effects of Salicylate Concentration on the Uptake of Salicylate and Cefmetazole into Rat Isolated Small Intestinal Epithelial Cells

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Abstract—The uptake of salicylate into rat isolated small intestinal epithelial cells reached an equilibrium within 15 min, but that of cefmetazole alone did not. The presence of salicylate at concentrations greater than 50 mM accelerated the uptake of cefmetazole, which reached equilibrium within 5 min. At equilibrium, the uptake clearance of salicylate (μmol (g protein)$^{-1}$ M^{-1} initial salicylate concn) was greater than that of cefmetazole. The uptake clearance of salicylate during the first several minutes was concentration-dependent, and a 'super-uptake' clearance of salicylate, greater than equilibrium values, was observed when 100 mM salicylate was present. This indicates that some mechanism, other than a simple diffusion process, may be involved in salicylate uptake into these cells.

It has been reported that intestinal salicylate absorption predominantly occurs by an unsaturable transport process (Schanker 1962; Yamashita et al 1984; Takahata et al 1986). However, it has been reported that colonic absorption of salicylate is suppressed by ouabain (Fix et al 1983) and that salicylate absorption from both the small intestine and the colon is facilitated by sodium ions (Mayersohn & Gibaldi 1969; Nishihata et al 1984c). Kunz et al (1971) and Yamashita et al (1984) have reported that a penetration pathway through the tight junctions of the intestinal epithelium might be the predominant transport process for salicylate. From those findings, the effects of sodium ion and ouabain on intestinal salicylate absorption may be explained thus: a high osmolarity in the epithelial intercellular fluid, induced by the efflux of sodium ions through the basolateral membrane of the epithelial cells as a result of Na^+, K^+-ATPase activity following the uptake of sodium ions from the brush border membrane, may lead to a solvent drag effect (Schultz 1981), facilitating the penetration of salicylate through the tight junctions. The effect of ouabain in suppressing salicylate absorption may be due to its effect in inhibiting Na^+, K^+-ATPase activity.

It has been reported (Nishihata et al 1986) that salicylate decreased the levels of non-protein thiols which are physiological components involved in maintaining cell integrity (Szabo et al 1981; Jewell et al 1982) in the intestinal tissue. It has also been reported that salicylate enhanced the absorption of a hydrophilic compound from the rectum (Nishihata et al 1980, 1984a) and from the small intestine (Nishihata et al 1983). It has been suggested that the enhancing effect of salicylate in increasing intestinal absorption of hydrophilic compounds is displayed only when salicylate itself passes through the cell membrane (Nishihata & Higuchi 1984; Kajii et al 1985), or when salicylate is taken into epithelial cells (Nishihata et al 1986), even though no morphological changes in the epithelium have been observed (Sithigorngul et al 1983). Those effects were significant only at relatively high concentrations of salicylate. Although the predominant pathway of intestinal salicylate absorption may be through epithelial tight junctions, it is necessary to clarify the effect of concentration-dependence of salicylate uptake into epithelial cells.

We have investigated salicylate uptake into rat isolated intestinal epithelial cells from incubating medium containing 2 to 100 mM. We have also examined the effect of salicylate on the uptake of cefmetazole, a hydrophilic drug, into isolated epithelial cells.

Materials and Methods

Sodium salicylate was obtained from Nakarai Chemicals (Kyoto, Japan) and sodium cefmetazole was supplied by Sankyo Co. (Tokyo, Japan). Other reagents were of analytical grade.

Isolation of small intestinal epithelial cells from male Wistar rats, 180–220 g, was carried out in a Krebs-Henseleit buffer, pH 7·4, by the method of Levine & Weintraub (1970). The isolated epithelial cells included both columnar tip cells and rounded crypt cells in approximately equal numbers, and the present study was undertaken without distinction between cell types. After separation of cells from Krebs-Henseleit buffer by rapid centrifugation (3000 rev min^{-1} 30 s) through a suspension of colloidal PVP-coated silica (final density, 1·06 g mL^{-1}), cells were suspended in 0·05 M phosphate 0·9% NaCl saline buffer (pH 7·4) for the uptake study. However, when higher sodium salicylate concentrations were used, the concentration of sodium chloride was reduced to maintain the same osmolarity of the medium.

In a separate study, the isolated epithelial cells (approximately 3×10^6 mL^{-1}) were suspended in a rotating, rounded-bottomed flask at 37°C in the phosphate-saline buffer (when sodium salicylate was added, concentration of sodium was decreased), pH 7·4, in the presence of salicylate and/or

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cefmetazole. The viability of the cells was assessed by counting the number of isolated cells which excluded trypan blue, according to Hanks & Wallace (1958).

The uptake of salicylate and cefmetazole into isolated cells was measured at designated times after the addition of salicylate and cefmetazole to the cell suspension. After incubation, the cells were separated from the medium by the method described above, and 5 mL of distilled water was added to the collected cells, before homogenization. Furthermore, to investigate the effects of rinsing, the cells that were collected were resuspended in 1 mL of the 0·05 M phosphate-saline buffer (4 °C) for 30 s. After rapid centrifugation, 5 mL distilled water was added to the collected cells which were then homogenized, and the concentrations of salicylate and cefmetazole measured by high performance liquid chromatography (Nishihata et al 1984b). Protein in the homogenate was assayed using the method of Lowry et al (1951).

The apparent uptake of drug was represented as the amount of drug per gram of protein because the apparent uptake includes the amount of drug adsorbed on the cell surface membrane. To analyse the effect of salicylate concentration further, uptake was represented as 'uptake clearance (mL (g protein)$^{-1}$)', by dividing the amount of salicylate uptake (μmol (g protein)$^{-1}$) by the initial salicylate concentration (M).

In a separate study, cells were treated with salicylate for 5 and 15 min, and were subsequently homogenized for assay of non-protein and protein thiols. Non-protein thiol concentration was determined as acid-soluble thiol, and protein thiol concentration was determined as acid-insoluble thiol by the colorimetric method of Di Monte et al (1984). Glutathione was used as a standard thiol.

Statistical analyses were performed using Student's t-test.

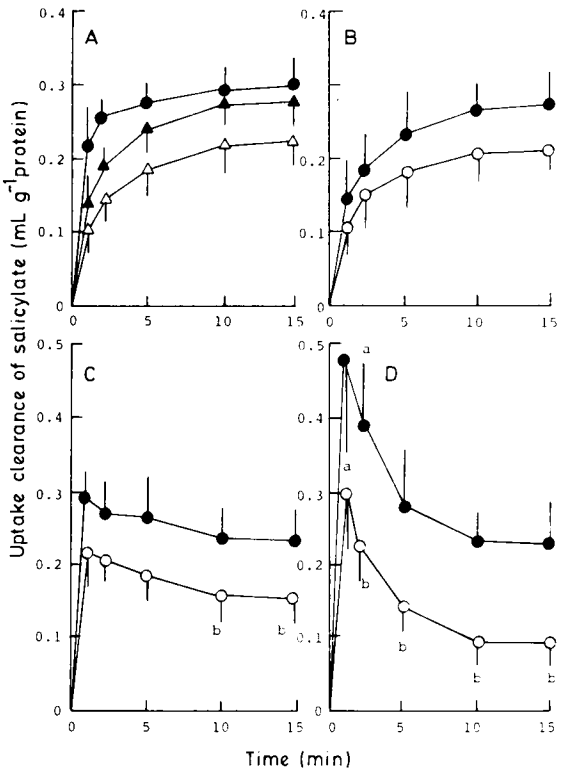

FIG. 1. Salicylate uptake into rat isolated small intestinal epithelial cells at 37 °C as a function of time. Initial salicylate concentrations were 2 mM (A circles), 5 mM (A triangles), 25 mM (B), 50 mM (C) and 100 mM (D). Closed symbols and open symbols represent uptake clearance of salicylate before and after rinsing cells, respectively, following separation from the drug solution by rapid centrifugation. Each value represents the mean ± s.d. (n = 5). a, $P < 0.05$ vs the result of 5 mM salicylate. b, $P < 0.05$ vs the result before rinsing..

Results

The uptake of salicylate into rat isolated small intestinal epithelial cells reached equilibrium within 15 min when the salicylate concentration ranged from 2 to 100 mM (Fig. 1). The uptake clearance of salicylate at equilibrium did not show a marked concentration dependence on salicylate, although a tendency to decrease the uptake clearance was observed at higher salicylate concentrations (Fig. 2B). Salicylate uptake increased with the incubation period up to 10 min with salicylate concentration up to 25 mM in the medium. Salicylate uptake at 2 mM seemed to occur rapidly compared with that at 5 or 25 mM (Fig. 1A, B). When the initial salicylate concentration was 100 mM, 'super-uptake' of salicylate over the equilibrium values was observed at earlier times, followed by a decrease in uptake (Fig. 1D). Those results were observed even without the process of cell-rinsing following separation from the salicylate solution. In the process of rinsing cells, uptake clearance of salicylate decreased significantly, especially when 50 or 100 mM salicylate was used (Figs 1C, D, 2A, B).

Cefmetazole uptake into the isolated cells increased with incubation time and did not reach equilibrium within the 15 min experimental period (Fig. 3A). The uptake of cefmetazole was not accelerated in the presence of salicylate at concentrations up to 25 mM (Fig. 2C). In comparison with salicylate uptake, cefmetazole uptake occurred slowly at lower concentrations of salicylate (Fig. 3A). However, when the salicylate concentration was more than 50 mM, cefmetazole uptake was accelerated, and reached equilibrium within 5 min (Fig. 3C, D). Super-uptake of cefmetazole over the equilibrium concentration was not observed. As with salicylate uptake, uptake of cefmetazole decreased significantly during the process of rinsing after treatment with 100 mM salicylate (Figs 2D, 3C, 3D).

The uptake clearance of salicylate at equilibrium could always be determined at 15 min, but that of cefmetazole could only be determined when 50 or 100 mM salicylate was used. Uptake clearances of salicylate and cefmetazole into the isolated epithelial cells were 0·268 ± 0·041 mL (g protein)$^{-1}$ (n = 25, salicylate concentration of 5 to 100 mM) and 0·182 ± 0·021 mL (g protein)$^{-1}$ (n = 10, cefmetazole concentration of 4 mM) ($P < 0.05$ vs uptake clearance of salicylate), respectively.

As shown in Table 1, salicylate did not influence protein thiol content in the isolated epithelial cells. Salicylate at 100 mM caused a decrease in non-protein thiol content, but salicylate up to 50 mM had no effect. Cell viability did not change markedly in the presence of salicylate (Table 1).

Discussion

Salicylate, as well as being an anti-inflammatory drug, also acts as an antiplatelet agent by inhibiting glycosylation of

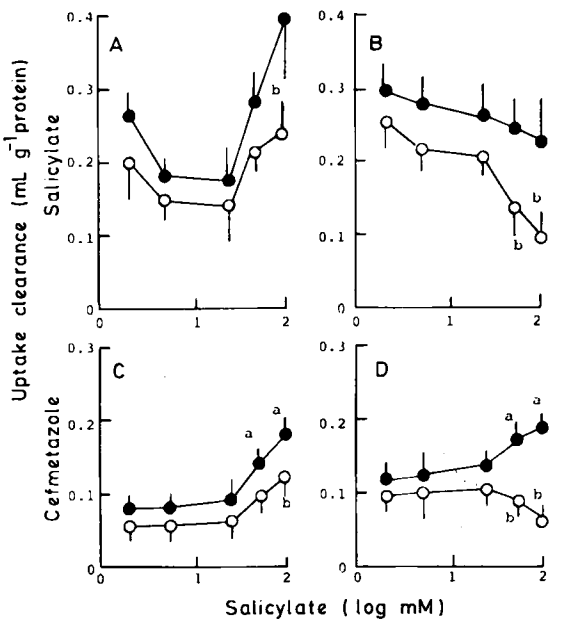

FIG. 2. The effect of salicylate concentrations on uptake clearances of salicylate (A and B) and cefmetazole (C and D) after incubation for 2 min (A and C) and for 15 min (B and D). The concentration of cefmetazole in the medium was 4 mM. Closed symbols and open symbols are as described in Fig. 1. In the absence of salicylate, the uptake clearances of cefmetazole at 2 min were 0·088 ± 0·003 mL (g protein)$^{-1}$ before rinsing and 0·060 ± 0·002 mL (g protein)$^{-1}$ after rinsing, and those at 15 min were 0·136 ± 0·029 mL (g protein)$^{-1}$ before rinsing and 0·122 ± 0·21 mL (g protein)$^{-1}$ after rinsing. Each value represents the mean ± s.d. (n = 5). a, $P < 0·05$ vs results in the presence of 5 mM salicylate. b, $P < 0·05$ vs the result before rinsing.

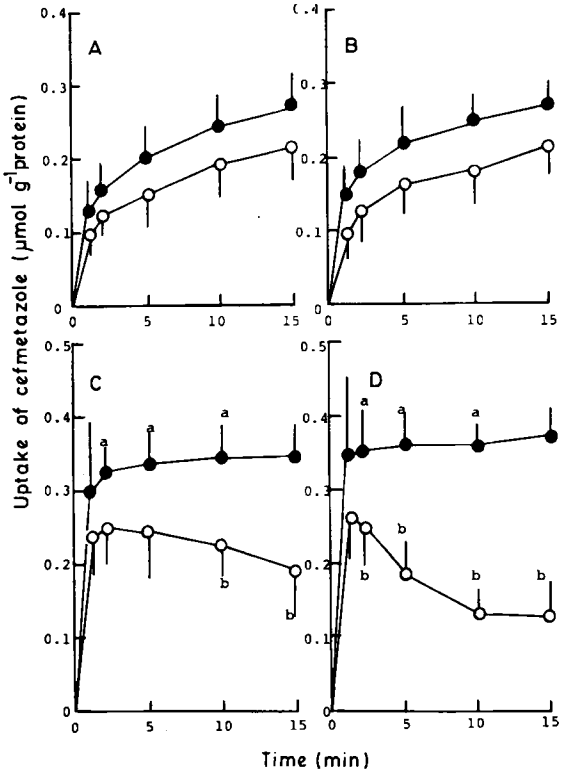

FIG. 3. The effect of salicylate on cefmetazole uptake into isolated epithelial cells at 37 °C as a function of time after the cells were suspended in a drug solution containing 4 mM cefmetazole alone (A), with 25 mM salicylate (B), with 50 mM salicylate (C), and with 100 mM salicylate (D). The closed symbols and open symbols are explained in Fig. 1. Each value represents the mean ± s.d. (n = 5). a, $P < 0·05$ versus in the absence of salicylate. b, $P < 0·05$ versus the result before rinsing.

collagen (Yue et al 1984) and by inhibiting cyclo-oxygenase activity (Yue et al 1985). Its action in uncoupling oxidative phosphorylation in mitochondria (Brody 1956) may relate to its effect in decreasing non-protein thiol concentrations (Table 1). NAD(P)H oxidation has been shown to occur in the presence of uncoupler (Powis et al 1984) or as a result of the conversion of GSSG (oxidized form of glutathione) to GSH (reduced form of glutathione) (Moore et al 1985); i.e. a decrease in NAD(P)H concentration in the presence of an uncoupler, results in a failure to reduce GSSG to GSH. Since GSH is a major non-protein thiol in cells (Szabo et al 1981), the uncoupling effect could lead to a reduction in the amount of non-protein thiol in isolated cells exposed to 100 mM salicylate (Table 1). However, during the experimental period, salicylate at the concentrations employed did not reduce cell viability (Table 1).

In terms of the uptake clearance of salicylate at equilibrium (measured at 15 min), no significant differences were observed between any of the salicylate concentrations studied. The clearance decreased only slightly as salicylate concentration increased (Fig. 2B). These findings suggest that uptake of salicylate into the isolated cells occurs predominantly by a simple diffusion process.

The super-uptake clearance of salicylate above and beyond the equilibrium value in the isolated epithelial cells was observed at an early stage (2 min) when salicylate was present at 100 mM (Fig. 1). Cefmetazole uptake was also significantly accelerated in the presence of 100 mM salicylate and rapidly reached equilibrium, but super-uptake of cefmetazole did not occur (Fig. 2). Within the context of this paper, it is difficult to speculate about the mechanism of salicylate super-uptake clearance, but it may involve non-protein thiols.

The higher uptake clearance of salicylate, in comparison with that of cefmetazole at equilibrium, suggests that salicylate associate not only with the cytosol, but also with

Table 1. Thiol concentration in isolated small intestinal epithelial cells of rats.

	Incubation period, min	Thiols, μmol (g protein)$^{-1}$		
		Non-protein	Protein	Viability
No additive	0	1·57 ± 0·16	33·4 ± 3·9	86·5 ± 4·2
	15	1·42 ± 0·07	34·6 ± 4·1	84·2 ± 3·0
Salicylate 25 mM	5	1·69 ± 0·21	31·6 ± 4·1	89·2 ± 2·9
	15	1·51 ± 0·08	30·7 ± 1·7	83·1 ± 5·4
50 mM	5	1·62 ± 0·09	30·2 ± 4·3	83·2 ± 2·7
	15	1·36 ± 0·17	32·7 ± 3·2	84·6 ± 3·9
100 mM	5	1·46 ± 0·14	35·9 ± 3·1	88·2 ± 5·6
	15	1·09 ± 0·08*	33·7 ± 2·7	87·1 ± 7·1
Cefmetazole 4 mM	5	1·62 ± 0·21	30·7 ± 2·9	89·4 ± 4·6
	15	1·57 ± 0·10	32·6 ± 4·1	86·2 ± 3·5

Each value represents the mean ± s.d. (n = 5).
*$P < 0·05$ results in the absence of salicylate.

the cell membrane, and that the association of salicylate with the membrane is greater than that of cefmetazole. Recently, we found (Nishihata et al, unpublished data) that salicylate displaced adsorbed arsenazo III on surface of small intestinal epithelial cells. This observation supports the idea that the apparent uptake of salicylate includes adsorption to the cell surface. Adsorption of salicylate to cell surfaces helps to explain the slight decrease in apparent uptake clearance at higher salicylate concentrations, because the adsorption process saturates at concentrations lower than 100 mM.

The uptake clearance of both salicylate and cefmetazole decreased significantly following the buffered-saline rinse, especially after treatment with 100 mM salicylate (Fig. 1). These observations suggest that the cell membrane permeability is increased in the presence of 100 mM salicylate, and, during the quick rinsing process, release of salicylate and cefmetazole from the cells occurred rapidly compared with release after exposure to salicylate at lower concentrations. Since it has also been reported that enhancement of the intestinal absorption of cefmetazole by diethyl maleate might involved depletion of non-protein thiol (Nishihata et al 1986), the accelerating effect of 100 mM salicylate may also be related a decrease in non-protein thiol content. At present, it is difficult to explain how non-protein thiol loss could induce an increase in cell membrane permeability. However, it may be that a loss of glutathione, a major non-protein thiol (Szabo et al 1981), allows membrane lipid to be oxidized, resulting in an increased permeability, because glutathione is known to inhibit the accumulation of lipid peroxide (Di Monte et al 1984).

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