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The effect of adjuvants on the colonic absorption of cefmetazole and [Asu^{1,7}]-eel calcitonin in rats: concentration dependent absorption pathways

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Summary

Rat colonic absorption of cefmetazole and [Asu^{1,7}]-eel calcitonin was enhanced by coadministration of sodium salicylate, disodium ethylenediaminetetraacetic acid, diethyl ethoxymethylenemalonate or trifluoperazine. Colonic absorption of cefmetazole and eel calcitonin, enhanced by various concentrations of either EDTA or trifluoperazine, appeared to occur via a paracellular pathway. Diethyl maleate did not enhance colonic absorption of eel calcitonin, but it did significantly enhance colonic absorption of cefmetazole, demonstrating the importance of a paracellular absorption pathway for eel calcitonin. Although low concentrations of DEEMM and salicylate enhanced the colonic absorption of only cefmetazole (having a low molecular weight of 471), those adjuvants at high concentrations remarkably enhanced the colonic absorption of both cefmetazole and the macromolecular peptide, eel calcitonin (mol. wt. 3363). This observation suggests two different adjuvant mechanisms, depending on the concentration of the adjuvant.

Introduction

Since our reports that sodium salicylate increased the rectal absorption of drugs (Nishihata, et al., 1980, 1981a and b; Caldwell et al., 1982), investigations on absorption-promoting adjuvants have been carried out by many scientific groups. It has been demonstrated that among the non-surfactant adjuvants, disodium ethylenediaminetetraacetic acid (EDTA) increases intestinal mucosal permeability by depleting calcium ions from the tight junctional areas, thereby opening the normally tight junctions (Martinez-Paloma et al., 1980). However, the underlying mechanisms of other adjuvants are still under investigation. It has been reported that one effect of diethyl maleate is a decrease of non-protein sulfhydryl concentration in intestinal tissue in vitro, and that this decrease might be involved in the mechanism of the absorption-promoting action of diethyl maleate (Nishihata et al., 1985). The mechanism behind such adjuvant action may involve a modification of the mucosa's physiological function(s), so it is relevant to investigate the effect of agents which can modify such physiological function(s).

Because it was demonstrated that strong chelat-

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ing agents allowed transmucosal transport of even macromolecular compounds in vitro (Martinez-Paloma et al., 1980), the actions of several different adjuvants were compared in the present study to that of a strong chelating agent with respect to colonic absorption in the rat in vivo. The target compounds for absorption were cefmetazole (relatively small molecular weight compound, 471) and [Asu^{1,7}]-eel calcitonin (relatively large molecular weight compound, 3363). The different molecular sizes were selected so as to differentiate two possible absorption pathways, ie. transcellular vs paracellular. As adjuvants, diethyl maleate, diethyl ethoxymethylenemalonate, and salicylate were examined, since it has been reported that they decrease the concentration of reduced non-protein sulfhydryls in rat intestinal tissue in vitro (Nishihata et al., 1985). Trifluoperazine was also examined in the concentration range of 1–100 μM, in which it can act as calmodulin inhibitor. As a strong chelating agent, disodium ethylenediaminetetraacetic acid was used.

Materials and Methods

Chemicals

Trifluoperazine (THP) was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Sodium cefmetazole and [Asu^{1,7}]-eel calcitonin (eel calcitonin) were supplied by Sankyo Co., Ltd. (Tokyo, Japan) and Toko Jozo Co., Ltd. (Tokyo, Japan), respectively. Sodium salicylate, diethyl ethoxymethylenemalonate (DEEMM), diethyl maleate (DEM) and disodium ethylenediaminetetraacetic acid (EDTA) were purchased from Nakarai Chemical Co., Ltd. (Kyoto, Japan). Other reagents used were of analytical grade.

Animals

Wistar male rats, 200–225 g, were fasted for 16 h prior to experimentation. During the experiments, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and were maintained on a warm surface at 38°C.

Absorption study

The rat colonic drug absorption study was performed according to the following procedure. The colonic absorption of cefmetazole, with or without adjuvant, after the administration of a microenema into the rat colonic loop (about 4 cm in length, 436 ± 26 mg of tissue wet weight) was determined by two methods. One method is based on the area under the plasma concentration–time curve (AUC) for 120 min after administration (Dittert and Bourne, 1979). The other method consisted of measuring the residual amount of cefmetazole in the colonic loop 120 min after administration (Nishihata et al., 1984b). The microenema used in this study was prepared with 0.2 M sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride. We used such a high buffer concentration because it has been reported that the adjuvant action of EDTA is strongly dependent on pH (Suzuka et al., 1985), and a large buffer capacity was needed to maintain the microenema containing disodium EDTA at pH 7.0. The use of such a high ionic strength solution is to remove the mucin layer to achieve a complete diffusion of compounds to the surface of the brush border membrane, as reported previously (Miyake et al., 1985). The reason why the loop method was used in this study was to avoid dilution of the initial concentrations by spreading the microenema over a large surface area. Colonic absorption of cefmetazole based on the AUC method was determined by comparing the AUC after colonic administration (AUC_{col}) with the AUC after intravenous administration (AUC_{iv}). A linear relationship between the dose of cefmetazole [$(Dose_{iv})_{cmz}$] (ranging from 1 to 20 mg · kg^{-1}) and AUC of cefmetazole after intravenous administration [$(AUC_{iv})_{cmz}$] (μg · min · ml^{-1}) was obtained in rats (Eqn. 1).

$$(AUC_{iv}) = 71.7\,(Dose_{iv})_{cmz} + 9.6 \qquad (1)$$

$r = 0.9895$, $n = 21$.

From the AUC_{col} value shown in Table 1, the $Dose_{iv}$ for cefmetazole needed to obtain the equivalent AUC_{iv} value was determined using Eqn. 1. Additionally, using a cefmetazole dose of 10 mg/kg ($Dose_{col}$), the absorption clearance of cefmetazole after colonic administration (A_{cmz}) was calculated using Eqn. 2, because we con-

cluded that drug absorption is influenced by the colonic surface area, which is probably related to the loop weight.

Absorption clearance =

$$(\text{Dose}_{iv})/[(\text{Dose}_{col}) \times (\text{loop weight}) \times (2\text{h})] \quad (2)$$

Disappearance clearance of cefmetazole from the loop (D_{cmz}) was determined using Eqn. 3.

Disappearance clearance =

$$[(\text{Dose}_{col}) - (\text{remaining amounts})]$$
$$/[(\text{Dose}_{col}) \times (\text{loop weight}) \times (2\text{h})] \quad (3)$$

The colonic absorption of eel calcitonin was determined only by comparing the AUC_{col} with the AUC_{iv}, since eel calcitonin was largely adsorbed to colonic tissue (around 15% of eel calcitonin was adsorbed when 400 U/kg was administered in the colonic loop). A correlation as shown in Eqn. 4 was obtained between the dose of eel calcitonin $[(\text{Dose}_{iv})_{ect}]$ (ranging from 5 to 150 U/kg) and AUC after intravenous administration $[(AUC_{iv})_{ect}]$ ($mU \cdot min \cdot ml^{-1}$).

$$\log (AUC_{iv})_{ect} = 1.505 \log(\text{Dose}_{iv})_{ect} + 0.998 \quad (4)$$

$r = 0.9848$, $n = 32$.

Using the AUC_{col} values (Table 1), the Dose_{iv} for eel calcitonin needed to obtain the equivalent AUC_{iv} value was determined. The absorption clearance of eel calcitonin after colonic administration was calculated using Eqn. 2. Cefmetazole and eel calcitonin were administered simultaneously.

Assay

Cefmetazole was assayed by an HPLC method with reverse-phase column material (RP-18) and with a detection limit of 0.2 μg/ml, as described previously (Nishihata et al., 1984c). Assay of eel calcitonin was performed by an enzyme immunoassay with a detection limit of 1 mU/ml (Aikawa et al., 1985). Concentration of reduced non-protein sulfhydryls in colonic tissue was determined according to the method described by Riddles et al. (1979) using glutathione as a standard compound.

Results

Comparison of individual adjuvant effect on rat colonic absorption of cefmetazole and eel calcitonin

Although we have already reported on the adjuvant action of EDTA, DEEMM, salicylate and DEM on the rat colonic absorption of cefmetazole (Nishihata et al., 1984a; Suzuka et al., 1985), we re-examined their absorption enhancing action in order to compare the strength of their effects on rat colonic absorption of cefmetazole and eel calcitonin. We also investigated the effect of TFP on the colonic absorption of both drugs.

The colonic absorption of cefmetazole was enhanced by coadministration of EDTA, TFP, DEEMM, salicylate or DEM, as indicated by plasma drug concentration (Fig. 1 and Table 1). Colonic absorption clearance of cefmetazole (A_{cmz}) increased with an increase in concentration of each adjuvant in the microenema (Fig. 2A–F). Colonic absorption of eel calcitonin also increased with an increase in the concentration of EDTA, TFP, DEEMM or salicylate, as shown with AUC (Table 1) and with absorption clearance (A_{ect}) (Fig. 2A–F). However, despite that colonic absorption of cefmetazole was enhanced to a similar degree when 15 mM EDTA, 0.05 mM TFP, 7.5 mM DEEMM or 100 mM salicylate was coadministered, neither DEEMM nor salicylate increased the colonic absorption of eel calcitonin at those concentrations (Fig. 2A–E). DEM did not significantly enhance the colonic absorption of eel calcitonin within the concentration range used in this study (Fig. 2F).

To study the enhancing efficacy of each adjuvant on colonic absorption of cefmetazole with respect to eel calcitonin, the ratio of A_{cmz} over A_{ect} was examined. As shown in Fig. 2A′–F′, the ratio of A_{cmz}/A_{ect} when either EDTA or TFP was coadministered remained constant at about 5 which is a somewhat small value in comparison with the ratio in the absence of adjuvant. When either DEEMM or salicylate was coadministered at low concentration, the ratio was significantly

TABLE 1

AREA UNDER THE CURVE OF PLASMA CEFMETAZOLE AND EEL CALCITONIN CONCENTRATION (AUC) FOR 120 min AFTER ADMINISTRATION OF MICROENEMA * INTO RAT COLONIC LOOP AS DESCRIBED IN FIG. 1

Adjuvant (mM)	Cefmetazole			Eel calcitonin		
	Dose (mg/kg)	AUC (μg min/ml)		Dose (U/kg)	AUC (μg min/ml)	
		without cysteamine **	with cysteamine **		without cysteamine **	with cysteamine **
No adjuvant	10	43 (12)		800	207 (45)	212 (54)
	20	80 (16)	84 (11)	1600	456 (197)	
EDTA						
15	10	108 (12)	111 (17)	400	629 (58)	658 (72)
30	10	234 (32)		400	1648 (101)	
50	10	469 (57)	447 (41)	400	4701 (375)	5206 (472)
TFP						
0.025	10	67 (20)	71 (16)	400	269 (52)	228 (42)
0.05	10	117 (21)		400	764 (104)	
0.10	10	220 (34)	242 (31)	400	1877 (262)	1826 (311)
DEEMM						
7.5	10	101 (31)	46 (7) ***	400	39 (11)	34 (7)
12.5	10	231 (29)		400	278 (41)	
25	10	349 (42)	207 (51) ***	400	1458 (124)	983 (101) ***
50	10	473 (48)		400	3651 (262)	
Salicylate						
100	10	104 (21)	49 (9) ***	800	113 (21)	106 (37) ***
200	10	256 (36)		800	1242 (159)	
400	10	397 (52)	248 (46) ***	800	5937 (616)	4213 (419) ***
DEM						
1.25	10	74 (10)	38 (6) ***	1600	216 (38)	207 (29)
1.25	20	114 (27)				
2.5	10	106 (19)		1600	237 (62)	
2.5	20	152 (44)				
3.75	10	182 (34)	42 (9) ***	1600	242 (53)	219 (62)
3.75	20	241 (79)				

Each value in the Table represents the mean ($\pm$S.D.), $n \geqq 6$.

* The microenema used in this study also contained eel calcitonin.

** The concentration of cysteamine in microenema was 5 mM.

*** $P < 0.01$ vs no cysteamine. (Statistical analysis by Student's t-test.)

higher compared to the ratio obtained without any adjuvant. However, an increase in the concentration of either DEEMM or salicylate resulted in a decrease in the value of the ratio, approaching that obtained without any adjuvant. On the other hand, when DEM was coadministered, the ratio was always a significantly higher value due to the selective action of DEM in increasing the colonic absorption of only cefmetazole.

The effect of each adjuvant on the reduced non-protein sulfhydryl concentration in rat colonic tissue and the influence of cysteamine on each adjuvant action

It has been reported (Nishihata et al., 1985) that the decrease in the reduced non-protein sulfhydryl concentration in the colonic tissue, after the colonic tissue was treated with DEM in vitro, might be related to the increased permeability of the colonic mucosal membrane. Therefore, we ex-

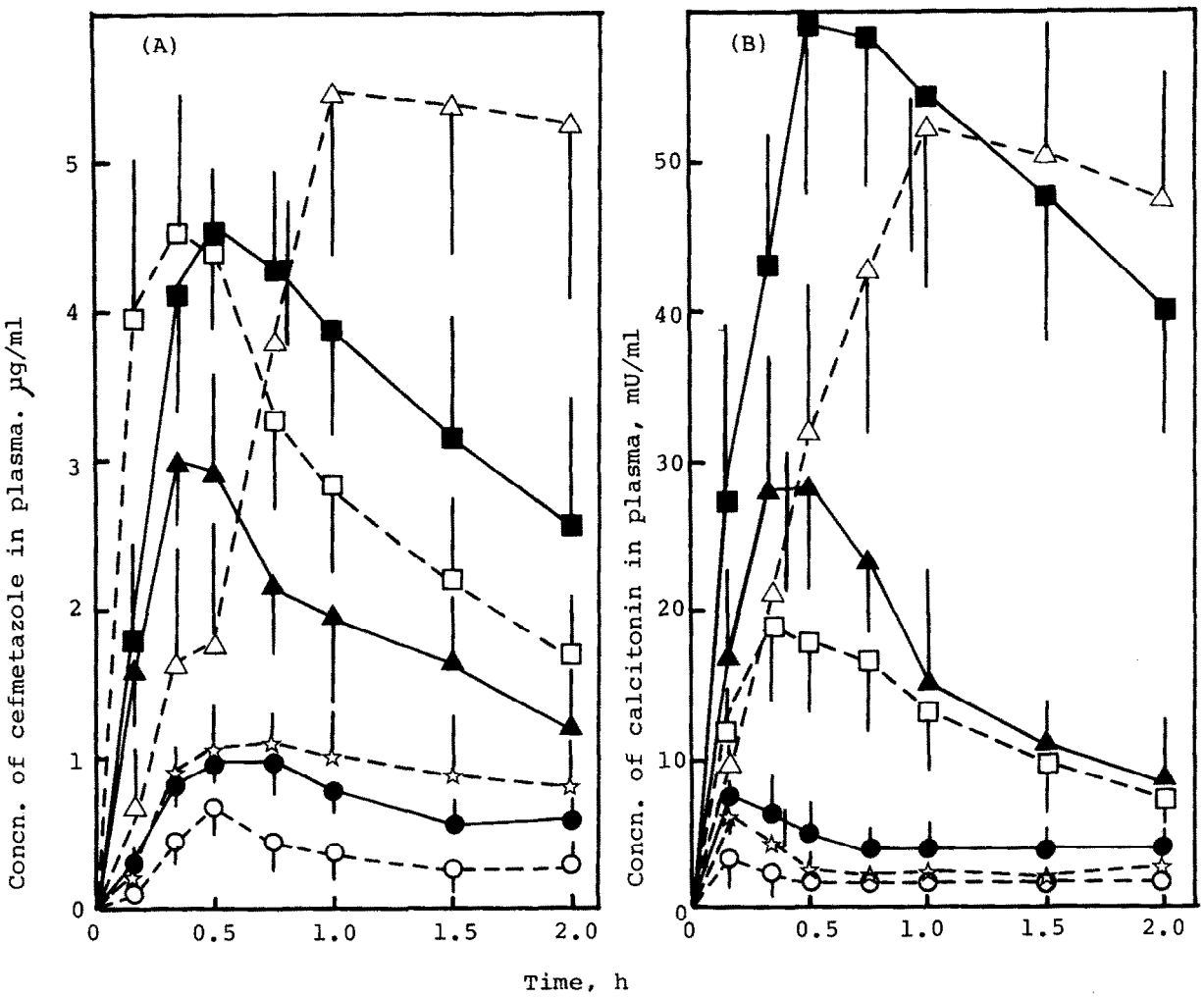

Fig. 1. Plasma concentrations of cefmetazole (A) and eel calcitonin (B, IR-eel calcitonin) as a function of time after administration of a microenema containing both drug and adjuvant at a volume of 1 ml/kg into rat colonic loop. In A: concentrations of cefmetazole and adjuvant were as follows: 10 mg of cefmetazole/ml and no adjuvant (○); 20 mg and no adjuvant (●); 10 mg/ml and 50 mM EDTA (△); 10 mg/ml and 0.10 mM TFP (▲); 10 mg/ml and 25 mM DEEMM (□); 10 mg/ml and 400 mM sodium salicylate (■); and 10 mg/ml and 2.5 mM DEM (☆). B: concentrations of eel calcitonin and adjuvant were as follows: 800 U of eel calcitonin/ml and no adjuvant (○); 1600 U/ml and no adjuvant (●); 400 U/ml and 50 mM EDTA (△); 400 U/ml and 0.1 mM TFP (▲); 400 U/ml and 25 mM DEEMM (□); 800 U/ml and 400 mM sodium salicylate (■); and 1600 U/ml and 2.5 mM DEM (☆). Each value represents the mean ± S.D., $n \geqq 6$.

amined the effect of each adjuvant on the reduced non-protein sulfhydryl levels in the colonic loop tissue and also investigated the effect of cysteamine, a non-protein sulfhydryl, on the enhancing action of various adjuvants.

As shown in Fig. 3A, the disappearance clearance of cefmetazole from the colonic loop (D_{cmz}) correlated well with the decrease of reduced non-protein sulfhydryl concentration in the tissue when DEM was coadministered. The adjuvant action of either DEEMM or salicylate at higher concentration was greater than expected

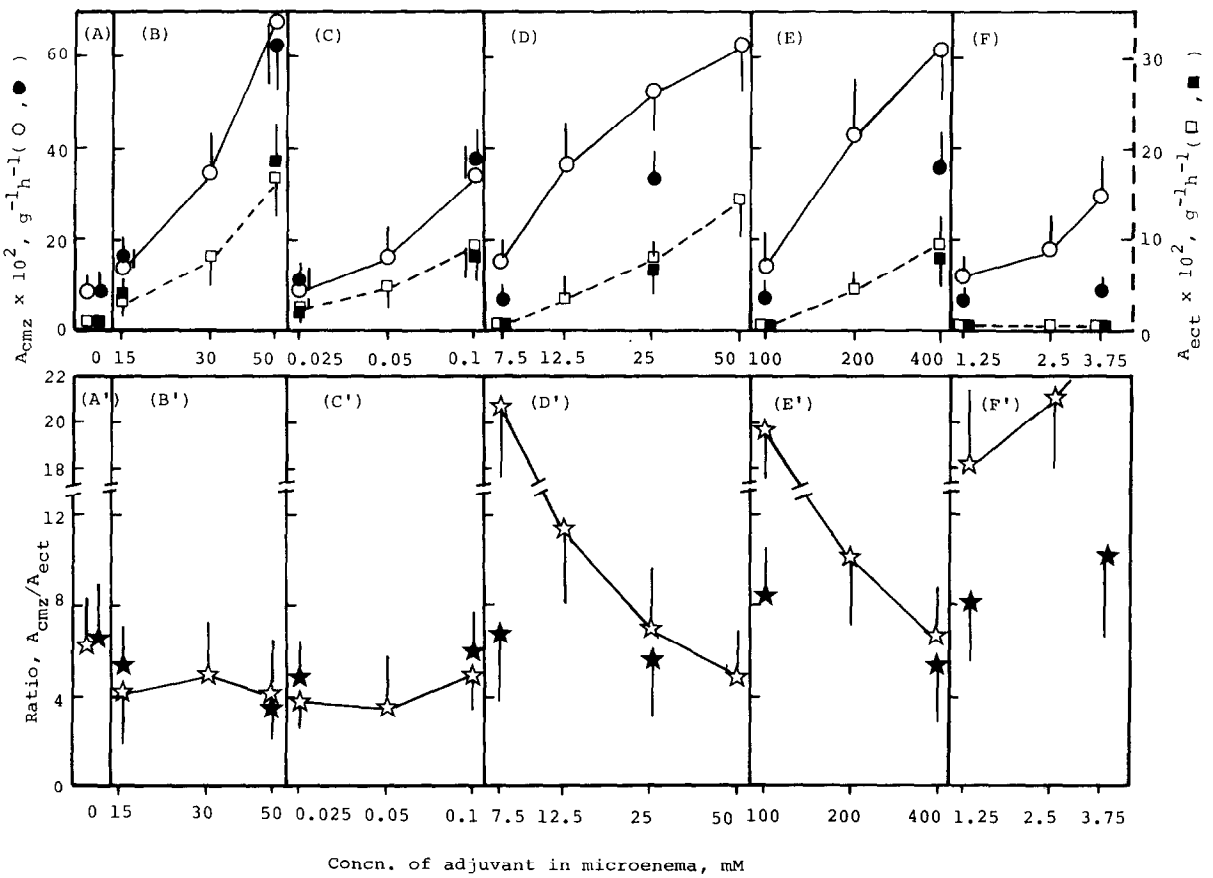

Fig. 2. Effect of varying the concentration of each adjuvant in the microenema on the absorption clearance of cefmetazole (A_{cmz}, ○ and ●) and eel calcitonin (A_{ect}, □ and ■) (A–F) and on the ratio of A_{cmz}/A_{ect} (A′–F′) determined by the AUC method for 120 min represented in Table 1. Open symbols represent the result without cysteamine coadministration and closed symbols represent the result with 5 mM cysteamine coadministration. A and A′, no adjuvant; B and B′, EDTA; C and C′, TFP; D and D′, DEEMM; E and E′, sodium salicylate; and F and F′, DEM. Each value represents the mean ± S.D., n ≧ 6. a, $P < 0.05$ vs no adjuvant; b, $P < 0.05$ vs no cysteamine (Student's *t*-test).

from a decrease of reduced non-protein sulfhydryl levels alone. EDTA and TFP increased the D_{cmz} from the colonic loop without significantly decreasing the reduced non-protein sulfhydryl concentration in the colonic tissue.

Coadministration of cysteamine in the microenema almost completely suppressed the enhancing action of DEM on A_{cmz} and D_{cmz} (Figs. 2 and 3B, respectively), and maintained a high concentration of reduced non-protein sulfhydryl in the tissue (Fig. 3). Although cysteamine almost completely suppressed the enhancing action of DEEMM and salicylate on A_{cmz} and D_{cmz} when both adjuvants were administered at low concentrations (Figs. 2 and 3), only partial suppression was achieved when either adjuvant was administered at higher concentration, in spite of the significantly increased reduced non-protein sulfhydryl concentration in the tissue (Figs. 2 and 3). Furthermore, cysteamine did not influence the enhancing action of DEEMM and salicylate at high concentration on the colonic absorption of eel calcitonin (Fig. 2). The adjuvant action of EDTA and TFP on A_{cmz} and A_{ect} was not influenced by the coadministration of cysteamine (Figs. 2 and 3).

The effect of calcium gluconate on the enhancing action of each adjuvant

As mentioned earlier, it has been reported that the increase in permeability induced by strong chelating agents involves deprivation of calcium ions. We therefore examined the effect of calcium gluconate on each adjuvant action.

As shown in Fig. 4, an increase in the concentration of calcium gluconate in the microenema inhibited the EDTA-induced increase in A_{cmz} and A_{ect}. A concentration of calcium gluconate greater than that of EDTA completely suppressed the adjuvant action. The enhancing action of TFP and DEM was not influenced, however, by the coadministration of calcium gluconate. The action of both DEEMM and salicylate on A_{ect} was suppressed to a greater degree by calcium gluconate in comparison with their action on A_{cmz}.

Discussion

The present study has compared the enhancing action of each adjuvant on colonic drug absorp-

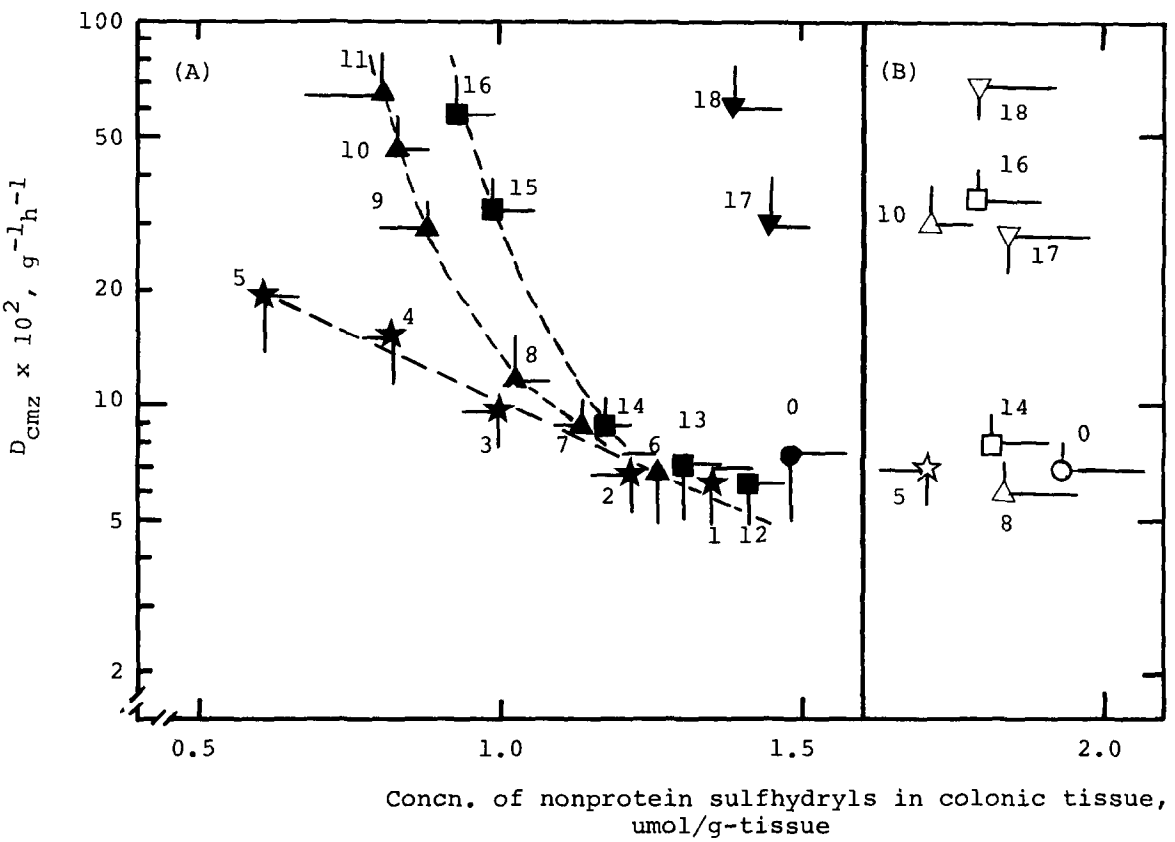

Fig. 3. The effect of varying concentration of each adjuvant on the disappearance clearance of cefmetazole (D_{cmz}) from the rat colonic loop and on reduced non-protein sulfhydryl levels in colonic tissue. The microenema containing cefmetazole and adjuvant was administered at a volume of 1 ml/kg. The concentration of cefmetazole in microenema was 10 mg/ml or 20 mg/ml, and at least 3 experiments were performed for each cefmetazole dose. Each number in (A) represents the concentration of DEM, DEEMM, sodium salicylate, EDTA or TFP as follows: 0, no adjuvant; 1, 0.25 mM DEM, 2, 0.5 mM DEM, 3, 1.25 mM DEM; 4, 2.5 mM DEM; 5, 3.75 mM DEM; 6, 1.5 mM DEEMM; 7, 3.75 mM DEEMM; 8, 7.5 mM DEEMM; 9, 12.5 mM DEEMM; 10, 25 mM DEEMM; 11, 50 mM DEEMM; 12, 50 mM salicylate; 13, 75 mM salicylate; 14, 100 mM salicylate; 15, 200 mM salicylate; 16, 400 mM salicylate; 17, 50 mM EDTA; and 18, 0.1 mM TFP. The effect of cysteamine on the adjuvant action was represented in (B). Cysteamine was added at 5 mM in microenema in (A) as described above. Each value represents the mean ± S.D., $n \geqq 6$.

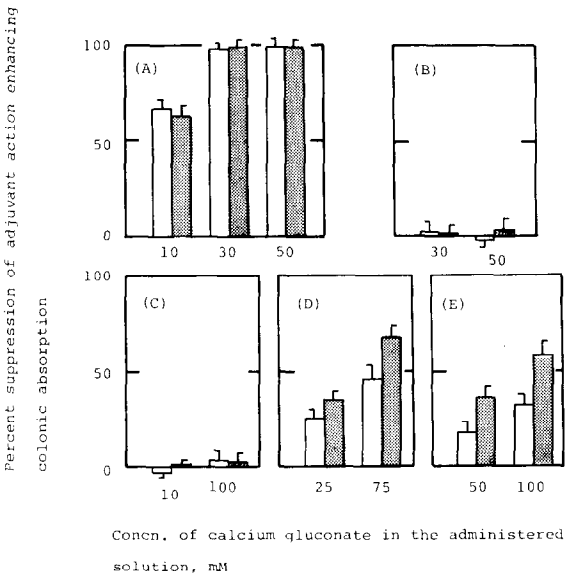

Fig. 4. The effect of calcium gluconate in the administered solution on the action of adjuvants such as EDTA (30 mM, A), trifluoperazine (0.05 mM, B), DEM (2.5 mM, C), DEEMM (25 mM, D) and sodium salicylate (200 mM, E), enhancing the colonic absorption of cefmetazole (□) and eel calcitonin (□). The dose of cefmetazole and eel calcitonin, and volume of administered solution for each adjuvant were described in Fig. 1. Effect of calcium gluconate was determined by the ratio of AUC of cefmetazole and eel calcitonin in the presence of calcium gluconate vs the ratio in the absence of calcium gluconate. Each value represents the mean ± S.D., $n \geqq 4$. [a] $P < 0.05$ vs cefmetazole study; [b] $P < 0.1$ vs cefmetazole study (Student's t-test).

tion using an in vivo rat colonic loop method. For the purposes of this study, the enhancing action of each adjuvant is classified as follows.

(1) The action of DEM predominantly increases the absorption of only relatively small molecules such as cefmetazole. This adjuvant action is accompanied by a significant decrease in the concentration of reduced non-protein sulfhydryls in the colonic tissue.

(2) The action of EDTA and TFP increases the colonic absorption of both cefmetazole and eel calcitonin without producing any significant effect on the reduced non-protein sulfhydryl concentration in the colonic tissue.

(3) The action of DEEMM and salicylate appears to involve either of the two processes mentioned above, depending on the concentration of either adjuvant in the microenema.

As mentioned earlier, it has been reported that strong chelating agents such as EDTA increase the in vitro intestinal mucosal permeability even to macromolecular substances such as albumin, via a paracellular route. Since this increased permeability via a paracellular route allows the transport of macromolecular substances, we suggest that the enhancing action of DEM is not operating via the paracellular route, but rather via an intracellular route. Therefore, the uptake of only relatively small molecules such as cefmetazole was seen. This suggested mechanism of DEM action correlated well with the decrease in reduced non-protein sulfhydryl concentration in the colonic tissue both in the present in vivo study and in the in vitro study reported previously (Nishihata et al., 1985).

It has further been demonstrated (Martinez-Paloma et al., 1980) that permeation of substances through cellular junctions, loosened by strong chelating agents, may depend on the diffusion coefficient which is determined primarily by molecular size and/or shape of the substances. Thus, the transport ratio between each substance via the paracellular route should be constant when the molecular size is less than albumin (mol. wt. ca, 75,000). This type of transport is mainly a diffusion process. From the present findings, we suggest that the enhancing action of TFP is operating via the paracellular route, since a constant ratio of A_{cmz}/A_{ect}, similar to that obtained with EDTA treatment, was obtained when TFP was coadministered. However, the enhancing action of TFP may occur by a somewhat different mechanism than EDTA, because the coadministration of calcium gluconate did not affect the action of TFP, but did suppress the action of EDTA. It should be interesting in future studies to investigate the relationship between the increase in paracellular transport induced by TFP and the calmodulin inhibiting activity of TFP, since the concentration of TFP used in the present study is sufficient for calmodulin inhibition (Farius et al., 1983).

Since the ratio of A_{cmz}/A_{ect} obtained without any adjuvants was very similar to that obtained by EDTA treatment, it would appear that colonic

absorption of cefmetazole and eel calcitonin normally occurs dominantly via paracellular route, but at a very low rate.

The enhancing action of both salicylate and DEEMM showed similar phenomena with their concentration dependency on the colonic absorption of cefmetazole and eel calcitonin and with partial suppression of either adjuvant by coadministration of calcium gluconate. Their action at lower concentrations may be explained in terms of an intracellular route as with DEM, but at high concentrations these adjuvants may also operate via the paracellular route. Both of these adjuvants at higher concentrations increase eel calcitonin absorption and decrease the ratio of A_{cmz}/A_{ect}.

We conclude from the present study, that the enhancing action of EDTA on colonic absorption of poorly absorbed compounds occurs paracellularly. The enhancing action of DEM occurs probably via an intracellular route, and is seen only with relatively low molecular weight compounds. The enhancing action of salicylate and DEEMM at low concentration appears to occur via the intracellular route, whereas at high concentration, it predominantly takes place via the paracellular pathway. TFP may also increase the absorption of poorly absorbed drugs via the paracellular route, but probably by a different mechanism than that of EDTA.

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