J.Pharmacobio-Dyn., **8**, 278—285 (1985)

INTESTINAL ABSORPTION MECHANISMS OF γ-BUTYROLACTONE-γ-CARBONYL-L-HISTIDYL-L-PROLINAMIDE CITRATE (DN-1417) AND THYROTROPIN-RELEASING HORMONE (TRH)

SHIGEHARU YOKOHAMA, TOSHIO YOSHIOKA, NOBUYUKI KITAMORI, TSUGIO SHIMAMOTO* AND AKIRA KAMADA**

Central Research Division, Takeda Chemical Industries, Ltd., Juso-honmachi, Yodogawa-ku, Osaka, 532, Japan and Faculty of Pharmaceutical Science, Osaka University,** 133-1 Yamada Kami, Osaka, 565, Japan*

(Received September 17, 1984)

The absorption mechanisms of γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417) and thyrotropin-releasing hormone (TRH) were studied in the rat. *In situ* absorption experiments were carried out by the radioimmunoassay, and experiments using everted sacs of small intestine were by radioactivity measurements with ^{14}C-labeled DN-1417 or ^{3}H-labeled TRH in the low concentration range of drug and by a high pressure liquid chromatography in the rather high concentration range of drug. The site specificity of absorption in the small intestine of rats could not be found with DN-1417, whereas TRH-T was absorbed from only the upper part of small intestine. Dose-proportional absorption of DN-1417 was observed in experiments of *in situ* as well as *in vitro*. Dose-proportional transfer of DN-1417 through the everted small intestine was also found within the concentration range from 120 ng/ml to 27 mg/ml, whereas the transfer ratio of TRH decreased with increase in the concentration of TRH. DN-1417 transfer from mucosal to serosal fluid was not inhibited by the replacement of medium Na ions by K ions, pretreatment of intestinal mucosa with $HgCl_2$, the existence of an oligopeptide, or the existence of β-lactam antibiotics which had been reported to be absorbed by active transport or carrier-mediated transport systems. While, TRH transfer was inhibited by the replacement of medium Na ions by K ions, pretreatment of intestinal mucosa with $HgCl_2$, the existence of an oligopeptide, and the existence of β-lactam antibiotics. From these results, it was concluded that DN-1417 transfer through the small intestine was mainly due to the simple diffusion, and the contribution of the carrier-mediated transport to TRH transfer through the small intestine was not disregarded.

Keywords — γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate; DN-1417; thyrotropin-releasing hormone; L-pyroglutamyl-L-histidyl-L-prolinamide-L-tartrate; radioimmunoassay; intestinal absorption mechanism; carrier-mediated transport; active transport; oligopeptide; β-lactam antibiotic

INTRODUCTION

Several previous studies on absorption of oligopeptides have suggested the possible existence of a specific carrier-mediated process which was different from the absorption mechanisms for amino acids.[1-4] Also, studies on absorption of antibiotics, which were regarded as one of oligopeptides, have revealed the existence of active transport for cyclacillin, of the facilitated diffusion mechanisms for amoxicillin, and no evidence of carrier-mediated transport for ampicillin.[5,6] Recently, we investigated the transport mechanisms of thyrotropin-releasing hormone tartrate (TRH-T), L-pyroglutamyl-L-histidyl-L-prolinamide-L-tartrate, in rats, and found the site specificity of absorption as well as the saturated absorption phenomenon in the *in situ* and everted sac experiments.[7-9] Then, a search was made for DN-1417, γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate, an analog of TRH and one of oligopeptides. Though the absorption of DN-1417 decreased

with food ingestion in the same manner as TRH-T, intestinal absorption of DN-1417 was somewhat different to that of TRH-T, *i.e.*, an apparent saturation of DN-1417 absorption was not observed.[10] The purpose of this paper is to characterize the absorption of DN-1417 compared with that of TRH-T through experiments of *in situ* and everted sacs of small intestine in rats.

MATERIALS AND METHODS

Animals —Experimental animals used in this study were male Sprague-Dawley (JCL: SD, SPF) rats weighing 180—220 g.

Materials — γ-Butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417), DN-1417-isobutylamide, the antiserum of DN-1417-isobutylamide, ^{125}I-labeled tracer were described previously.[10] L-Pyroglutamyl-L-histidyl-L-prolinamide-L-tartrate monohydrate, Takeda, (TRH-T), ^{125}I-TRH, anti-TRH serum, goat anti-rabbit γ-globulin serum and normal rabbit serum were prepared as described in our report.[7] ^{3}H-TRH (Fig. 1, B) was purchased from New England Nuclear, Boston, Mass., USA, and the specific activity was 90.0 Ci/mmol. ^{14}C-DN-1417 (Fig. 1, A) was synthesized in Chemical Laboratories of Takeda Chemical Ind. Ltd., and the specific activity was 33.97 μCi/mg. 2,5-Diphenyloxazole (DPO), 1,4-bis (2-methylstyryl) benzene (Bis-MSB) and 1,4-bis(2-(5-phenyloxazolyl))benzene (POPOP) were purchased from Wako Pure Chemical Ind. Ltd., Osaka, Japan. All other materials and solvents were of analytical reagent grade and used without further purification.

In Situ Absorption Studies in Rats —Rats were fasted 24 h prior to the experiment. Rats were injected intraperitoneally with 100 mg/kg of sodium phenobarbital and 50 mg/kg of sodium pentobarbital to induce anesthesia and used for the *in situ* absorption studies similarly as described by Noguchi.[11] The bile duct was ligated. One milli-liter of the drug solution was injected into the loop of upper part of small intestine (10 cm length from pylorus), middle part of small intestine (10 cm), or lower part of small intestine (10 cm). For the measurement of DN-1417 the heparinized arterial blood samples were periodically collected from the jugular artery and 50—100 μl of plasma was obtained by centrifugation at 4 °C, 3000 rpm for 5 min and poured into a test tube containing 2 ml of isobutylamine-isopropanol (1 : 4) mixture. The mixture was centrifuged at 3000 rpm, 4 °C for 5 min. The supernatant was kept at 25 °C for 2 h to convert DN-1417 to DN-1417-isobutylamide and then evaporated under dried N_2 gas at 40—50 °C. The sample was dissolved in 1 ml of distilled water. After the sample solution was treated by SEP-PAK™ C_{18} and washed twice with ethyl ether[10,12] the measurement of DN-1417 was performed by the radioimmunoassay.

Permeation through the Small Intestine —The everted sac (10 cm length) of the small intestine of rats was prepared according to the method of Wilson and Wiseman.[13] The everted sac was placed in a 50 ml flask with 30 ml of modified Ringer's solution. The flask was gassed with the mixture of 95% O_2 and 5% N_2 gas, and main-

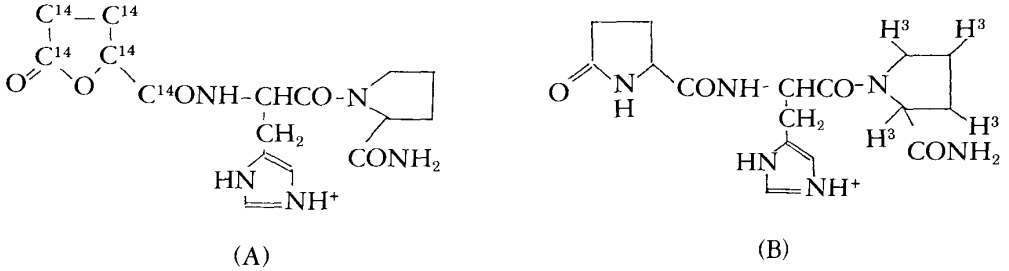

FIG. 1. *Chemical Structures of ^{14}C-Labeled DN-1417 (A) and ^{3}H-Labeled TRH (B)*

tained at 37 °C. The serosal initial volume was 1 ml. At the end of the incubation period (normally 30 min), the inner solution of each sac was poured into a test tube.

Assays — Radioimmunoassay of DN-1417 was performed by the procedure described previously,[10,12] and radioimmunoassay of TRH was detailed in another report.[7] The condition of the high pressure liquid chromatography (HPLC) for the measurements of DN-1417 and TRH was as follows. HPCL, LC-4A (Shimadzu, Kyoto, Japan) equipped with an ultraviolet detector, SPD-2A, was used in a reversed phase with a μ Bondapak C_{18} stainless column (30.5 cm × 4.0 mm i.d.; Waters Assoc., Milford Mass., USA). A mobile phase of methanol-water mixture containing 0.02 M KH_2PO_4 for the assay of DN-1417 and TRH was 5 : 95 (by volume) and the flow rate was 1 ml/min. The wave length of the detector was 225 nm. The drug concentration was calculated from the peak height using the calibration curve. The counting samples for ratioacitiviy were burned by Automatic Sample Combustion System Aloka (Aloka-ASC-113, Tokyo, Japan) and scintillation mixture were added. The scintillation mixtures for ^{14}C-samples consisted of 12 ml of toluene solvent (15 g of DPO and 1 g of Bis-MSB were dissolved in 1 l of toluene) and 6 ml of Oxisorb™-NEN (New England Nuclear). That for ^{3}H-samples was 20 ml of dioxane solvent (100 g of naphthalene, 12 g of DPO, 0.3 g of POPOP were dissolved in the mixtures of 720 ml of dioxane, 135 ml of toluene and 45 ml of methanol). The radioactivity was counted in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Curves of DN-1417 plasma concentration and

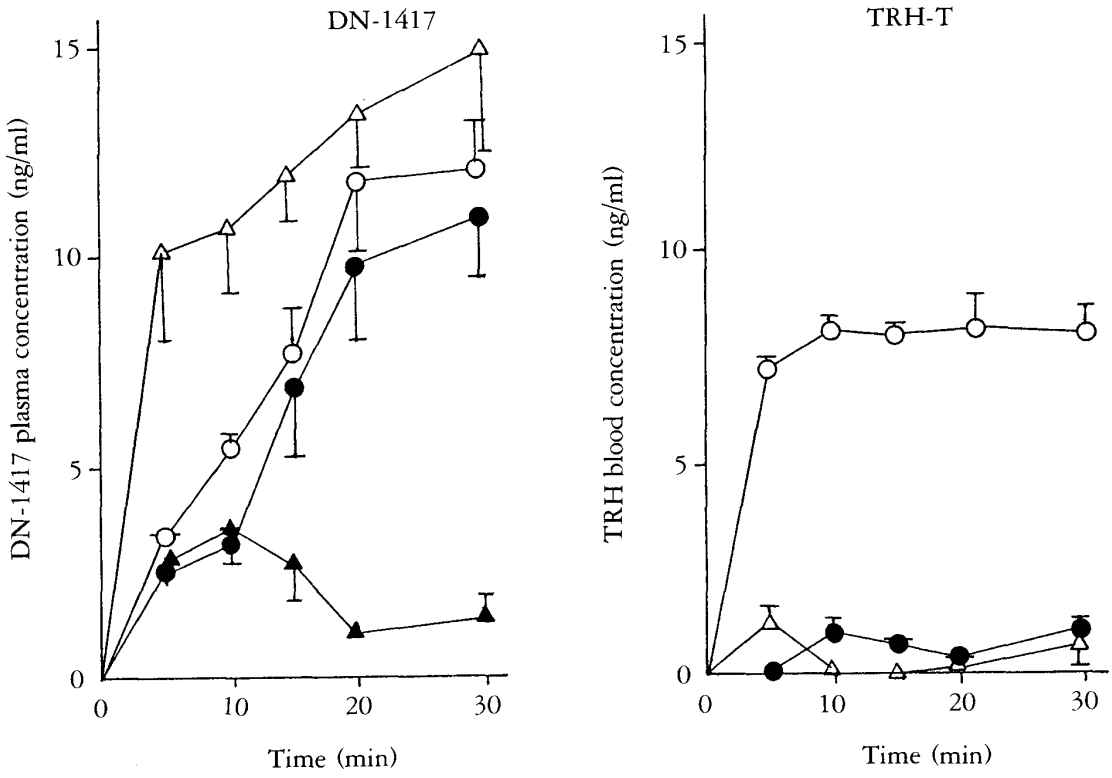

FIG. 2. *Plasma DN-1417 and Blood TRH Levels on in Situ Absorption Experiments from Rat Small Intestinal Loop*

○, *upper part;* ●, *middle part;* △, *lower part;* ▲, *stomach.*
Each point represents the mean and vertical bar indicates S. E. (n= 4).

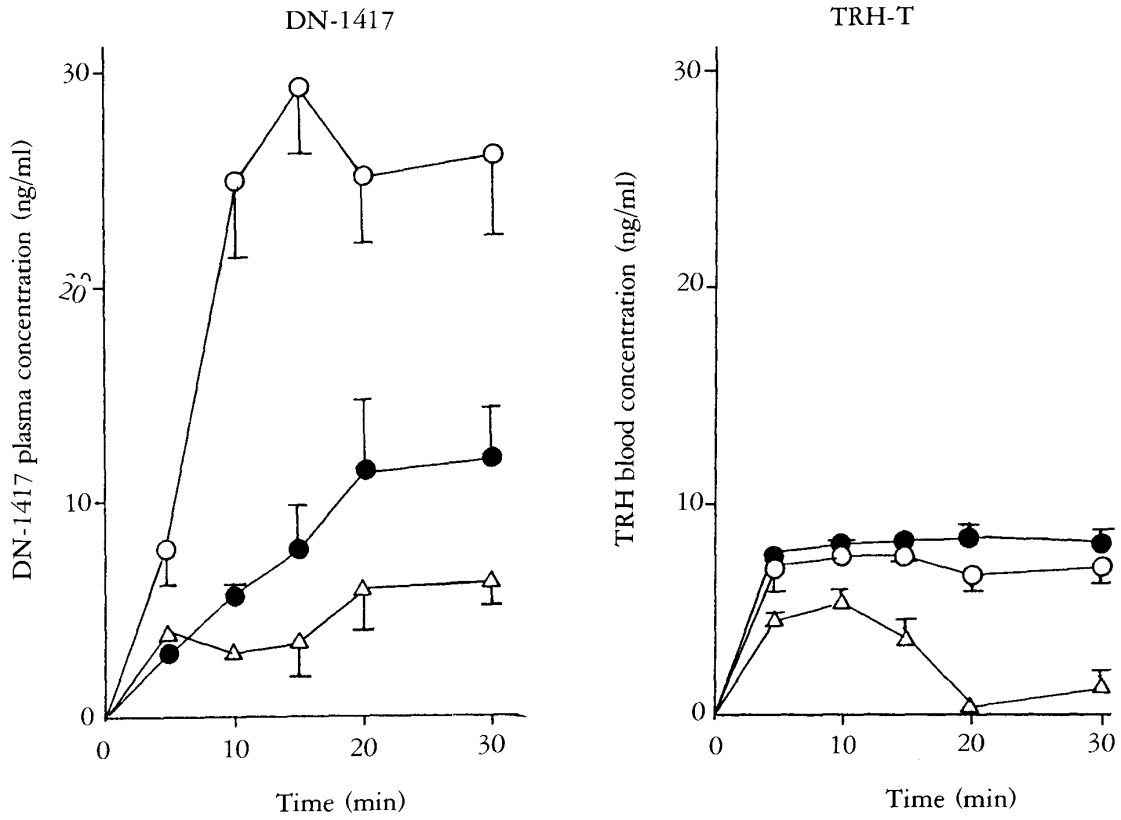

FIG. 3. *Plasma DN-1417 and Blood TRH Levels on in Situ Absorption Experiments from Rat Small Intestinal Loop*
○, *1.0 mg/kg;* ●, *0.2 mg/kg;* △, *0.04 mg/kg.*
Each point represents the mean and vertical bar indicates S. E. (n=4).

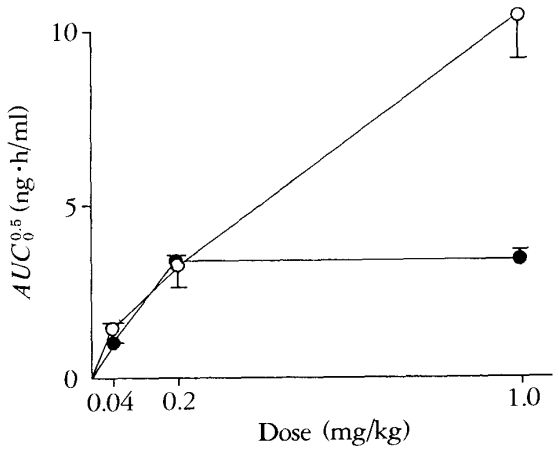

FIG. 4. *The Relation between Dose and AUC of DN-1417 and TRH in the in Situ Intestinal Loops (n=4)*
○, *DN-1417;* ●, *TRH.*
Each point represents the mean and vertical bar indicates S. E. (n=4).

TRH blood concentration obtained in the *in situ* absorption experiments using small intestine loops of rats are shown in Figs. 2 and 3. Does of 0.04, 0.2, and 1.0 mg/kg DN-1417 or TRH (eq. 0.06, 0.29, and 1.46 mg of TRH-T, respectively) were injected into the loop of the upper small intestine, and dose of 0.2 mg/kg of DN-1417 or TRH was also injected into the loop of the middle or lower part of small intestine or stomach.

It was found that TRH-T was absorbed mainly from the upper small intestine, but little or no TRH-T was absorbed from the middle and lower part of small intestine.[9] Whereas, DN-1417 was absorbed from all over the small intestine though it was hardly absorbed from stomach. Oligopeptides, both di-and tripeptides were reported to be absorbed from all parts of small intestine even if the absorption rate was slightly different.[14] DN-

1417 absorption was similar to the absorption of oligopeptides (di-or tripeptides) but TRH-T absorption was quite different. TRH-T showed the apparent saturation phenomenon of the absorption from the upper small intestine, while DN-1417 showed the dose-proportional absorption (Fig. 4).

Results of DN-1417 and TRH transfer from

TABLE I. *The Effect of Drug Concentration on the Transfer of DN-1417 and TRH through Everted Sacs of Rat Small Intestine*

Drug concentration	DN-1417 transfer S/M ratio (%)[a] and SE		TRH transfer S/M ratio (%)[a] and SE	
120 ng/ml	8.5	0.7	17.5	1.5
400	9.8	0.8	18.8	1.3
1 μg/ml	9.0	1.2	15.8[b]	1.1
10	—	—	14.6[b]	1.6
100	—	—	13.6[c]	1.2
3 mg/ml	10.4	1.3	—	—
9	9.6	1.7	—	—
27	10.3	1.5	—	—

Each datum represents the average of six to twenty experiments. a) shows S/M ratio: final serosal-to-mucosal fluids concentration ratio. The significant difference to the S/M ratio in the medium concentration of 120 ng/ml is expressed with b) ($p < 0.05$) and c) ($p < 0.01$).

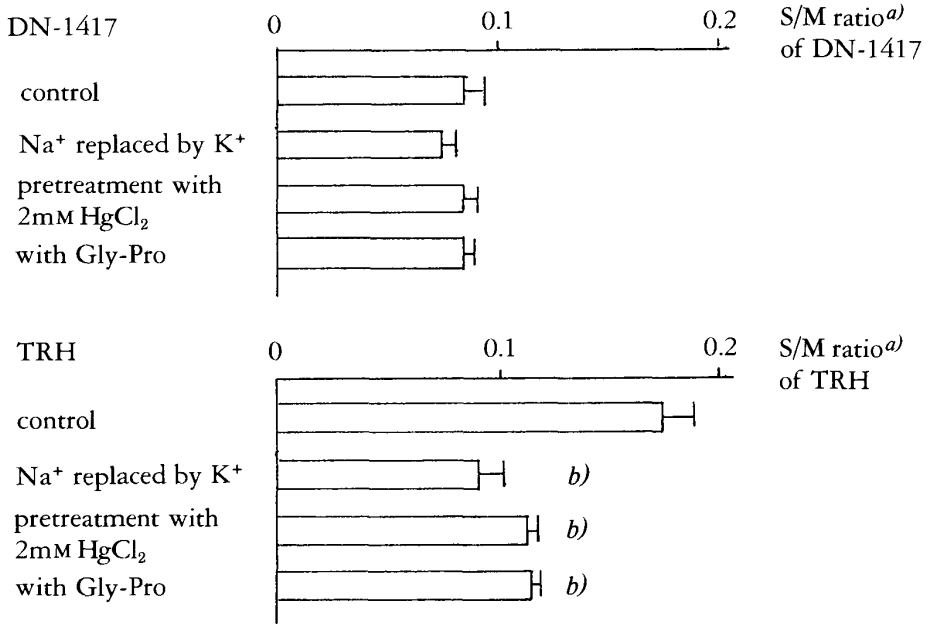

FIG. 5. *Transfer of DN-1417 or TRH through Everted Sac of Rat Small Intestine*
Initial DN-1417 or TRH concentration in mucosal side is 120 ng/ml. Each column represents the mean and horizontal line indicates S.E. (n= 6–12).
a) shows S/M ratio: final serosal-to-mucosal fluids concentration ratio. The significant difference against the control is expressed with b) ($p < 0.01$).

mucosal to serosal fluids in everted sac experiments were shown in Table I. The experiments were performed using isotopic substances at the dose below 1 μg/ml and cold substances, which were analyzed by an HPLC method, at the dose above 10 μg/ml. Tsuji[15] studied the dose dependency on the disappearance of aminopenicillins and aminocephalosporins from the rat intestinal perfusate, and reported the saturable absorption of some aminopenicillins and aminocephalosporins. However, values of K_m (Michaelis-Menten kinetic parameter) varied with comporins. However, values of K_m (Michaicillin, cyclacillin and cephalexin, respectively.[16] Therefore, it was necessary to investigate the absorption behaviour over a wide range of drug concentration in order to confirm the existence of saturable absorption. In this paper, the dose-dependent transfer of TRH and DN-1417 were examined by the everted sac experiments ranging in concentration from 120 ng/ml to 100 μg/ml for TRH and from 120 ng/ml to 27 mg/ml for DN-1417. The transfer of TRH through the everted sac of rat small intestine showed a slight decrease with an increase of TRH concentration. This result coincided with the previous report.[9] Whereas, that of DN-1417 showed a dose-proportion over the entire concentration range.

Fig. 5 shows the effects of the replacement of Na ions by K ions in the medium, pretreatment of the everted sac of small intestine with $HgCl_2$ and the existence of an oligopeptide (Gly-Pro) on the transfer of ^{3}H-TRH and ^{14}C-DN-1417. A certain degree of inhibition was found for the transfer of TRH, but no apparent inhibition by these factors was observed for the transfer of DN-1417. Moreover, Fig. 6 shows the effects of the existence of some β-lactam antibiotics on the transfer of these drugs. The transfer of TRH was inhibited by these β-lactam antibiotics significantly, whereas, that of DN-1417 was not inhibited apparently. These results of TRH also coincided with previous report.[9]

Rubino *et al.*[23] showed the Na ions depen-

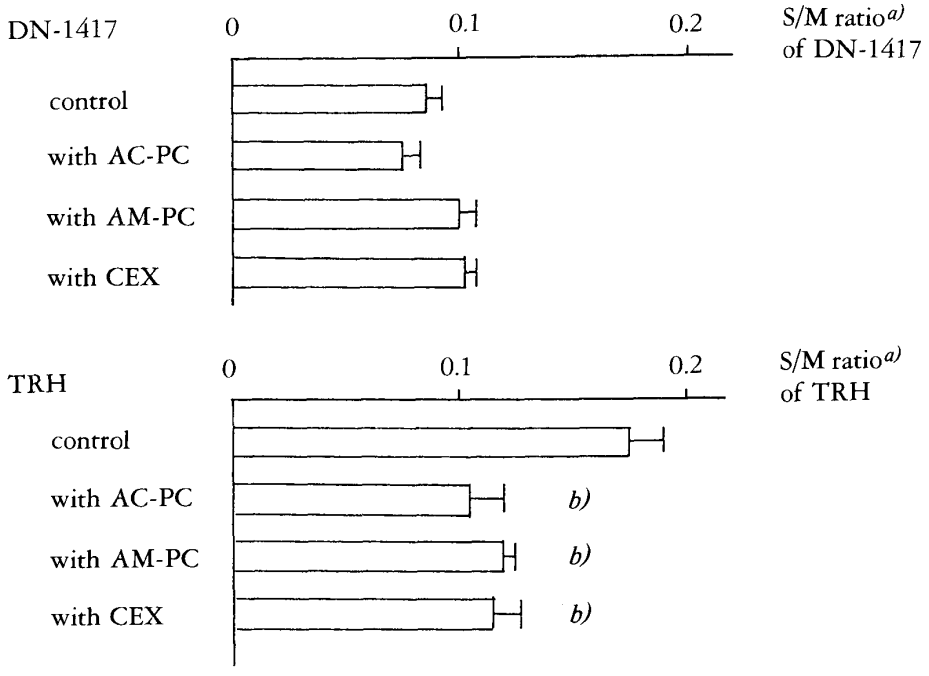

FIG. 6. *Transfer of DN-1417 and TRH through Everted Sac of Rat Small Intestine*
Initial DN-1417 or TRH concentration in mucosal side is 120 ng/ml. Each column represents the mean and horizontal line indicates S.E. (n= 6– 12) a) shows S/M ratio: final serosal-to-mucosal fluids concentration ratio. The significant difference against the control is expressed with b) ($p < 0.01$).

dence of oligopeptides influx into rabbit ileal mucosa *in vitro*. Also, Berteloot *et al.*[24] examined the Na ions gradient dependence to the transfer of an amino acids *in vitro* and reported the over shoot phenomenon by Na ions.

Klip *et al.*[19] reported the effects of short term pretreatment of intestinal mucosa with a low concentration of $HgCl_2$ on the sulfhydryl groups of the apical plasma membrane of intestinal epithelial cells, and showed the strong inhibition of sugar transport. Furthermore, Kimura found the inhibition of aminopenicillins transport by the treatment of intestinal mucosa with $HgCl_2$.[6] As is evident from Fig. 5, the transfer of DN-1417 was not inhibited but that of TRH was inhibited significantly by the treatment with $HgCl_2$, suggesting the contribution of protein or sulfhydryl groups of the small intestine to the transfer of TRH but not to that of DN-1417.

Absorption mechanisms of some β-lactam antibiotics have been examined by some investigators,[5,6,15-18,20] and the existence of carrier-mediated or/and active transport systems have been proposed. The phenomena of TRH transfer being inhibited by the replacement of Na ions by K ions in the medium, the treatment of intestinal mucosa with $HgCl_2$, and the existence of an oligopeptide or some β-lactam antibiotics revealed the existence of carrier-mediated transport systems in the small intestine. On the other hand, the contribution of carrier-mediated transport systems to DN-1417 transfer was much smaller than that of TRH or none.

The reason of these phenomena is not clear now. However, in the studies of absorption mechanisms for oligopeptides by Agar,[1] Wiggans,[2] Craft,[3] and Adibi,[4,21] the influence of molecular structure on the transport of peptides through the small intestine was described. If the amino- or carboxy-terminal group in the molecule is substituted for other group, its affinity to transport carrier is reduced or abolished. Recently, Amidon described in his report the importance of a free α-amino group in amino acids derivatives of drugs for absorption.[22] DN-1417, γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate, is substituted at both the amino- and carboxy-terminal groups. Therefore, DN-1417 would have very poor or no affinity to the oligopeptide transport systems.

Acknowledgements The authors would like to acknowledge the continuing guidance of Dr. Y. Nagawa, Takeda Chemical Industries Ltd.

REFERENCES

1) W. T. Agar, F. J. R. Hird and G. S. Sidhu: The active absorption of amino-acids by the intestine, *J. Physiol.*, **121**, 252–263 (1953).
2) D. S. Wiggans and J. M. Johnstone: The absorption of peptides, *Biochim. Biophys. Acta*, **32**, 69–73 (1959).
3) I. L. Craft, D. Geddes, C. W. Hyde, I. J. Wise and D. M. Matthews: Absorption and malabsorption of glycine and glycine peptides in man, *Gut*, **9**, 425–437 (1968).
4) S. A. Adibi and D. W. Mercer: Protein digestion in human intestine as reflected in luminal, mucosal and plasma aminoacid concentrations after meals, *J. Clin. Invest.*, **52**, 1586–1594 (1973).
5) J. M. Addison, D. Burston, J. A. Dalrymple, D. M. Matthews, J. W. Payne, M. H. Sleisenger and S. Wilkinson: A common mechanism for transport of di- and tripeptides by hamster jejunum *in vitro*, *Clin, Sci. Mol. Med.*, **49**, 313–322 (1975).
6) T. Kimura, H. Endo, M. Yoshikawa, S. Muranishi and H. Sezaki: Carrier-mediated transport systems for aminopenicillins in rat small intestine, *J. Pharmacobio-Dyn.*, **1**, 262–267 (1978).
7) S. Yokohama, K. Yamashita, H. Toguchi and N. Kitamori: Radioimmunoassay of thyrotropin-releasing hormone (TRH) in rat, dog and human blood, *J. Pharmacobio- Dyn.*, **6**, 627–636 (1983).
8) S. Yokohama, K. Yamashita, H. Toguchi, J. Takeuchi and N. Kitamori: Absorption of thyrotropin-releasing hormone (TRH) after oral administration of TRH tartrate monohydrate in the rat, dog and human, *J. Pharmacobio- Dyn.*, **7**, 101–111 (1984).
9) S. Yokohama, T. Yoshioka, K. Yamashita and N. Kitamori: Intestinal absorption mechanisms of thyrotropin-releasing hormone, *J. Pharmacobio- Dyn.*, **7**, 445–451 (1984).
10) S. Yokohama, T. Yoshioka and N. Kitamori: Absorption of γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate (DN-1417), an analog of thyrotropin-releasing hormone, in rats and dogs, *J. Pharmacobio- Dyn.*, **7**, 527–535 (1984).
11) T. Noguchi, C. Takahashi, T. Kimura, S. Muranishi and H. Sezaki: Mechanisms of the intestinal absorption of drugs from oil-in-water emulsion. IV. Absorption of

lipid-soluble dyes from tributyrin and triolein emulsions in rat small intestine, *Chem. Pharm. Bull.*, **23,** 775—781 (1975).
12) K. Shiota, A. Miyake, C. Tsunoda, Y. Oka, Y. Nagawa and R. Nakayama: Radioimmunoassay for an analog of thyrotropin-releasing hormone, γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide (DN-1417), *J. Immunol. Methods,* **51,** 69—80 (1982).
13) T. H. Wilson and G. Wiseman: The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface, *J. Physiol.*, **123,** 116—125 (1954).
14) R. F. Crampton, M. T. Lis and D. M. Matthews: Sites of mucosal absorption and hydrolysis of two dipeptides by rat small intestine *in vitro, Clin. Sci.*, **44,** 583—594 (1973).
15) A. Tsuji, E. Nakashima, A. Asano, R. Nakashima and T. Yamana: Saturable absorption of amino-cephalosporins by the rat intestine, *J. Pharm. Pharmacol.*, **31,** 718—720 (1979).
16) E. Nakashima, H. Mizuo, A. Tsuji and T. Yamana: Carrier-mediated transport mechanism in the rat intesinal absorption of amino-β-lactam antibiotics, *J. Pharmacobio- Dyn.*, **6,** s-93 (1983).
17) J. F. Quay: Transport interaction of glycine and cephalexin in rat jejunum, *Physiologist,* **15,** 241 (1972).
18) K. Umeniwa, O. Ogino, K. Miyazaki and T. Arita: Intestinal absorption of several β-lactam antibiotics. II. Absorption characteristics of amino-penicillins and amino-cephalosporins, *Chem. Pharm. Bull.*, **27,** 2177—2182 (1979).
19) A. Klip, S. Grinstein, J. Biber and G. Semenda: Interaction of the sugar carrier of intestinal brush-border membranes with $HgCl_2$, *Biochim. Biophys. Acta,* **598,** 100—114 (1980).
20) T. Kimura, T. Yamamoto, M. Mizuno, S. Suga, S. Kitade and H. Sezaki: Characterization of amino-cephalosporin transport across rat small intestine, *J. Pharmacobio- Dyn.*, **6,** 246—253 (1983).
21) D. M. Matthews and S. A. Adibi: Progress in gastroenterology, "peptide absorption", *Gastroenterology,* **71,** 151—161 (1976).
22) G. L. Amidon, M. Chang, D. Fleisher and R. Allen: Intestinal absorption of amino acid derivatives: Importance of the free α-amino group, *J. Pharm. Sci.*, **71,** 1138—1141 (1982).
23) A. Rubino. M. Field and H. Shwachman: Intestinal transfer of amino acid residues of dipeptides. 1. Influx of the glycine residue of glycyl-L-proline across mucosal border, *J. Biol. Chem.*, **246,** 3542—3548 (1971).
24) A. Berteloot, A. H. Khan and K. Ramaswamy: K^+- and Na^+-Gradient-dependent transport of L-phenylalanine by mouse intestinal brush border membrane vesicles, *Biochim. Biophys. Acta,* **691,** 321—333 (1982).