[Chem. Pharm. Bull. 22(5)1029—1034(1974)]

UDC 547.466.1.09 : 615.36.015.4

Studies on the Passage of α-Chymotrypsin across the Intestine. II.[1] Enzymic Activity and Radioactive Macromolecule recovered in the Mesenteric Perfusate

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(Received July 18, 1973)

A perfusion with Krebs–Ringer solution containing dextran through the mesenteric vascular system was performed on rat to which ^{131}I-labelled α-chymotrypsin was administered in the jejunum. This method sufficed for the purpose of eliminating various blood components, such as the inhibitors for the enzyme, which interfer with quantitative assay of a small amount of the enzyme absorbed. Using the method, the non-diffusible ^{131}I-bound substance(s) and N-acetyl-L-tyrosine ethyl ester(ATEE)-hydrolyzing activity were found in the mesenteric perfusates of the rats, and the amount of the enzyme absorbed during 90 min was estimated to be about 0.6% of the infused dose. Furthermore, when the immunoreactivity with the antiserum against α-chymotrypsin was examined on the mesenteric perfusate, a precipitin band sharply indicating the enzyme was formed with the perfusate collected during the first 30 min after the administration. These results strongly suggest that small but significant amount of α-chymotrypsin is transmitted across the intestinal wall into the mesenteric vascular system as retaining its enzymic and immunochemical properties.

In the previous paper,[1] the authors reported that the radioactivity distribution in rat tissues after the administration of ^{131}I-chymotrypsin in the intestine was different from that of ^{131}I-KI, and that radioactive macromolecule(s) was also found in the serum of ^{131}I-chymotrypsin-given rat. Furthermore, the passages of ^{131}I-tagged macromolecule(s) and a substance which possessed N-acetyl-L-tyrosine ethyl ester-hydrolyzing activity through the intestinal wall were demonstrated in *in vitro* experiment using a sac of everted intestine.

From these results, it was supposed that a small amount of α-chymotrypsin seemed to be transferred across the rat intestine without losing its enzymic property. Further experiment has failed in an attempt of demonstration of the enzymic activity and immunological reactivity of α-chymotrypsin in the portal blood after the intestinal administration of the enzyme, probably being due to the presence of its inhibitors in the blood. The mesenteric perfusion performed on a rat to avoid this interference could demonstrate the enzymic activity and radioactive macromolecule in the perfusate recovered from the mesenteric vein after giving ^{131}I-chymotrypsin in the jejunum.

In the present paper, details of the mesenteric perfusion and the results of transmission of α-chymotrypsin from the intestine to the mesenteric vascular system are described.

Material and Method

α-Chymotrypsin (abbr. as CT)——A purified preparation extracted from hog pancreas according to Kunitz's method[3] (5.74 CT unit/mg protein) was kindly supplied from Dr. Matsuoka, Eizai Co. Ltd., Tokyo.

1) Part I: H. Moriya, C. Moriwaki, S. Akimoto, K. Yamaguchi, and M. Iwadare, *Chem. Pharm. Bull.* (Tokyo), **15**, 1662 (1967).
2) Location: *Funakawara-cho, Ichigaya, Shinjuku-ku, Tokyo.*
3) M. Kunitz, J.H. Northrop, and R. Herriott, "Crystalline Enzyme," 2nd ed., Columbia Univ. Press, New York, 1948.

Determination of the Esterolytic Activity of CT——As described in the previous paper,[1] the digestive activity upon N-acetyl-L-tyrosine ethyl ester (ATEE, Sigma Chemical Co.) was measured by the method of Kabacoff[4] with slight modifications.

Preparation of ^{131}I-labelled CT——CT was labelled with ^{131}I by a minor modification of the chloramine T method.[5] While stirring, 1 mCi ^{131}I-NaI (Dainabott RI Lab.) was added to 3.0 mg CT in 0.1 ml of 0.05 M phosphate buffer, pH 7.5, and the mixture was exposed to 100 μg chloramine T in 0.05 ml buffer. This mixture stood for 30 sec before addition of 120 μg sodium metabisulfite in 0.05 ml buffer. Into the reaction mixture 60 mg CT as carrier was added. It was then applied on a Sephadex G-50 column (1.5×30 cm) and eluted with the buffer to separate ^{131}I-CT from non-reacted ^{131}I-iodide.

The protein fractions were combined in a Visking tube (Visking Co.) and concentrated to 1/5 volume by covering the tube with dry Sephadex powder at 4°, This concentrated solution ($1-10\times10^6$ cpm/ml) containing 50 mg/ml of CT was used as ^{131}I-CT. More than 90% of the radioactivity in this ^{131}I-CT preparation was proved to be derived from radioactive macromolecule by a dialysis and a quantitative radio-paper-chromatography,[6] while the labelling processes caused no decrease of the enzymic activity. Radioactivity was counted by Aloka scintillation counter with a well type sodium iodide crystal (2″φ×2).

Anti-CT Rabbit Serum——A 0.5% CT solution in physiological saline was emulsified with an equal volume of Freund's complete adjuvant (Iatron Lab., Tokyo). About 2 ml of this emulsion was injected intracutaneously in all the foot pads and various positions on the back of a rabbit weighing about 2.5 kg. After 30 days the booster injections of the same emulsion were performed either intra- or subcutaneously once a week for 3 weeks, and whole blood was collected 5 days later the last injection. The antiserum was incubated at 56° for 30 min and stored at −20°. This antiserum formed only one precipitin band against CT in immunoelectrophoresis and was able to detect the antigen at the concentrations from 20 μg/ml upwards by a micromethod of double immunodiffusion.[7]

Double Immunodiffusion on Agar-Dextran Media[8]——A micromethod of Ouchterlony technique was employed. A solution of 1.5% agar in 0.05 M Veronal buffer containing dextran, pH 7.4, was poured on a glass plate (2.6×7.5 cm) and allowed to gellify to give 1 mm thick layer. The wells of 2 mm in diameter were filled with the antigen and the antiserum, and the plates were kept in a humid chamber for 5 hr at 4°. Thereafter, the precipitates were inspected directly or stained by 1% Amidoschwarz 10 B after the elimination of soluble proteins.

Mesenteric Perfusion——Male Wistar-strain rats, 140 g body weight, which were fasted from the preceding day were anesthetized by intraperitoneal injection of pentobarbital (62.5 mg/kg). Cannulation into the superior mesenteric artery was carried out by placing a polyethylene tubing at the position of the artery branched out from the aorta, and perfusion with Krebs–Ringer bicarbonate buffer (pH 7.4) containing 6% dextran gassed with 95% O_2 and 5% CO_2 was performed with a peristaltic pump (LKB, Sweden) through this cannula (15 ml/hr). The perfusate was recovered from a polyethylene catheter (PE 50, Clay Adams, USA) inserted into the superior mesenteric vein. Cannulation to the blood vessel was accomplished by direct puncture with a beveled segment of polyethylene tubing. The *in situ* mesenteric perfusion system is illustrated in Fig. 1. After preliminary perfusion for 30 min, 0.3 ml of ^{131}I-CT ($3-40\times10^5$ cpm) was injected into a segment of the jejunum ligated at both ends where the cannulated mesenteric vein distributed. The perfusion was continued for 90 min after the sample administration and the perfusate was pooled every 30 min.

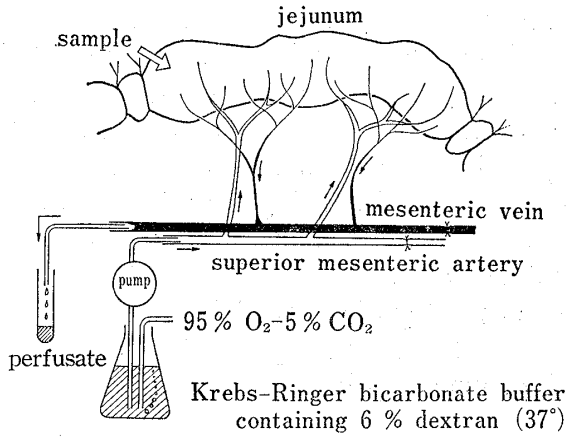

Fig. 1. Scheme of *in situ* Mesenteric Perfusion System

Collected perfusate samples were determined their radioactivities and ATEE-digestive potencies, and were dialyzed against distilled water adjusted pH to 8.0 to separate the radioactive macromolecule(s).

Concentration of the Perfusate——To some of the perfusate samples, casein (Hammarsten) was added to make 1% of the final concentration and an equal volume of saturate solution of $(NH_4)_2SO_4$ was then added. The precipitate formed was dissolved in a small amount (1/10—1/20 of the initial volume) of

4) L. Kabacoff, M. Umhey, and S. Avakian, *J. Pharm. Sci.*, **52**, 1188 (1963).
5) F.C. Greenwood and W.M. Hunter, *Biochem. J.*, **89**, 114 (1963).
6) Y. Ito, C. Moriwaki, and H. Moriya, *Endocrinol. Japon.*, **13**, 448 (1966).
7) O. Ouchterlony, *Acta Path. Microbiol. Scand.*, **25**, 186 (1948).
8) M. Ceska and F. Grossmuller, *Experientia*, **124**, 391 (1968).
9) K. Hellsing, *Acta Chem. Scand.*, **20**, 1251 (1966).

0.9% NaCl solution containing dextran.[9] This concentrated samples were served for the immunochemical analysis.

Result

Immunoreactive Substance in Mesenteric Perfusate

On the ring test[10] in which the antigen was layered over the antiserum in a capillary tube, the perfusate collected after CT administration formed a precipitate at the interface. Furthermore, one clear band which fused with that of administered CT was formed on the immunodiffusion plate only by the concentrated perfusate collected during 30 min after CT administration (Fig. 2).

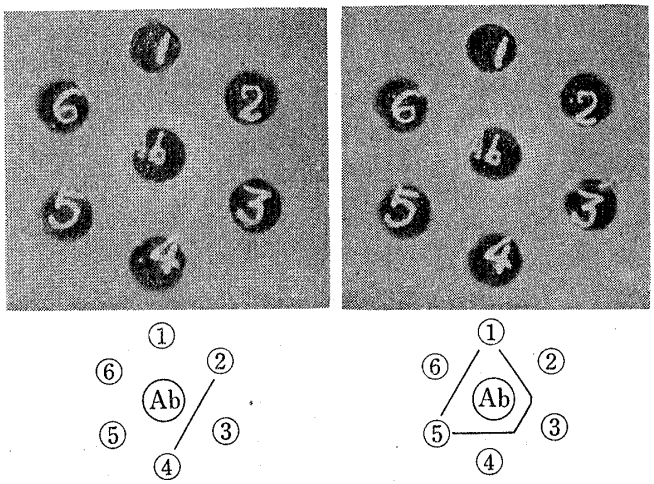

Fig. 2. Detection of α-Chymotrypsin in the Mesenteric Perfusates by Double Immunodiffusion
Ab: antiserum against α-chymotrypsin
1: preliminary perfusate
3: 0—30 min perfusate
5: 30—60 min perfusate
2, 4, 6: right; 50 μg/ml α-chymotrypsin left; saline solution

Radioactivity in the Perfusate

The radioactivities in the perfusates from ^{131}I-CT-administered rats are shown in Table I. The highest radioactivity influx (about 1.4% of the total radioactivity administered) was found in the initial 30 min perfusate. The value progressively decreased with time; 1.1% in the second and 0.7% in the third 30 min. In the control experiment with ^{131}I-KI, 4.2, 0.3, and 0.1% of the given radioactivity were recovered in the first, second, and third perfusates, respectively, indicating that the decrease of iodide influx was much more remarkable than of ^{131}I-CT administration.

More than 90% of the radioactivity in ^{131}I-CT solution was found to be retained in Visking tube even after 3 hr dialysis. Non-diffusible radioactivity in the 0—30, 30—60, and 60—90 min perfusates from ^{131}I-CT-given rats was 26, 16, and 15%, respectively (Fig. 3), while that of the control was negligible. This result showed that there was ^{131}I-bound macromolecule(s) in the perfusates from ^{131}I-CT-administered rats and that there was little possibility of *de novo* binding of ^{131}I to a macromolecule in the intestinal epithelial cell. The decrease of non-diffusible radioactivity in the perfusate collected every 30 min may be attributable to rapid degradation of CT in the intestine by the digestive juices as indicated in our previous paper.[1]

Referring to the result of dialysis, recovery of the radioactive macromolecule in each perfusate was calculated. In the case of the first 30 min perfusate, where the recovery of radioactivity was 1.4% (Table I) and 26% of it was non-diffusible, ^{131}I-bound macromolecule corresponded to 0.36% of the administered ^{131}I-CT. The values in the 30—60 and 60—90 min perfusates were 0.17 and 0.10%, respectively. Thus about 0.6% of the given ^{131}I-CT was recovered in the mesenteric perfusate as the radioactive macromolecule during 90 min.

Esterolytic Activity in the Perfusate

Recovery of ATEE-hydrolyzing activity in the perfusates is shown in Fig. 4. As the result of radioactive macromolecule determination, the first 30 min perfusate possessed the most potent activity which corresponded to 0.32% of the administered and 60% of the total

10) C.A. Kabat and M.M. Mayer, "Experimental Immunochemistry," 2nd ed., Charles C. Thomas Publisher, Springfield, 1967.

Table I. Radioactivity in the Mesenteric Perfusates from the Rat Administered ^{131}I-CT in Intestine

Exp. No.	Administered radioactivity (cpm × 10^3)	Percent of the administered radioactivity		
		0—30 min	30—60 min	60—90 min
1	723	0.89	0.13	0.00
2	722	0.71	0.61	0.14
3	1105	0.79	0.35	0.02
4	379	0.54	0.41	0.32
5	381	3.17	0.90	0.47
6	378	1.51	1.22	0.59
7	366	2.04	0.57	0.13
8	384	1.98	1.60	1.09
9	287	0.45	0.65	0.13
10	277	0.75	0.66	0.57
11	3330	2.44	2.10	1.57
12	3525	2.40	2.70	1.83
13	3623	2.19	2.18	1.32
14	3692	1.39	1.70	1.38
15	3111	2.01	2.21	1.71
16	3538	0.90	1.01	0.81
17	3478	0.99	0.90	0.37
18	3530	0.45	1.06	0.76
19	3642	1.44	0.64	0.06
20	3471	0.57	0.44	0.26
Mean± S.E.		1.38±0.18	1.10±0.16	0.68±0.13

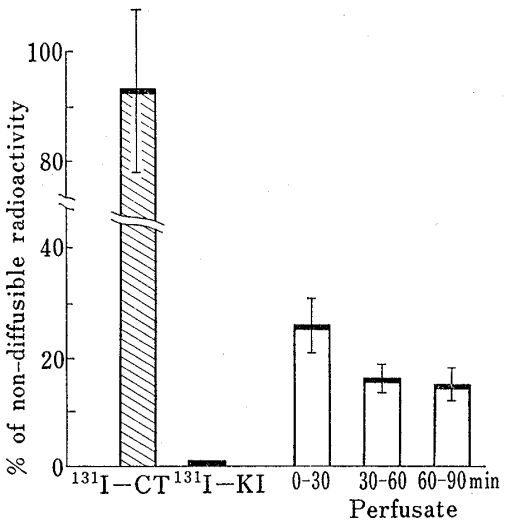

Fig. 3. Non-diffusible Radioactivity in the Mesenteric Perfusates after 3 hr Dialysis

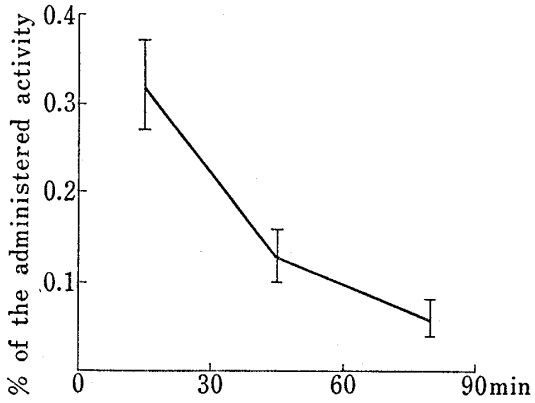

Fig. 4. ATEE-Hydrolyzing Activity in the Mesenteric Perfusate from the Rat Administered CT in Intestine

Table II. Radioactivity and Esterase Activity in the Mesenteric Perfusate (% of the total administered)

Perfusate collected (min)	−30—0	0—30	30—60	60—90
Radioactivity	0	1.38±0.18	1.10±0.16	0.68±0.13
Non-diffusible radioactivity	0	0.37±0.11	0.17±0.04	0.10±0.04
Esterase activity	0	0.32±0.05	0.13±0.03	0.06±0.02
Volume of perfusate (ml)	6.7±0.5	6.6±0.4	5.4±0.4	4.2±0.3

Each value represents the mean± S.E. of 20 experiments.

recovered activity in 90 min, and the activity decreased in the subsequent perfusates. The recovery of the esterase activity in each 30 min perfusate was in good agreement with the transmission of the radioactive macromolecule (Table II).

Discussion

Though there are quite a few papers[11] on the intestinal absorption of CT, most of them presented merely quantitative evidence on the presence of CT in the vascular system. It is quite desirable to get a quantitative result for this research, and the authors employed ^{131}I-CT in both *in vitro* and *in vivo* experiments. Our previous *in vivo* tracer experiment showed that the ^{131}I-bound macromolecule corresponding to about 0.20% of CT given in the jejunum was found in blood plasma.[1] This finding, however, was still indirect and insufficient as the reliable proof of the intestinal absorption of CT.

Although demonstration of the enzymic activity is one of the most direct evidence of this investigation, the difficulties encountered in determining the activity are that only an infinitesimal amount of CT is transmitted and that inhibitors for CT are present in the blood plasma. Kabacoff, *et al.*[11c] measured ATEE-degrading activity in plasma after rectal and intestinal administration of CT and observed the elevation of this activity, but they did not referred to the amount absorbed. The decrease of the inhibitor level in blood was found after the administration of pronase or bromelain in intestine.[12] These investigations, however, were still hard to be recognized as the quantitative experiments.

Hence we developed an *in situ* mesenteric perfusion technique, and succeeded in avoiding the interference of the inhibitors in blood and the confusion with the endogenous enzymes possessing the same enzymic properties. By this method it becomes possible to estimate the amount of the enzyme appeared in the vascular system and also to detect its immunoreactivity.

Esterolytic activity upon ATEE as a specific substrate for CT could be detected in the mesenteric perfusate collected from ^{131}I-CT-given rat, and the recovery percent during 90 min after CT administration was about 0.5%, whilst the preliminary perfusate and the perfusates from the control rats did not possess this activity.

The immunochemical analysis is an excellent method to identify a protein, and the double immunodiffusion was employed in this investigation. The substance which was reactable with the anti-CT serum was found in the 0—30 min perfusate, but the subsequent perfusates, even when concentrated, did not form any percipitin band. It might be due to the fact that the amount absorbed was under the sensitivity of this method (20 μg/ml).

In addition, after giving ^{131}I-CT, but not ^{131}I-KI, radioactive macromolecule was demonstrated in the mesenteric perfusate. Its recovery in the perfusate was in good agreement with that of the enzymic activity (Table II). Unlike the results of *in vitro* experiment, the influx of both activities decreased gradually after rising to the maximum within 30 min. Decline of the perfusate volume also occurred with duration of the perfusion, but it did not run parallel with the decrease of the activities. Although the dosage of the enzyme was huge, it does not seem to be responsible for the decline of perfusate volume, because the same observation was obtained in the control rat received ^{131}I-KI in the intestine. It might be possible to suppose a regulation mechanism on the transmission of macromolecules through the intestinal absorptive cells.

11) *a*) S. Avakian, *New Engl. J. Med.*, **264**, 764 (1961); *b*) R.L. Bogner and C.C. Snyder, *J. Int. Coll. Surg.*, 3, 289 (1962); *c*) B.L. Kabacoff, A. Wohlman, M. Umhey, and S. Avakian, *Nature*, **199**, 815 (1963); *d*) S. Avakian, *Clin. Pharm. Therap.*, **5**, 712 (1964); *e*) B.L. Kabacoff, *Exp. Med. Surg. Suppl.*, **1965**, 63; *f*) J.L. Ambrus, H.B. Lassman, and J.J. Marchi, *Clin. Pharm. Therap.*, **8**, 362 (1967).

12) T. Hayashi, *Pharmaceuticals Monthly*, **10**, 1503 (1968).

The results of the present investigation lead to a conclution that a little portion of CT administered in the intestine can be transmitted into the mesenteric vein without losing the enzymic and the immunochemical properties. Further investigation should be carried out on the absorption mechanisms of the macromolecules, and the mesenteric perfusion described here is expected to be one of useful experimental methods in this field as is such with the cytochemical observation by a electron microscopy.

Acknowledgement The authors are indebted to Eizai Co. Ltd. for the supply of α-chymotrypsin and the synthetic substrate, and thanks are given to Messers H. Hasegawa and H. Yamamoto for their collaboration through this work.