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211. Hiroshi Moriya, Chiaki Moriwaki, Setsuko Akimoto, Keiko Yamaguchi, and Masanori Iwadare*1 : Studies on the Passage of α-Chymotrypsin across the Intestine.

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^{131}I-labelled α-chymotrypsin was administered into the ligated intestine of adult rats, and ^{131}I distribution was studied. Differences were found in the distribution in the kidney from the control experiment of ^{131}I-KI administration in the same way. Furthermore, ^{131}I-bound protein was surely observed in the serum after the intraintestinal administration of ^{131}I-labelled α-chymotrypsin, but not in the serum of the control rats.

^{131}I-labelled α-chymotrypsin was comparatively stable in natural human intestinal and gastric juices under the addition of 0.033*M* Ca ion, but without Ca ion, it was rapidly degraded and lost its esterase activity with both juices.

In *in vitro* experiment with the sac of everted intestine of rats, it was found that chymotrypsin was transferred from the mucosal to the serosal side with its esterase activity.

From these experimental results, the possibility of the transfer of α-chymotrypsin across the intestine was discussed.

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The intestinal absorption of proteins is insignificant from the nutritional standpoint, however, it has been recently reported that a small amount of some enzyme proteins was selectively absorbed from the digestive tract and absorbed proteins gave some physiological effects. Namely, proteinases, such as trypsin,[1,2] α-chymotrypsin,[3,4] bromelain,[5,6] orally administered in the form of enteric coating, are known to be clinically efficacious as anti-inflammatory or anti-edematic agents,[7] and many reports on their intestinal absorption have been presented. Besides those proteinases, insulin,[8,9] saliva-parotin-A,[10,11] hog pancreatic kallikrein,[12] etc., have also been studied.

This paper deals with the investigation about the stability of α-chymotrypsin against the human natural digestive juice, and *in vivo* and *in vitro* experiments concerning its passage through the rat intestine as the fundamental investigation on the oral administration of α-chymotrypsin.

Materials and Methods

α-Chymotrypsin (abbr. as CT)——This was extracted from hog pancreas according to Kunitz's method[13] and lyophilized after dialysis against 0.001*N* HCl (6.8 CT unit-casein 35°/mg.,[14] 1310 NF CT unit/mg.,[15] kindly supplied from Dr. Matsuoka, Enzyme Lab. of Eizai Co. Ltd., Tokyo).

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Determination of the Esterase Activity of CT——Esterase activity of CT preparation was estimated by using N-acetyl-L-tyrosine ethyl ester (ATEE, Sigma Chemical Co.) as the substrate. The method was modified from Kabacoff.[16] TCA-HCl solution (100 ml. of 30% w/v trichloroacetic acid and 165 ml. of conc. HCl were mixed and diluted with distilled water to 500 ml.) was used in stead of $4N$ HCl. If precipitate was formed, it was removed by centrifugation and the supernatant was developed color with ferric chloride solution. The optical absorbance was read at 525 mμ.

^{131}I-Labelled Chymotrypsin (abbr. as ^{131}I-CT)——This was prepared by the following two methods.

Preparation of ^{131}I-CT for *in vivo* experiment: Thirty milligrams of CT were mixed with 5 mCi of ^{131}I-NaI (Dainabott Radio Isotope Lab.) according to the method of Moriwaki,[17] and was reacted with $^{131}I_2$ being converted by oxidation with HNO_2 from $Na^{131}I$. The inorganic radioactive iodine which was mixed with protein was removed by Sephadex G-25 column (1.3×30 cm.) with 0.1 ammonium formate buffer (pH 8.0) and protein fractions were lyophilized. About 10% of radioactive iodine was combined with the protein. No significant decrease of the esterase activity was found after this labelling procedure.

Preparation of ^{131}I-CT for *in vitro* experiment: According to Greenwood's method,[18] 30 mg. of CT was dissolved in 0.4 ml. of $0.05M$ phosphate buffer (pH 7.5) containing 1 mCi of ^{131}I-NaI and 100 μg. of chloramine T in 0.1 ml. of the phosphate buffer was added. After mixing for 2 min., 0.1 ml. of $0.0125M$ sodium metabisulfite and 0.1ml. of $0.1M$ KI solution were added. The mixture was dialysed against distilled water at pH 8.0 for 30 min. and then gel filtered through Sephadex G-50 column (1.3×30 cm.) with $0.05M$ phosphate buffer (pH 8.0). In this case radioactive iodine combined with CT was found about 50%. The esterase activity of ^{131}I-CT prepared by this method was 70% of the initial activity and the decomposed products were removed to the external water on the dialysis.

Determination of Radioactivity——Radioactivity was measured with a well-type scintillation counter of γ-radiation in NaI crystal ($2''\phi \times 2$).

Human Mixed Gastric and Intestinal Juices——Pooled gastric and intestinal juices collected from normal person fasted for 24 hr. were kindly supplied from Dr. Kudo, Laboratory of Clinical Chemistry, School of Medicine, Keio-Gijuku University.

Incubation of CT with Human Mixed Gastric and Intestinal Juices——One tenth milligram of ^{131}I-CT and 0.1 to 0.3 mg. of CT were dissolved in 0.4 ml. of $0.001N$ HCl and in the same solution containing $0.1M$ $CaCl_2$ (final conc. $0.033M$ $CaCl_2$/ml.), 0.4 ml. of gastric juices and 0.4 ml. of $0.1M$ citrate buffer (pH 2.0) were added, then incubated at 37°. The incubation with the intestinal juice was carried out using $0.1M$ borate buffer (pH 8.0) in the same method.

As the control, the buffer solution was added instead of digestive juice. At every designated incubation time, an aliquot was taken out, submitted to radio-paperchromatography and estimated its esterase activity in order to examine the degradation of CT.

Intraintestinal Administration of ^{131}I-CT——Female Donryu strain rats, weighing 130 to 150 g., were used as the experimental animals. Rats were intraperitoneally injected with 0.5 ml. of saline containing 0.001 M KI every day for 4 days and fasted for 24 hr. before the experiment.

Under pentobarbital anaesthesia, rats were given 2 mg. of ^{131}I-CT (2.5×10^6 c.p.m.) dissolved in 0.2 ml. of saline solution into the 10 cm. length of ligated loop of jejunum according to Danforth and Moore.[9] At 1, 2 and 3 hr., after intraintestinal administration, rats were sacrificed by exanguination from the carotid artery. Then the total radioactivity in their liver, kidney, spleen, thyroid, serum and ligated loop of jejunum was counted. As the control experiment, ^{131}I-KI (2.5×10^6 c.p.m.) was given in the same method.

Analysis of ^{131}I-compound in Serum——a) Dialysis of serum: The rat serum was put into cellulose tube and dialysed against 1 L. of distilled water for 2 hr. under continuous stirring. The external water was renewed every 30 min. The radioactivity of the tube was counted at 0, 1 and 2 hr. dialysis. The remaining radioactivity in the tube was regarded as non-dialysable radioactivity, and its percentage to initial radioactivity was calculated.

b) TCA precipitation of serum protein: Radioactivity in 0.5 ml. of serum was counted, then 0.2 ml. of $0.002M$ KI and 0.5 ml. of 10% TCA were added. The protein precipitate was collected by centrifugation and dissolved in 0.2 ml. of $2N$ NaOH. After repeated TCA precipitation, radioactivity in the precipitate was counted and its percentage to initial radioactivity was calculated.

c) Gel filteration of serum: About 0.5 ml. of rat serum was gel filtered through Sephadex G-25 column (1.3×40 cm.) with $0.1M$ phosphate buffer (pH 7.5). The radioactivity in protein fraction was counted, and its percentage to the total radioactivity in gel filtered serum was calculated. The protein elution was detected with optical density at 280 mμ.

***In Vitro* Experiment with the Everted Intestine**——Female rats of Donryu strain, weighing 130 to 150 g., were fasted for 24 hr. and killed by bleeding from the carotid artery. The intestine was immediately

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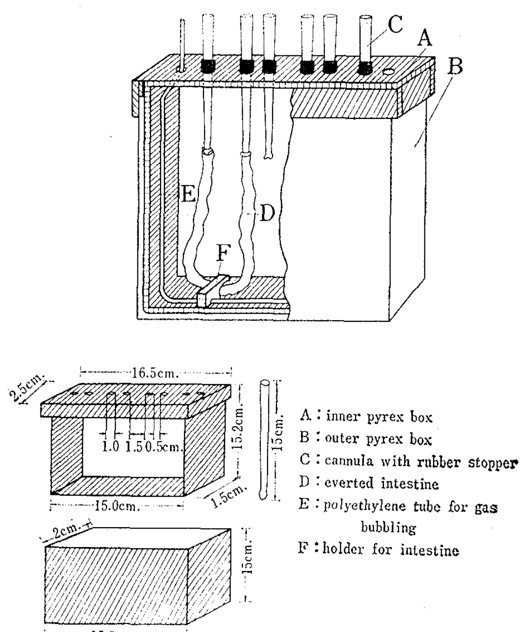

Fig. 1. Apparatus for the *in vitro* Experiment

removed and washed with saline solution and everted by a glass rod. Then the intestine was cut into 2 or 3 fragments of 15 cm. length from the upper side of the jejunum. Each fragment was jointed to a glass-cannula, and the jointed part was sticked with Alone-α (biological paste, Tooa Gosei, Co., Ltd.). As the control, cellulose tube was used instead of the sac of the intestine. The apparatus is shown in Fig. 1.

Into the sacs of the intestine and the cellulose tube, 1 to 2 ml. of Krebs-Ringer-bicarbonate buffer (pH 7.4) saturated with 95% O_2 and 5% CO_2 was poured. The sacs of the everted intestine and the cellulose tube were placed in the box in which the same buffer (150 ml.) containing ^{131}I-CT (100 μg./ml., 40,000 c.p.m./ml.) and CT (100 μg./ml. or 1 mg./ml.), or ^{131}I-KI was poured and the box was incubated at 37° under gas bubbling. At 1 and 2 hr. incubation, small amount of the serosal and the mucosal fluids were taken out, and their radioactivity and esterase activity were estimated. After that, each sample was submitted to one-dimensional radio-paperchromatography on No. 51 Toyo filter paper using 95% ethanol : 2N ammonia (9:1).

Results

Stability of Chymotrypsin against Human Gastric and Intestinal Juices :

The results of the quantitative radio-paperchromatographic analysis of ^{131}I-CT incubated with human gastric and intestinal juices were shown in Fig. 2 and 3. In the cases of incubation at pH 2.0, pH 8.0 and with gastric juice, the rate of the degradation of ^{131}I-CT was slight. Meanwhile, after the 30 min. incubation with intestinal juice, radioactivity remaining in the area around the starting line remarkably decreased (Fig. 3), and it increased along Rf 0.1 to 0.3. Therefore, it was supposed that ^{131}I-CT was

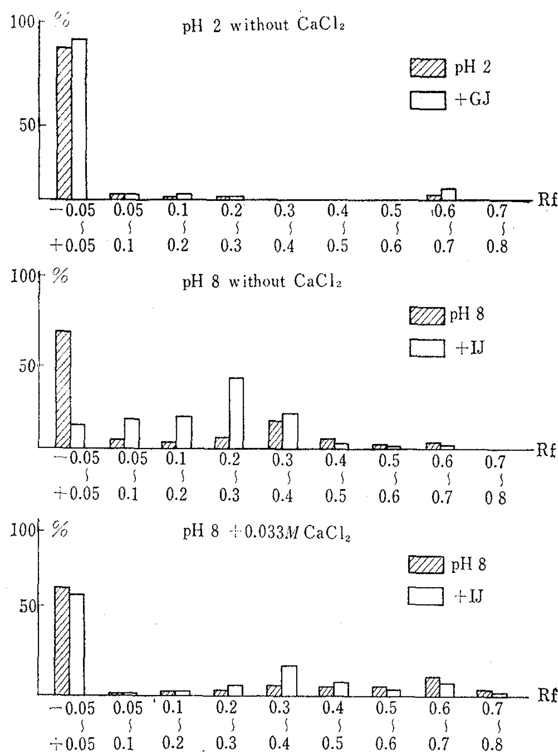

Fig. 2. Quantitative Radio-paperchromatographic Analysis of the Mode of Degradation of ^{131}I-CT incubated with Digestive Juice for 60 min.

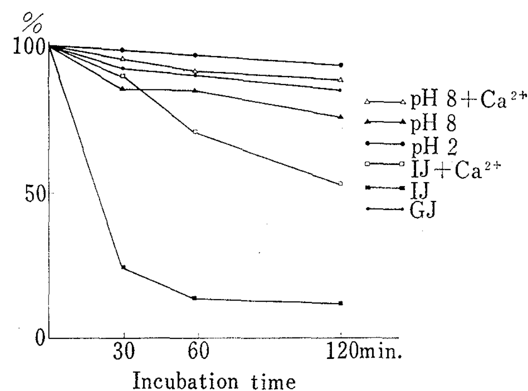

Fig. 3. Percentage of the RA remaining around the Starting Line (−0.05 +0.05) on the Radio-paperchromatography of ^{131}I-CT incubated with Digestive Juice

rapidly degraded with intestinal juice. Such degradation of CT with intestinal juice, however, was fairly prevented by addition of 0.033M $CaCl_2$ in the incubation system.

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The esterase activity of CT incubated with gastric and intestinal juices for 60 min. decreased to 31.3% and 21.3% of the initial, and to 44.6% and 60.7% for 60 min. at pH

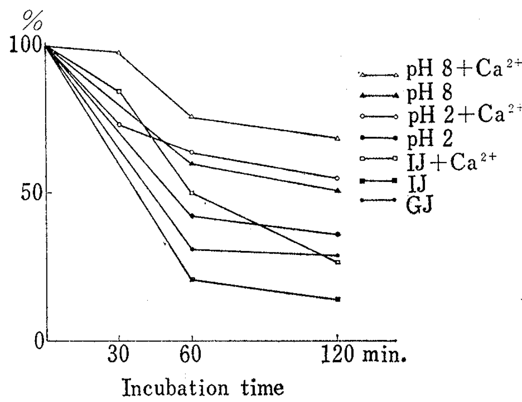

Fig. 4. The Esterase Activity (ATEE) of CT after Incubation with Digestive Juice

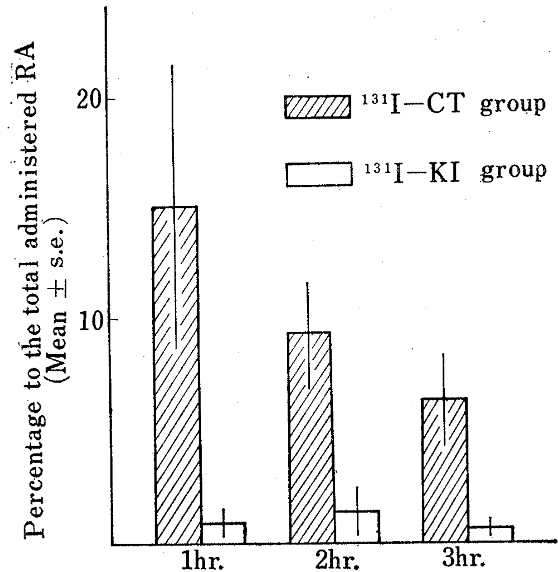

Fig. 5. ^{131}I remained in Intestine after the Intraintestinal Administration of ^{131}I-CT and ^{131}I-KI

2.0 and pH 8.0 respectively (Fig. 4). The degradation by intestinal juice was very rapid, *e.g.* after 3 min. esterase activity decreased to about 70%. However Fig. 2, 3 and 4 showed that the addition of Ca ion prevented the loss of both radio and esterase activities.

Distribution of the Radioactivity in Tissues after the Intraintestinal Administration of ^{131}I-CT :

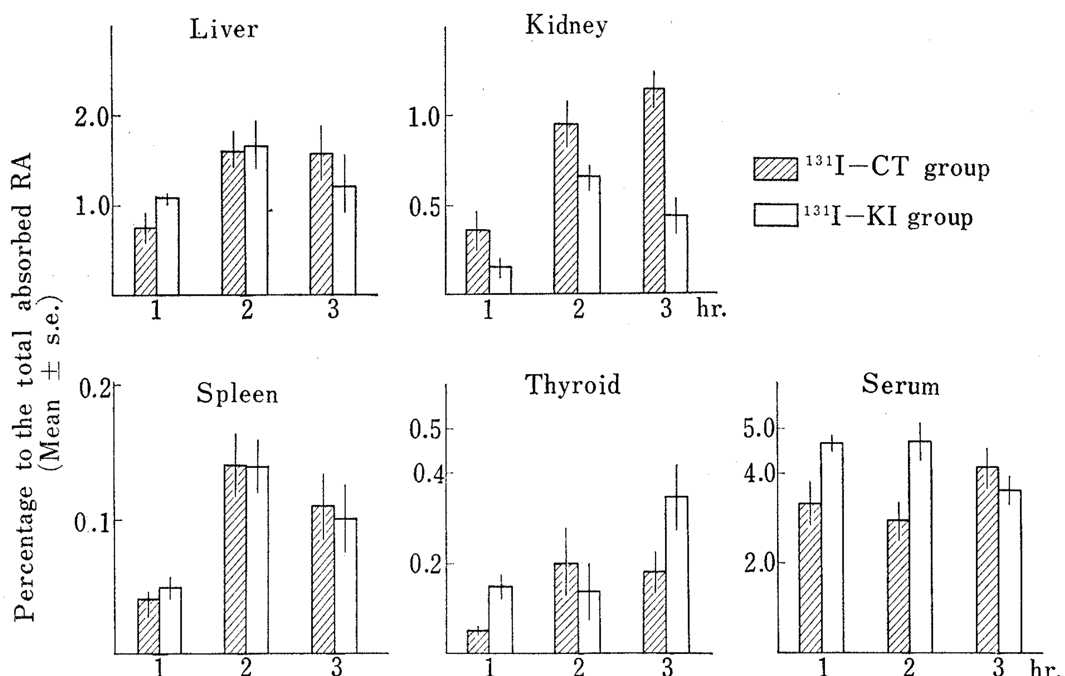

Fig. 6. ^{131}I Distribution in Tissues after the Intraintestinal Administration of ^{131}I-CT and ^{131}I-KI

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Fig. 5 shows the radioactivity remained in the intestine after the intraintestinal administration of ^{131}I-CT and ^{131}I-KI. The disappearance of radioactivity from the ligated intestine was much slower in the case of ^{131}I-CT administration than that of ^{131}I-KI. The total absorbed radioactivity was calculated by subtraction of the remained in the intestine from the total administered radioactivity.

The percentage of the radioactivity distributed in various tissues to the total absorbed one are shown in Fig. 6.

The different distribution was observed only in the kidny between the ^{131}I-CT and ^{131}I-KI administration. The radioactivity in the kidney increased gradually in the administration of ^{131}I-CT, whereas, there was no special significance to be observed in the case of ^{131}I-KI.

Non-dialysable Radioactive Substance in Serum :

The non-dialysable radioactivity in the rat serum administered ^{131}I-CT was 5.7% (2 hr. group) and 2.9% (3 hr. group) (Table I). On the other hand, only 0.2% was recognized as the non-dialysable radioactivity in serum of ^{131}I-KI received rats. Hence, this difference might be due to the administration of high molecular ^{131}I-CT.

Table I. Non-dialysable Radioactivity in Serum through Cellulose Membrane

Treatment	Time of dialysis	Percentage to the Initial RA Mean ± s.e. 1 hr.	2 hr.
2 hr. group	^{131}I-CT	7.3±2.1	5.7±1.5
	^{131}I-KI	0.6±0.3	0.2±0.5
3 hr. group	^{131}I-CT	3.8±0.4	2.9±0.9
	^{131}I-KI	0.6±0	0.2±0.6

TCA-insoluble Compound in Serum :

The radioactivity in the serum protein precipitated by TCA was 3.6% (2 hr. group) and 3.2% (3 hr. group) in the ^{131}I-CT administered group, 1.4% and 1.6 % in the ^{131}I-KI group respectively. This difference was made sure to be statistically significant (p=0.05) (Fig. 7).

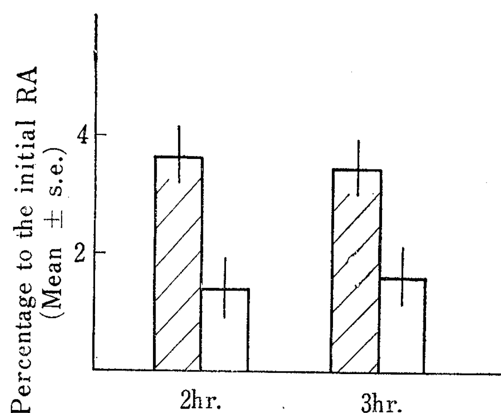

Fig. 7. Radioactivity in TCA Insoluble Fraction of Rat Serum

▨ ^{131}I-CT group □ ^{131}I-KI group

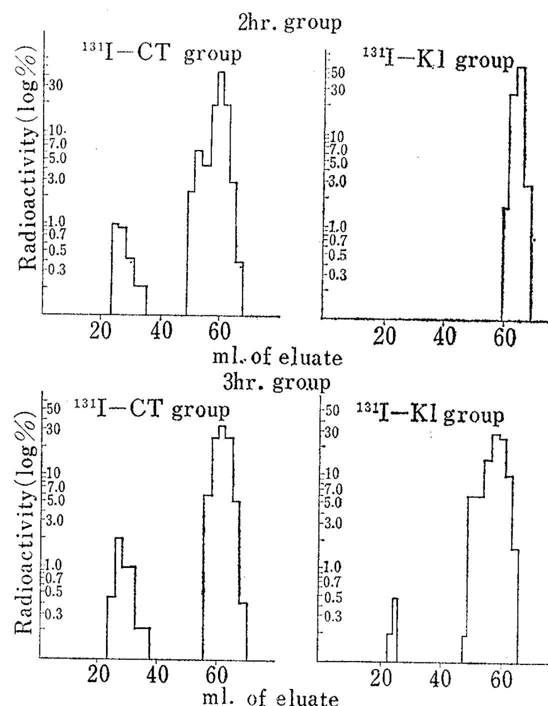

Fig. 8. ^{131}I Eluate Pattern of Serum with Gel-filteration

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Gel Filtration of Serum:

The serum was gel filtered through Sephadex G-25 column. The serum protein was eluted in 24 to 37 ml. of the effluent and the radioactivity in it was 3.5% (2 hr. group) and 4.8% (3 hr. group) in ^{131}I-CT administration, while it was 0% and 0.7% after the ^{131}I-KI administration respectively (Fig. 8).

Transfer of CT across the Everted Intestine:

The percentage of the radioactivity in 1 ml. of the serosal fluid to those of the mucosal and the esterase activity equivalent to chymotrypsin (μg./ml.) in 1 ml. of the

Table II. ^{131}I-CT and CT, and ^{131}I-KI Transfer through the Everted Intestine

	Time	1 hr.			2 hr.		
	External sol.	CT		KI	CT		KI
	Tube	100 μg./ml.	1000 μg./ml.		100 μg./ml.	1000 μg./ml.	
Radioactivity	I	10.9±1.5	15.8±1.7	72.5±2.3	23.3±4.1	46.5±9.1	85.6±7.1
passage[a]	II	17.4±1.9	18.8±1.6	71.1±4.1	25.5±3.7	47.5±0.5	80.1±1.8
Mean±s.e.	III	6.9±2.5	8.3	80.6±6.0	20.4±7.9	28.7	89.5±4.5
	cellulose tube	14.0±1.2		97.3±6.2	23.9±4.4		100.7±2.9
Esterase activity	I	3.5±1.0	116±1.0	0.43±0.22	15.0±5.2	333±15	7.8±2.5
passage (μg./ml.)	II	5.1±1.2	146±16	0.27±0.13	12.0±5.1	349±15	5.6±3.1
Mean±s.e.	III	2.5	174	0.15±0.17	6.8±3.2	263	0
	cellulose tube	0.2±0.2			0.2		

a) (RA in 1 ml. of serosal fluid / RA in 1 ml. of mucosal fluid)×100

serosal fluid were shown in Table II. The mucosal fluid containing 100 μg./ml. of CT and the serosal fluid obtained after the 2 hr. incubation were developed to one-dimensional paperchromatography and their autoradiogram were shown in Fig. 9.

Even in the control experiment of ^{131}I-KI, the esterase activity in the external fluid was slightly recognized and was equivalent to 1.1 ± 0.8 μg. of CT/ml. after 1 hr. and 2.7 ± 1.1 μg./ml. after 2 hr., while in the serosal fluid of No. I and No. II intestine incubated for 2 hr. to 7.8 ± 2.5 and 5.6 ± 3.1 μg. of CT/ml. In this case, on the radio-paperchromatogram the radioactivity was found only about Rf. 0.7, therefore such esterase activity was probably eluated from the intestinal wall.

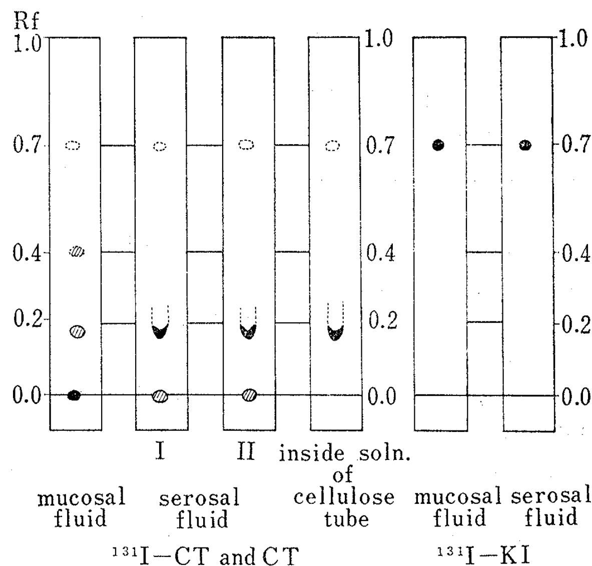

Fig. 9. Autoradiogram of the Serosal and the Mucosal Fluids in *in vitro* Experiment Using Intestine
(CT: 100 μg./ml., 2 hr. incubation)

On the other hand, in 1000 μg/ml. of CT experiment, the passage of the esterase were 333 ± 15 in No. I and 349 ± 15 μg./ml. in No. II intestine for 2 hr. incubation, and even in 100 μg./ml., 15.0 ± 5.2 in No. I and 12.0 ± 5.1 μg./ml. in No. II and there were almost radioactivity remained at the starting line of paperchromatogram.

However the cellulose tube was hardly transferred any esterolytic compound, and on the paperchromatograph the radioactivity did not appear at the starting line, but about Rf. 0.2 and 0.7 (Fig. 9).

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Discussion

In 1957, Martin[1] and others observed that experimental egg white edema of the rat was disappeared with the oral administration of trypsin, and they supposed that trypsin was absorbed through the intestinal wall with its enzymatic activity. Moreover, Avakian[3,19] found the increase of the esterase activity against ATEE in blood after the oral administration of the enteric coated CT to persons.

Kabacoff[4] and others administered CT into the intestine or the rectum of the rabbit, then determined the esterase appearing in the blood using two synthetic substrates. They found that the hydrolytic activity of plasma against ATEE and BPNE (benzoylphenylalanine naphthyl ester) were increased after its administration and they concluded the orally administered CT passed across the intestinal wall and retained its activity.

In the present investigation in which ^{131}I-CT was administered into rat intestine, the radioactive distribution in tissues was different from that of the control experiment with ^{131}I-KI. The presence of ^{131}I-bound protein in the serum was also proved from the results of the dialysis, protein precipitation and gel-filtration of the serum. There is almost no possibility of the biosynthesis of ^{131}I-bound protein after injection of inorganic iodine except in the thyroid[20,21] and moreover such thyroid function of our experimental animals was blocked by the KI pre-treatment. Therefore, ^{131}I-bound protein estimated in the serum might be the protein based on the administered ^{131}I-CT.

In the incubation of CT with natural gastric and intestinal juices, it was evident that CT was decomposed by the treatment with intestinal juice and its esterase activity decreased to 20% after 60 min. However, CT was stabilized under the presence of 0.033*M* Ca ion and 50% of its potency was remained after the 60 min. incubation. These results suggest that CT might be stable in some extent in the intestinal tract if the adequate condition is provided.

In order to use ^{131}I-CT having adequate radioactivity for our *in vitro* experiment, ^{131}I-CT was prepared also according to Greenwood's method. Although some breakdown products were formed, they were removed by the gel filtration with Sephadex G-50.

In *in vitro* experiment using ^{131}I-CT and CT in the mucosal fluid, the radioactivity was found in the serosal fluid, and the esterase activity in it was much higher than that of the control experiment using only ^{131}I-KI. On the radio-paperchromatogram of the serosal fluid, there were 2 kinds of ^{131}I-compounds, which were found in the area of the starting line and Rf 0.1 to 0.3. Comparing the result with that of the inside of the cellulose tube, the former was supposed to be as ^{131}I-CT for the reason of being non-dialysable through cellulose tube and still having esterase activity, and the latter would be regarded as the decomposed product of ^{131}I-CT.

From the results of *in vivo* and *in vitro* experiments above mentioned, it might be possible to conclude that CT was partially transferred across the rat intestine as the active form.

The authors have already reported some papers concerning the transfer across the intestine of saliva-parotin-A and hog pancreatic kallikrein, and further studies on the mechanism of the protein absorption are now being under investigation.

The authors thank for Eisai Co., Ltd. to be supplied α-chymotripsin and the synthetic substrate through the courtesy.

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