[Chem. Pharm. Bull. 15(4) 403~410 (1967)]

UDC 612.015.1-084 : 612.343

48. Hiroshi Moriya, Chiaki Moriwaki, and Setsuko Akimoto : Studies on Kallikreins. II. Passage of ^{131}I-Labelled Hog Pancreatic Kallikrein across the Rat Intestine.*1

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The hog pancreatic kallikrein was labelled with radioactive iodine (^{131}I) and was administered to rats in the tied loop of the jejunum. Relatively high distribution of radioactivity was found in the kidney and the liver and the existence of the ^{131}I-labelled macromolecules in serum was also observed after the intraintestinal administration of ^{131}I-kallikrein.

The sac of everted jejunum was incubated in the Krebs-Ringer bicarbonate buffer containing ^{131}I-kallikrein and it was observed that the radioactive macromolecule having the vasodilator activity was transferred to the serosal side from the mucosal side.

From these results, the possibility of the intestinal absorption of pancreatic kallikrein was discussed.

(Received September 2, 1966)

In recent years it has been investigated that the kallikrein-kallidin system was related to the regulation of the function of various tissues. The authors have presented proceeding papers on the physiologically active peptides and the enzymatic system releasing them.[1~8]

Kallikreins which release the active peptides, are found in the various kinds of tissues, particularly abound in the pancreas, and there was some kallikrein activity in the pancreatic juice.[9] It is reasonable to consider that the pancreatic kallikrein takes part in the regulation of the function of the pancreas, but the physiological meaning of the kallikrein secreted into the intestine with the pancreatic juice still remains unexplained. If the kallikrein has physiological meaning, it would play some role on the intestinal epithelium or on the circulating system distributed under the epithelial layer. From this conception, it becomes interesting to study the permeation or the behavior of the kallikrein in the intestinal epithelium.

Pancreatic kallikreins are protein whose molecular weight were found to be about 33,000.[4] Though the intestinal absorption of protein is not widely accepted, not only the observations about the immunolacto-globulin in the colostrum in new born animals,[10,11] but also some reports on the intestinal absorption of the various proteins

*1 This investigation was supported in part by Public Health Service Research Grant No.5 ROI HEO7641 (1965) from the National Heart Institute, U. S. A.

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including proteinase enzymes have been presented.[12~23] Though the proteins absorbed from the intestine might be meaningless as the nutritives, it is still conceivable that the small amounts of proteins absorbed give their own effects in the body. Therefore, the investigation on the intestinal absorption of pancreatic kallikrein seems to be necessary for the study on the physiological role of the kallikrein in the pancreatic juice.

In the previous paper,[8] it was reported that the biological activity of hog pancreatic kallikrein was almost stable against the digestive juice and the liver. In this paper, the results of the investigation on the transport of ^{131}I-labelled hog pancreatic kallikrein through the intestinal wall are described.

Experimental

The preparation of the standard kallikrein and the determination of its vasodilator and esterase activities were the same as described in the previous papers.[6,7] The hog pancreatic kallikrein used in this experiment was prepared in our laboratory and its vasodilator activity was estimated as 68.5 FU*3/mg.

Labelling of Kallikrein with Radioactive Iodine (^{131}I)——To 3 mc. of NaI-^{131}I (Dainabott Radioisotope Laboratory) 0.5 ml. of 0.002*M* KI, 0.1 ml. of 0.1*M* $NaNO_2$, 0.1 ml. of 0.1*N* H_2SO_4 and 0.1 ml. of 1.0*M* $NH_4SO_3NH_2$ were added. After neutralization with 0.2 ml. of 0.1*N* NaOH, 50 mg. of kallikrein dissolved in 1.0 ml. of 0.05*M* ammonium formate buffer (pH 8.0) was added. The mixture was incubated at 37° for 1.5 hrs. and was stood for in refrigerator over 1 night. The iodination was stopped by addition of 0.1 ml. of 0.03*M* $Na_2S_2O_3$ and this solution was submitted to gel filtration with Sephadex G-25 (1.5×40 cm. column) using 0.05*M* ammonium formate buffer (pH 8.0). Lyophilizing the protein fraction ^{131}I-labelled kallikrein (^{131}I-kallikrein) was obtained.

The prepared ^{131}I-kallikrein showed 2 μc./mg. To determine its purity, it was submitted to paper-chromatography on Toyo Roshi No. 51 filter paper with 95% ethanol - 2*N* ammonia (9:1), and 90% of the radioactivity was found at Rf −0.05 to 0.05, then it was evaluated that the prepared ^{131}I-kallikrein was 90% purity. Furthermore, the vasodilator activity of the labelled kallikrein was 66.7 FU/mg. and there was no significant decrease of the activity.

***In Vivo* Experiment on the Intestinal Absorption of ^{131}I-Kallikrein**——The experimental animals were 31 male *Donryu* strain rats, weighing 120 g. The rats were given 0.01*M* KI aqueous solution as drinking water for 4 days and fasted 24 hr. before experiment. They were incised the abdomen and 10 cm. length to tied loop was made on the jejunum under pentobarbital anesthesia by the method of Danforth and Moore.[14] Into the tied loop, 3 mg. of ^{131}I-kallikrein (9.6×10^6 c.p.m.) dissolved in 0.2 ml. of saline solution was given, and at 2 and 3 hr. after administration the rats were sacrificed by exsanguination. As the control experiment, rats were given KI-^{131}I solution (1.2×10^6 c.p.m.) in the same manner and were also intravenously injected 1.5 mg. of ^{131}I-kallikrein (4.7×10^6 c.p.m.) and KI-^{131}I (1.2×10^6 c.p.m.), and in this case sacrificed 1 hr. after injection. The liver, kidney, spleen, thyroid, serum and small intestine were removed from each rat and the radioactivity in those tissues were counted with a well type scintillation counter ($2''\varphi\times2''$).

Adding 0.2 ml. of 0.002*M* KI as the carrier, 0.5 ml. of serum was dialysed against distilled water for 1 hr. with cellulose tube, and the ratio of the non-dialysable radioactivity was estimated.

Serum protein was precipitated by the Somogyi's method.[24] The precipitate was well washed and its radioactivity was counted.

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*3 FU : Frey unit

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Radio-paper chromatographic analysis of the serum was performed as the estimation of ^{131}I-kallikrein purity. The paper chromatogram was cut into 11 fragments along the Rf value and the radioactivity in each fragment was estimated.

The serum was gel filtered with Sephadex G-25 under the same condition as that of the preparation of ^{131}I-kallikrein, and the optical density at 280 mμ and the radioactivity in each fraction were measured. The protein fractions were dialysed and radioactive substance in them were confirmed as non-dialyzable.

***In Vitro* Experiment on the Intestinal Transfer of ^{131}I-Kallikrein**——Male rats of *Donryu* strain, weighing 150 g. fasted for 24 hrs., were killed by a blow on head. The jejunum was immediately removed and the sac of everted intestine of 10 cm. length was made according to Wilson and Wiseman.[25] Then 1 ml. of Krebs-Ringer bicarbonate buffer was poured in the sac, and the sac was placed in 30 ml. of the same solution containing ^{131}I-kallikrein or KI-^{131}I (as the control) and incubated at 37° for 1 hr. with gas bubbling (5% CO_2 and 95% O_2). After the incubation the serosal fluid was removed and radioassayed. Then the serosal fluid was carried out the dialysis against water, radio-paper chromatography and determination of the vasodilator activity in dog.

Results

The percentages of radioactivity in each organ to the total absorbed radioactivity are shown in Table I. About 54% of radioactivity remained in the intestine of the rats administered ^{131}I-kallikrein, whereas only a little radioactivity remain was found in the intestine of the control rats administered KI-^{131}I. There was also significant difference in the radioactivity distribution in the kidney between the ^{131}I-kallikrein administered and the control groups. Higher distribution of radioactivity was found in the liver removed at 2 hr. after ^{131}I-kallikrein administration. The same findings were observed in the rats intravenously injected these samples.

Table I. Radioactiviy Distribution in Tissues after the Intraintestinal Administration of ^{131}I-Kallikrein

Sample	Time (hr.)	Rat No.	Absorption[a] (%)	Tissue Radioactivity / Total Absorbed Radioactivity × 100				
				Liver	Kidney	Spleen	Thyroid	Serum[b]
^{131}I-Kallikrein	2	6	42.63±20.93[c]	3.19±0.90	1.65±0.33	0.22±0.07	0.25±0.07	11.75±2.72
KI-^{131}I	2	6	98.89± 0.23	1.97±0.81	0.85±0.24	0.26±0.12	0.17±0.05	9.02±2.63
^{131}I-Kallikrein	3	8	46.44±12.81	1.80±0.43	1.34±0.36	0.20±0.10	0.15±0.10	7.61±2.69
KI-^{131}I	3	7	98.49± 1.83	1.92±0.67	0.79±0.28	0.24±0.16	0.15±0.05	7.84±2.04
^{131}I-Kallikrein (i. v. injection)	1	2		16.40	2.07	0.29	0.14	39.40
KI-^{131}I (i. v. injection)	1	2		2.38	0.91	0.16	0.13	11.20

a) Absorption % = $\frac{\text{Total Administered RA} - \text{RA in the Intestine}}{\text{Total Administered RA}} \times 100$

b) Total Serum Weight: 4 g.

c) S.E.

Fig. 1 shows the percentage of the radioactivity found in the inside phase after 1 hr. dialysis of rat serum to the initial radioactivity. The contents of non-dialysable radioactivity in rats serum collected 2 and 3 hr. after ^{131}I-kallikrein administration were 6.1 ± 2.4% and 4.6 ± 1.7% respectively, whereas in the control rats the percentage was only about 1 %. Back ground count was 70 c.p.m., while the radioactivity in the inside phase of cellulose tube was about 500 to 1,000 c.p.m. On the other hand, 70.3% of the non-dialysable radioactivity was found in the serum of the rats injected intravenously ^{131}I-kallikrein, but in case of KI-^{131}I injection only 0.9% was found as non-dialysable.

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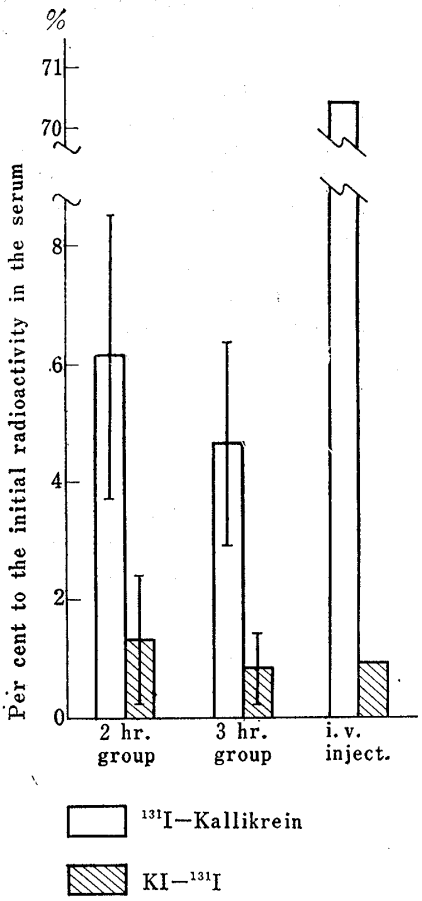

Fig. 1. Non-dialysable Radioactivity in Serum after 1 hr. Dialysis against Distilled Water.

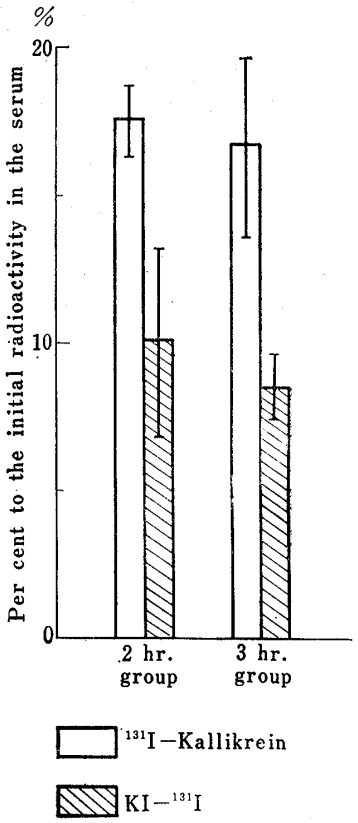

Fig. 2. Radioactivity in the Protein Precipitate of Serum.

There were 17.5% (2 hr. group) and 16.6% (3 hr. group) of the radioactivity in the protein precipitates in rat serum administered ^{131}I-kallikrein, while in the control KI-^{131}I group 10.0 and 8.4% of the radioactivity were found in the precipiates respectively (Fig. 2).

From the analytical results of the quantitative radio-paper chromatography, it was observed that the almost of all radioactivity of the ^{131}I-kallikrein was presented around the starting line (Rf−0.05 to 0.05), while KI-^{131}I was developed at Rf 0.55 to 0.75 under the same condition (Fig. 3-a). The serum obtained from the rats received the intravenous injection of these samples showed the similar radio-paper chromatograms as those of the injected samples (Fig. 3-b). In case of the rats serum received the intraintestinal administration of ^{131}I-kallikrein, there were higher radioactivity distribution in Rf −0.05 to 0.15 area than those of the serum of the rats administered KI-^{131}I (Fig. 3-c and -d).

^{131}I-Kallikrein was gel filtered with normal rat serum and 85% of the radioactivity was found in the serum protein fraction (effluent volume 20 to 40 ml.). Radioactive iodine (KI-^{131}I) was also gel filtered in the same way and the whole radioactivity was recovered in 55 to 80 ml. (Fig. 4). The protein and non-protein fractions were dialysed against water for 1 hr. with cellulose tube. In the former fraction, 75% of the radioactivity was non-dialysable and in the latter 99.4% of it was dialysable.

The serum of the rats received the samples in the intestine, were gel filtered and more radioactivity existence in the protein fraction was found in the serum of the rats given ^{131}I-kallikrein than those in the case of KI-^{131}I administration (Fig. 5).

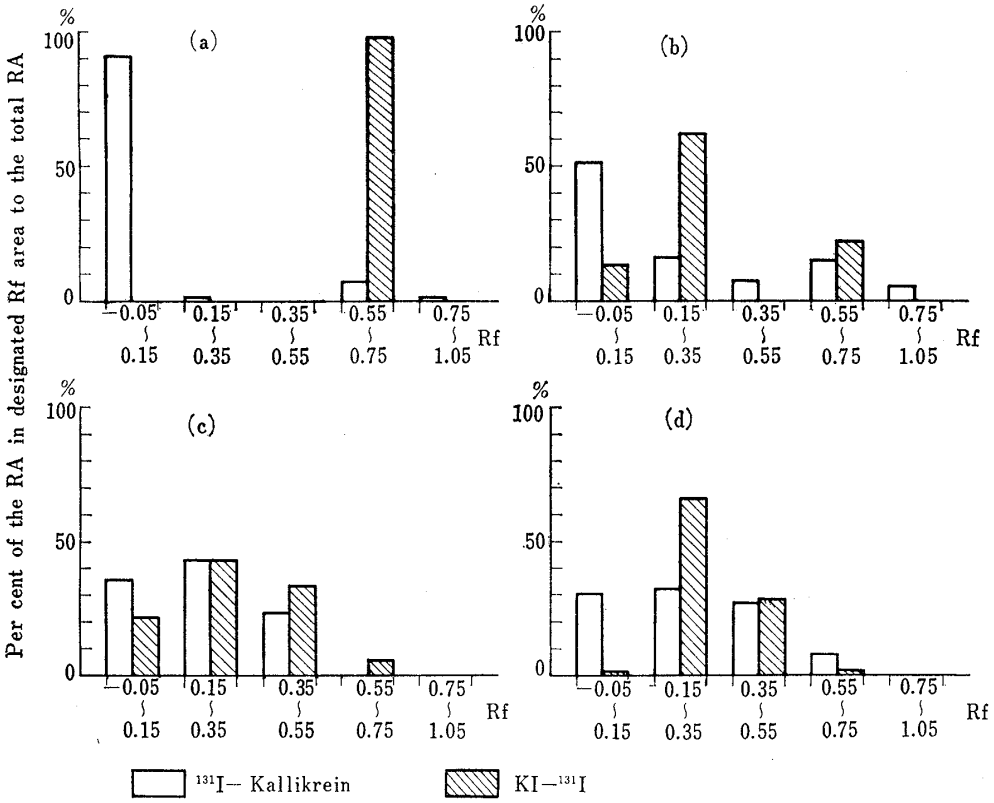

Fig. 3. Quantitative Radio-Paper Chromatographic Analysis of ^{131}I-Labelled Substances in Rats Serum.

In the serum of the rats injected intravenously ^{131}I-kallikrein, 73.2% of the radioactivity was ^{131}I-bound protein but in KI-^{131}I injection only 1% of it eluted in the protein fraction.

The results of *in vitro* experiments of the intestinal transfer of ^{131}I-kallikrein and radioactive iodine were shown in Table Ⅱ. As shown in the Table, the radioactivity transfer to the serosal side through the intestinal wall was recognized with ^{131}I-kallikrein, and the radioactivity ratio (v/v) of the seroral fluid to the mucosal fluid was 40%. In the control experiment with radioactive iodine, the radioactivities in 1 ml. of serosal and mucosal fluid were approximately equal (Table Ⅱ). ^{131}I-Kallikrein showed smaller transfer rate than the radioactive iodine *in vitro*. About 45% of the radioactivity remained in the inside phase after dialysis of the serosal fluid obtained in ^{131}I-kallikrein experiment (Table Ⅲ) and there was ^{131}I-labelled substance which was found on the starting line on the radio-paper chromatogram of the fluid (Fig. 6). In addition, as there was no change of the vasodilator activity before and after the dialysis, it was supposed that the non-dialysable ^{131}I-combined substance in the serosal fluid would be ^{131}I-kallikrein or the partially decomposed substance maintaining the physiological activity of kallikrein.

Discussion

Concerning the protein absorption in adult animals, Uhlenhus[12] reported that the intestinal absorption of egg white in adult dog was proved with the precipitate reaction. Laskowski, *et al.*,[13] Danforth and Moore[14] and Inouye and Vars[15] observed the blood sugar decrease in the animals after the administration of insulin and the various protease inhibitors into the intestine, and they[14] also observed, in *in vitro* experiment using the everted intestine, the transfer of insulin to the seroral side with the paper chromatographic analysis and the assay of the hypoglycemic effect of the serosal fluid.

Table II. Transfer of ^{131}I-Kallikrein and KI-^{131}I through the Everted Intestine *in vacuo*

Exp. No.	Original Solution			Mucosal Fluid		Serosal Fluid				RA Ratio[c] (%)	Relative Potency[d] (%)
	Total RA ($\times 10^4$c.p.m.)	RA/ml. (c.p.m.)	Activity (FU/ml.)	RA/ml. (c.p.m.)	Activity (FU/ml.)	Total Vol. (ml.)	Total RA (c.p.m.)	RA/ml. (c.p.m.)	Activity (FU/ml.)		
Kal[a]- 1	31.03	8870		7750		1.03	4090	3980		51.3	
- 2	29.87	9900	13.0	7730	11.3	0.82	2520	3070	1.8	39.8	16.0
- 3	30.62	9900		7830		0.98	2990	3090		39.5	
- 4	29.10	9900		7610		1.16	3300	2860		37.6	
- 5	155.55	53150		50840		0.56	17480	31110		61.2	
- 6	163.32	54210		52450		1.23	23390	18730		35.8	
- 7	118.95	49550		45950		1.13	20380	18110		39.4	
- 8	169.68	58280	33.2	54780	33.7	1.08	24810	22980	3.8	41.9	11.3
- 9	164.39	58280		54800		1.67	26050	24400		44.5	
-10	114.23	57370	8.0	56760	7.9	0.98	18290	18740	1.4	33.0	17.7
-11	144.97	58530		53830	8.8	0.96	18730	19550	2.1	36.3	23.8
-12	192.49	58530		53180	8.1	0.92	11600	12600	0.8	23.7	9.9
										(40.6±6.3)	(15.7±5.5)
KI[b] -1	21.88	6250		5780		0.65	2490	3810	0	66.0	
-2	67.76	22590		20010		0.96	18390	18570		92.3	
-3	175.01	58330		59760		1.00	51070	51200		85.7	
-4	146.04	47650		31090		0.83	26520	32690	0.1	103.2	
-5	135.98	44270		32820		1.20	38130	31710	0	96.6	
-6	125.22	45960		32510		1.47	43640	29630	0.1	91.1	
										(89.1±12.8)	

a) Kal: ^{131}I-Kallikrein
b) KI: KI-^{131}I
c) RA Ratio = $\frac{\text{Radioactivity in 1 ml. of Serosal Fluid}}{\text{Radioactivity in 1 ml. of Mucosal Fluid}} \times 100$
d) Relative Potency = $\frac{\text{FU in 1 ml. of Serosal Fluid}}{\text{FU in 1 ml. of Mucosal Fluid}} \times 100$

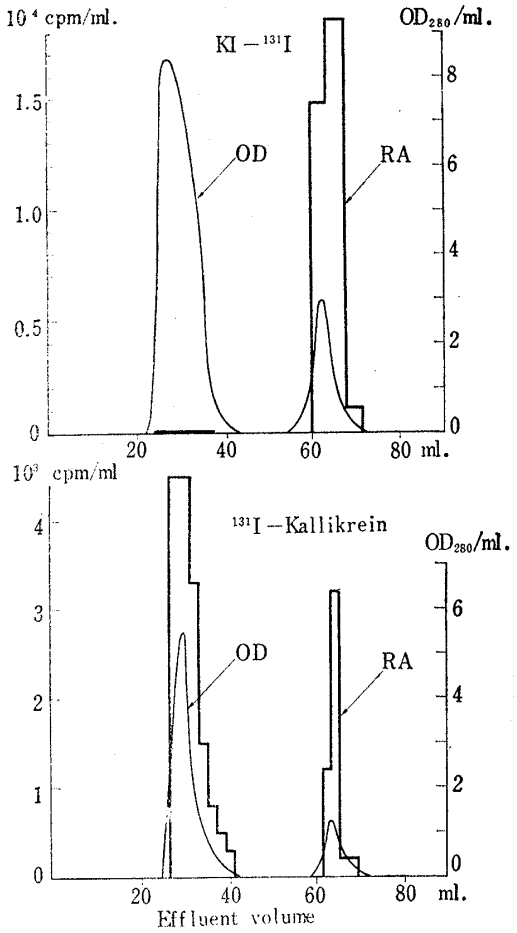

Fig. 4. Radioactivity and Protein Elution Patterns in the Gel Filtration of ^{131}I-Kallikrein and KI-^{131}I with Rat Serum.

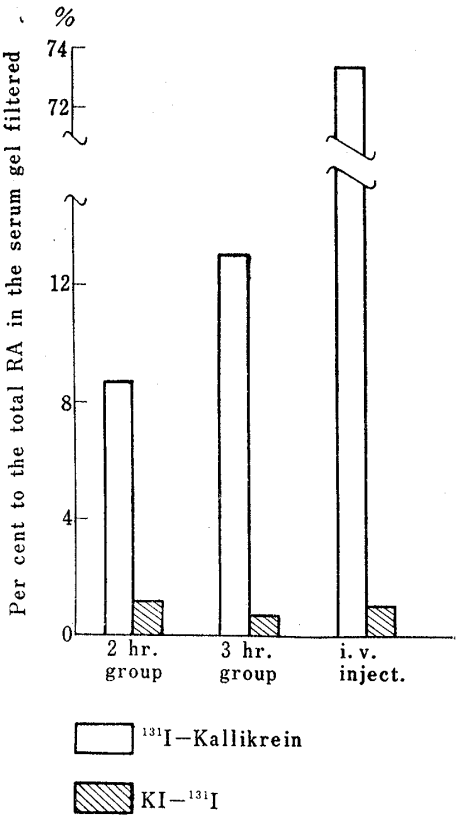

Fig. 5. Radioactivity in the Protein Fraction obtained by the Gel Filtration of Rat Serum.

TABLE III. Radioactivity and Vasodilator Activity Remain after the Dialysis of the Serosal Fluid

	Before dialysis	After dialysis	$\frac{\text{After}}{\text{Before}} \times 100$
Total radioactivity	6820 c.p.m.	3180 c.p.m.	46.5
Vasodilator activity	0.7 FU/ml.	0.7 FU/ml.	100

Moreover, there are many publications reported the absorption of the proteins, such as chymotrypsin,[16~18] bromelein,[19,20] saliva-parotin-A,[21,22] etc. One of the methods to study this problem, tracer technique has been widely employed, such as ^{131}I-serum and -colostral protein,[11] ^{131}I-trypsin,[23] ^{131}I-chymotrypsin,[16] ^{131}I-bromelein[20] and ^{131}I-saliva-parotin-A,[22] etc. In these investigations, various kinds of experimental animals, including human, were administered orally or intraintestinally the labelled protein and the observation of the biological responses or the radioactivity

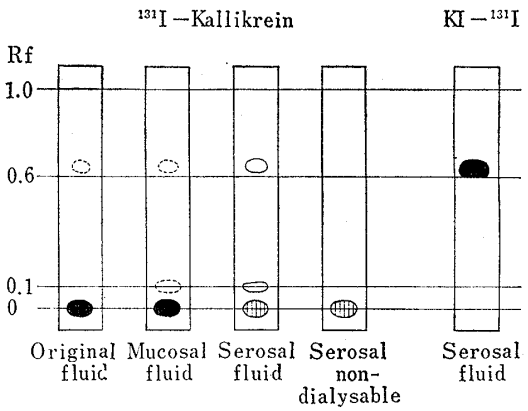

Fig. 6. Autoradiograms of the Original, Mucosal and Serosal Fluid in *in vitro* Experiment.

distribution and the analysis of the radioactive substances in the tissues were carried out.

In the present investigation, the rats were administered ^{131}I-kallikrein into the intestine and the results were compared with those of the control experiments used radioactive iodine. In case of ^{131}I-kallikrein, the smaller absorption rate and the different radioactivity distribution in the kidney and the liver from the control were observed. The similar differences in the radioactivity distributions were found in the case of the intravenous injection and it is presumed that not only radioactive iodide ion but also some other substances tagged ^{131}I were absorbed from the intestine.

It was elucidated from the experimental results that the macromolecules combined with ^{131}I was presented in the serum of the rats received ^{131}I-kallikrein. Namely, this substance was non-dialysable, insoluble in the protein precipitating reagent, analogous to the behavior of ^{131}I-kallikrein in paper chromatography and eluted in the serum protein fraction in Sephadex gel filtration. The presence of such macromolecules in serum was, however, hardly found in the rats serum administered KI-^{131}I as the control.

This difference also indicates that the substance absorbed from the intestine was not only radioactive iodide ion which might be liberated from ^{131}I-kallikrein through the digestive processes. Haurowitz and Crampton[26] reported that the biosynthesis of ^{131}I-protein after the injection of radioactive iodide ion was limited in the thyroid gland. As the thyroid function of the rats used in this experiment was blocked by the preliminary treatment with KI, there was little possibility of biosynthesis of ^{131}I-protein *in vivo*. Then, it is supposed that the macromolecules combined with ^{131}I and found in rats serum might be the protein relating ^{131}I-kallikein administered and absorbed from the intestine.

In *in vitro* experiment with the everted intestine, it was obvious that kallikrein was transferred from the mucosal side to the serosal side because of the findings of the vasodilator activity in the serosal fluid. The radioactivity in 1 ml. of the serosal fluid was 40% to that of the mucosal fluid and 16% of the relative biological potency. But 55% of the radioactive substance in the serosal fluid was dialysable and mobile in the paper chromatography. Therefore, omitting this percentage, 18% was the transfer rate of the macromolecules to the serosal side and this value is in good agreement with the relative potency.

The results obtained in this experiment does not prove the presence of the hog pancreatic kallikrein itself in *in vivo*. For this purpose, the immunochemical method will be investigated in future. However, as described above, ^{131}I tagged macromolecules were found in serum of the rats which were given ^{131}I-kallikrein in the intestine and it was also observed that ^{131}I-kallikrein was transferred to the serosal fluid maintaining its vasodilator activity in *in vitro* experiment with the everted intestine of rat. From these experimental results, it seems reasonable to conclude that ^{131}I-kallikrein was absorbed through the intestinal wall.

If the pancreatic kallikrein is thus absorbed from the intestine, it will be possible to assume that the physiological role of the kallikrein is to regulate the circulation around the digestive tract and others. The authors are now under the investigation from this aspect.

The authors thank Misses N. Inoue, N. Otaki and A. Tsuda and Messrs. M. Iwadare and K. Kawaguchi for their collaboration during this investigation.

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