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Studies on Kallikreins. III.[1] Intra-intestinal Administration of Hog Pancreatic Kallikrein and Its Appearance in the Perfusate from the Mesenteric Vein

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The perfusion of the mesenteric vascular system with Krebs-Ringer solution was carried out on the rats, and the vasodilator and BAEE-esterolytic activities in the perfusate were determined after the intestinal administration of hog pancreatic kallikrein. The recoveries of both kallikrein activities agreed well with each other and with that of ^{131}I-macromolecules in the case of a combined administration of ^{131}I-kallikrein. The transmission of kallikrein through the intestine seemed to reach its maximum within 30 min after the administration and it diminished rapidly thereafter. The mesenteric perfusate formed a few precipitin bands against the anti-kallikrein rabbit serum on the double immunodiffusion, and one of them was also positive in the activity-staining utilizing BAEE-esterolytic activity.

These results suggest that the pancreatic kallikrein is transmitted from the intestine to the vascular system, maintaining its enzymic properties and its immunoreactivity and that about 1% of the given kallikrein seems to be absorbed into the mesenteric vein under this experimental condition.

Transmission of pancreatic kallikrein through the intestinal wall has been investigated by the authors for elucidating the physiological role of the kallikrein in the pancreatic juice. In the previous papers,[1] it was reported that the esterolytic and vasodilator activities of the kallikrein were stable in the intestinal juice, and these activities were found to pass to the serosal side in the *in vitro* experiment with a sac of intestine. Following intraluminal administration of ^{131}I-labelled kallikrein, the existence of radioactive macromolecules was recognized in the rat serum. Though the possibility of the intestinal absorption of pancreatic kallikrein was discussed from these experimental results, more direct and conclusive evidence is still desirable.

The detection of kallikrein activity in blood serum after the intra-intestinal administration of it is one such direct evidence. However, such attempts failed because of the presence of kallikrein inhibitors or kininases in the blood. On the other hand, the amount of kallikrein transmitted from the intestine into the vascular system would be small and this also makes it very difficult to prove kallikrein absorption.

Considering these difficulties, a mesenteric perfusion was carried out on rats which received hog pancreatic kallikrein in the intestine, and its biological potency and the immunoreactivity in the perfusate collected from the mesenteric vein were determined. The experimental results of this investigation are dealt with in the present paper and the intestinal absorption of pancreatic kallikrein is discussed again.

Material and Method

Kallikrein Preparation——The kallikrein used was extracted and partially purified from hog pancreas by the method described previously.[3] The vasodilator activity of this preparation was 146 kallikrein unit

1) *a*) Part I: H. Moriya, C. Moriwaki, S. Akimoto, and K. Yamazaki, *Chem. Pharm. Bull.*, **15**, 399 (1967); *b*) Part II: H. Moriya, C. Moriwaki, and S. Akimoto, *ibid.*, **15**, 403 (1967).
2) Location: *Funakawara-cho, Ichigaya, Shinjuku-ku, Tokyo.*
3) H. Moriya, K. Yamazaki, H. Fukushima, and C. Moriwaki, *J. Biochem.*, **58**, 208 (1965).

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(KU) per mg and 1 mg of it hydrolyzed 35 μmoles of N-α-benzoyl-L-arginine ethyl ester (BAEE) in 1 min. Six protein bands were found in disc electrophoresis of this kallikrein preparation.

Preparation of ^{131}I-Labelled Kallikrein——According to Greenwood and Hunter,[4] 2 mg of kallikrein was labelled with 1 mCi of ^{131}I-iodide by oxidation with chloramine T. To the reaction mixture, 50 mg of the kallikrein was added as carrier and was gel-filtered through a column of Sephadex G-75 (1.5×30 cm) with 0.05 M phosphate buffer, pH 7.5, as the eluting solvent. The protein fractions which were found by the determination of the absorbancy at 280 nm were combined in a Visking dialysis tube (Visking Co.), and concentrated to 1/5 volume by covering with dry Sephadex powder in a refrigerator.

This concentrated solution, which was 1500 KU/ml and 3×10^6 cpm/ml, was immediately administered in intestine as ^{131}I-kallikrein. No decrease of enzymic activity was observed in the labelled preparation, but slight elevation of the activity occurred by gel filtration.

Radioactivity was counted by scintillation measurement of γ-radiation in a well-type NaI crystal.

Activity Assay——Vasodilator activity was determined by the blood flow increase response in dogs.[5] Esterolysis of BAEE was measured by the increase of the optical extinction at 253 nm according to Schwert and Takenaka.[6]

Immunochemical Analysis——A 1.0% solution of partially purified hog pancreatic kallikrein in saline was injected into the rabbit with an equal volume of Freund's complete adjuvant (Iatron Lab., Tokyo), and the antiserum was obtained by the same procedure as the anti-chymotrypsin serum.[7] It was possible to detect the kallikrein at the concentrations from 20 μg/ml upwards by this antiserum with a micromethod of double immunodiffusion.[8]

The immunodiffusion was performed on a 1 mm thick agar plate. After 5 hr of immunodiffusion in a refrigerator, the plates were dried under moist filter paper after washing in saline and then distilled water. Some plates were stained by 1% Amidoschwarz 10 B to demonstrate the precipitin band and the others were immersed in a medium containing neutral red as pH indicator and BAEE. By this procedure the esterolytic activity of the precipitate could be discerned by the colour change of pH indicator from yellow to red in the vicinity of the band.[9]

Mesenteric Perfusion——The mesenteric perfusion[7] with Krebs-Ringer bicarbonate buffer (pH 7.4) containing 6% dextran was carried out on the rats, weighing about 140 g, to which 0.3 ml of ^{131}I-labelled kallikrein or ^{131}I-KI (0.1 mg KI/ml) was given in the upper jejunum. During perfusion for 90 min, the perfusate effluxed from the mesenteric vein was pooled every 30 min. These perfusate samples were submitted to the assays of kallikrein activities after the radioactivity counting, and an aliquot of the perfusates was dialyzed against distilled water (pH 8.0) for 3 hr to separate ^{131}I-bound macromolecule. Some of the perfusates were concentrated to about 1/10 of the initial volume as described previously,[7] and was submitted to double immunodiffusion.

Results

The concentrated 0—30 and 30—60 min perfusate samples clearly formed a few bands with the anti-kallikrein serum in the double immunodiffusion using the Ouchterlony technique, while no band could be detected around the preliminary perfusate. The last perfusate (60—90 min) usually gave very faint bands in comparison with the preceding perfusates.

By the protein staining of the precipitin patterns more detailed inspection could be achieved. A rather broad band was observed around the kallikrein which was employed in this experiment, and the bands formed around the perfusate samples collected after the kallikrein administration were fused with that of the given sample (Fig. 1). "KK" was another kallikrein preparation which was not used in this experiment. Moreover, the red bands indicating the distribution of BAEE-esterolytic activity were found around the given kallikrein and all perfusate samples except the preliminary one, and they fused obviously with each other (Fig. 2). These immunochemical observations indicate that the mesenteric perfusate contains the kallikrein administered in the intestine.

The recovery of radioactivity in the perfusates was given in Fig. 3 in terms of the percent of the administered activity. Compared with the control experiment in which ^{131}I-KI was

4) F.C. Greenwood and W.H. Hunter, *Biochem. J.*, **89**, 114 (1964).
5) H. Moriya, K. Yamazaki, and H. Fukushima, *J. Biochem.*, **58**, 201 (1965).
6) G.W. Schwert and Y. Takenaka, *Biochem. Biophys. Acta*, **16**, 570 (1955).
7) C. Moriwaki, K. Yamaguchi, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **22**, 1029 (1974).
8) O. Ouchterlony, *Acta Path. Microbiol. Scand.*, **25**, 186 (1948).
9) J. Uriel and S. Avrameas, *Anal Biochem.*, **8**, 180 (1964).

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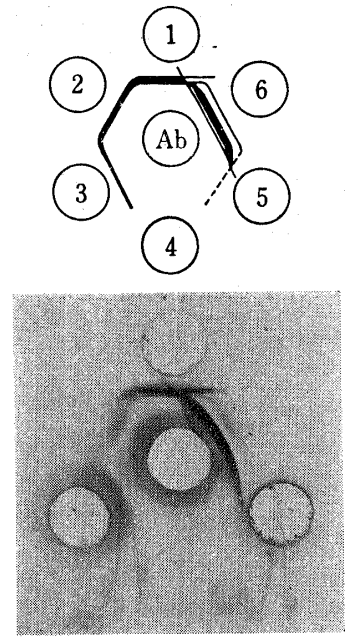

Fig. 1. Identification of Kallikrein in Mesenteric Perfusate

(amidoschwarz 10B staining)
Ab: anti-kallikrein rabbit serum
1: 0—30 min perfusate
2: used kallikrein
3: 30—60 min perfusate
4: preliminary perfusate
5: 60—90 min perfusate
6: "KK"

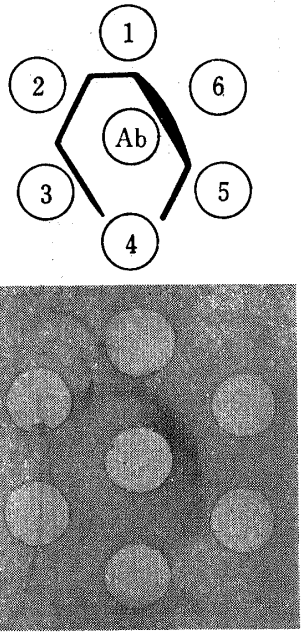

Fig. 2. Detection of Esterolytic Activity (BAEE) of the Precipitates formed by Kallikrein and Mesenteic Perfusate

(sample positions: *cf.* Fig. 1)

given in the intestine, slow but continuing transfer of radioactivity into the perfusate was observed in the case of ^{131}I-kallikrein administration.

The presence of macromolecule was also examined by dialysis of the perfusates. About 24% of the radioactivity was found as the nondiffusible radioactivity in all perfusates from the rat given ^{131}I-kallikrein, whilst less than 1% of the initial radioactivity remained in the Visking tube after 3 hr dialysis of all perfusates from the control rats which were given ^{131}I-KI (Fig. 4). Then, the recovery of nondiffusible radioactivity, namely ^{131}I-labelled macromolecule,

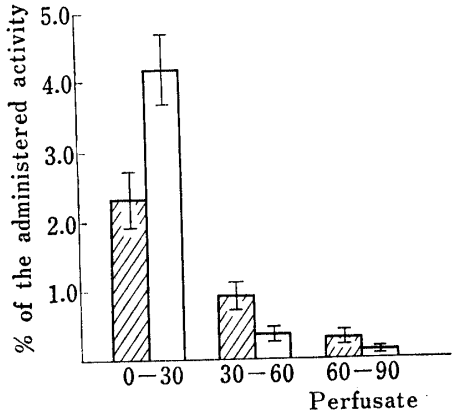

Fig. 3. Radioactivity transmitted into the Mesenteric Perfusate

(mean and s.e. of 20 experiments)
▨: ^{131}I-kallikrein administration
□: ^{131}I-KI administration

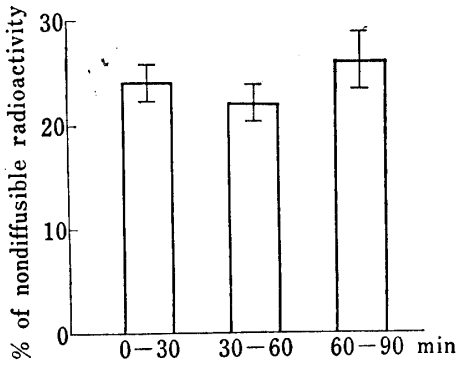

Fig. 4. Nondiffusible Radioactivity in the Mesenteric Perfusate from Rat Given ^{131}I-Kallikrein after 3 hr Dialysis

(mean and s.e. of 10 expeirments)

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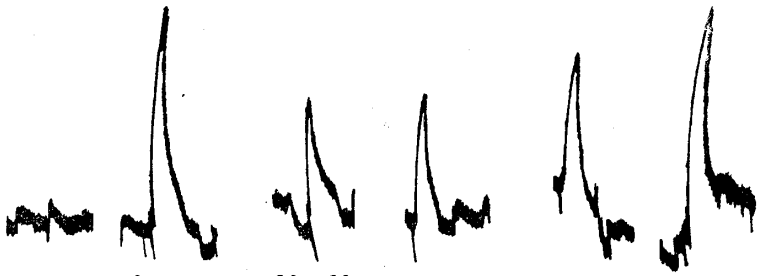
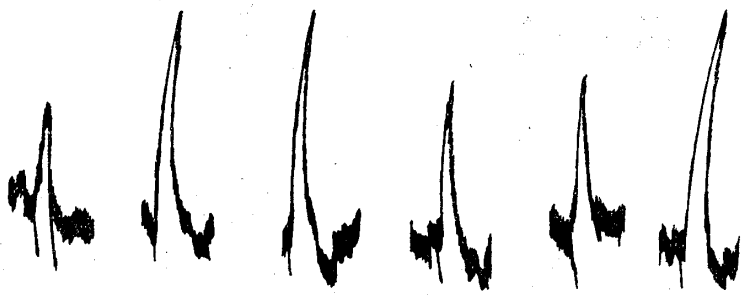
was calculated by multiplying the percent recovery of total radioactivity by that of the nondiffusible one in each perfusate. Consequently 0.58, 0.24, and 0.08% of labelled macromolecule were to be transmitted into the mesenteric vein during 0 to 30, 30 to 60, and 60 to 90 min, respectively.

The vasodilator activity as one of the representative actions of kallikrein could also be detected in the perfusate (Fig. 5). The perfusates after giving 820 KU of the kallikrein significantly increased the blood flow in the dog femoral artery, even being diluted 10 times, whereas the preliminary perfusate gave little or on response. The mean recovery of this activity in each perfusate was 0.53, 0.10, and 0.04% in the first, second, and third perfusates, respectively.



Perfusate(min)	−30−0	0−30	30−60	60−90	hog pancreatic kallikrein
Injected(ml)	0.2	0.02	0.02	0.04	
KU equivalent	0	0.019	0.011	0.012	0.01 0.02 *KU*
KU/ml	0	0.95	0.55	0.30	
KU/perfusate	0	3.0	1.0	0.51	



Perfusate(min)	−30−0	0−30	30−60	60−90	hog pancreatic kallikrein
Injected(ml)	0.2	0.01	0.01	0.01	
KU equivalent	0.004	0.009	0.009	0.006	0.005 0.01 *KU*
KU/ml	0.02	0.90	0.90	0.60	
KU/perfusate	0.13	5.87	3.39	1.45	

Fig. 5. Vasodilator Activity of the Mesenteric Perfusate

(blood flow increase response in dog by injection of 0.1 or 0.2 ml of the perfusate, with dilution if necessary, into the femoral artery)

Since the kallikreins split BAEE, the amount of kallikrein being transmitted into the mesenteric perfusate was also able to be determined by the esterolytic activity assay (Fig. 6). The perfusates which were collected prior to the administration of kallikrein or were obtained from the rat which was given the saline solution instead of the sample, did not have the potency, while the activity could be detected in all perfusates after giving kallikrein (50 to 150 μmoles/min, per head). The recovery of the activity in each perfusate agreed well with those of the nondiffusible radioactivity and of the vasodilator activity, and 0.8% of the administered activity was recovered during 90 min under this experimental condition (Table I). There were rough correlations between the amounts of kallikrein administered and the BAEE-esterolytic and vasodilator activities found in the mesenteric perfusate (Fig. 7).

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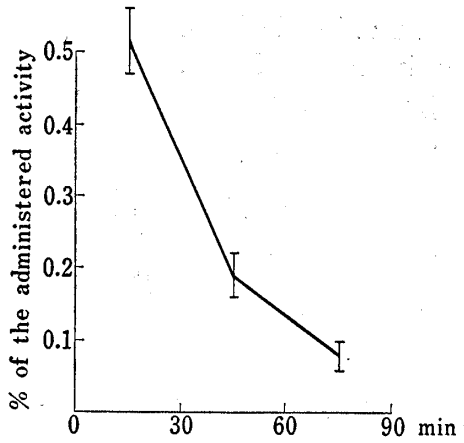

Fig. 6. Esterolytic Activity on BAEE in the Mesenteric Perfusate after Administration of Hog Pancreatic Kallikrein

(mean and s.e. of 31 experiments)

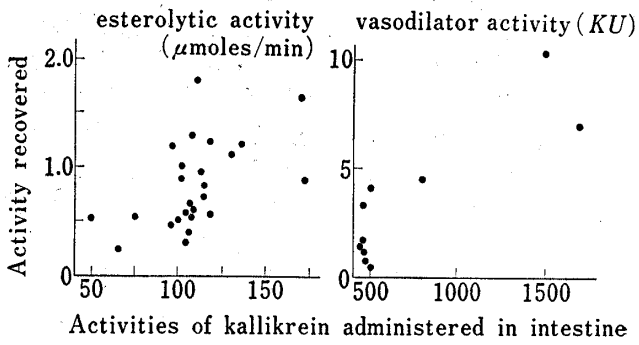

Fig. 7. Correlations between the Activities Administered in Intestine and Recovered in Mesenteric Perfusate (0—90 min)

Table I. Recoveries of Radioactivity and Kallikrein Activities in the Mesenteric Perfusate (% of the Total Administered)

Perfusate collected (min)	−30—0	0—30	30—60	60—90
Radioactivity	0	2.33±0.41	0.90±0.19	0.31±0.09
Nondiffusible radioactivity	0	0.58±0.10	0.24±0.05	0.08±0.01
Esterolytic activity	0	0.54±0.07	0.19±0.03	0.08±0.02
Vasodilator activity	0	0.53±0.13	0.10±0.03	0.04±0.01
Volume of perfusate (ml)	6.1±0.3	5.4±0.3	3.7±0.3	2.5±0.3

Each value represents the mean±s.e.

Discussion

As noted in our previous paper,[1b] the transmission of kallikrein through an everted sac of rat intestine *in vitro* or the finding of ^{131}I-tagged macromolecule(s) in serum are not enough to prove the absorption of the intestinally administered kallikrein. Demonstration of the typical actions of kallikreins in the blood plasma would be one direct evidence. However, this attempt is difficult because there are the endogenous kallikreins possessing almost identical properties with hog pancreatic kallikrein and the kallikrein inhibitors in the rat plasma. To avoid these interferences, the *in situ* mesenteric perfusion system has been established by the authors[7] in which the blood in the mesenteric vascular system is replaced by a suitable solution, and it becomes possible to determine the various activities of the sample enzyme effluxed into the mesenteric vein from the intestine.

The vasodilator and the synthetic arginine ester-hydrolyzing responses are the representative actions of the kallikreins.[10] As shown in the experimental results these activities were detected in the mesenteric perfusate from the rats which received hog pancreatic kallikrein in the bowel. It is reasonable that the experimental animals have their own kallikreins in the intestine and other places in the body, but it is obvious that these endogenous kallikreins do not participate in the present experimental results because there were only a few or no kallikrein activities in the control perfusate.

10) E.G. Erdös, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by O. Eichler, A. Farah, H. Herken, and A.D. Welch, Springer-Verlag, Berlin, Heidelberg, New York, 1970, p. 52.

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The recovery of these activities in the perfusate was summarized in Table I. Though less than 1% of the administered activity could be found in the perfusate, the biological responses of the perfusate samples were significant (Fig. 5), and there were rough correlations between the dossages and the recovered activities (Fig. 7).

The perfusion continued for 90 min, but most of the recovered activities were concentrated in the first 30 min perfusate. The same efflux patterns were obtained in the radioactivity and ^{131}I-macromolecule, but not in the perfusate volume. Hence it appears that the absorption of kallikrein occurred immediately after the administration, but it seems to be restrained after a little. It remains to be elucidated whether this restriction is caused by some mechanisms in the absorptive cells of the intestine or by the experimental conditions employed here.

In addition to the detection of the kallikrein activities, it was demonstrated that there were substances which reacted against the anti-kallikrein serum in the mesenteric perfusate. In the double immunodiffusion the perfusate samples formed the precipitin bands which fused with those of the given kallikrein and another more potent kallikrein preparation. However, it is still a dubious point as to whether the kallikrein itself was absorbed or not, because there were quite a few bands around each sample, even around the kallikrein preparations (Fig. 1).

A precipitin band, where BAEE-hydrolyzing activity is located, can be demonstrated on an agar plate.[9] On the immunoelectrophoresis of the sample kallikrein, only the fastest band toward the anode, which was identified as the kallikrein in comparing with a highly purified preparation, possessed this esterolytic activity.[11] One of the bands which were formed around each perfusate sample, except the preliminary one, gave a positive result in this activity-staining and these bands fused mutually (Fig. 2). These immunochemical findings also indicated the presence of the administered kallikrein in the mesenteric perfusate.

Thus, transmission of the hog pancreatic kallikrein from the rat intestine into the mesenteric vascular system was confirmed with the demonstration of the typical activities and the immunoreactivity of the kallikrein in the perfusate. Recently, the histochemical evidence has been presented on horseradish peroxidase absorption.[12] The reaction product of the enzyme was observed in the various epithelial cells, including the intestine, and in the blood and lymph vessels lying under those cells. These data seem to be decisive proof of the intestinal absorption of the macromolecules. However, the mechanisms of the absorption and its physiological rationales have to be investigated. Further experiment with this view is now being carried out in our laboratory on the pancreatic kallikrein.

Acknowledgement Thanks are due to Mr. N. Misaki and Miss Y. Kikuchi for their co-operation in this work.

11) Y. Fujimoto, H. Moriya, K. Yamaguchi, and C. Moriwaki, *J. Biochem.*, **71**, 751 (1972).
12) *a*) R. Cornell, W.A. Walker, and K.J. Isselbacher, *Lab. Invest.*, **25**, 42 (1971); *b*) A.L. Warshaw, W.A. Walker, R. Cornell, and K.J. Isselbacher, *ibid.*, **25**, 675 (1971).

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