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Studies on Kallikrein Inhibitors from Potatoes. III.[1] Some Pharmacological Actions of Potato-Kallikrein-Inhibitors (PKI)

Yoshio Hojima, Hiroshi Moriya, Chiaki Moriwaki[2a] and Tetsuya Tajima[2b]

Faculty of Pharmaceutical Sciences, Science University of Tokyo[2a] and Laboratory of Pharmacology, Eisai Co., Ltd.[2b]

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Potato-kallikrein-inhibitors (PKI) showed the anti-inflammatory potency in rat carrageenin edema and reduced the exudation of leucocytes into carboxymethylcellulose pouch in the dorsum of rats, but not the protein exudation. PKI was not effective against egg white edema, which was depressed by α-chymotrypsin to some extent.

PKI showed the inhibitory action on the release of vasodilative substances produced from rat serum (pH 4) and human plasma (pH 5) by acidification *in vitro*, which might be the products (kinin- and kallikrein-like, respectively) from the activation of kallikrein-kinin system. PKI also inhibited the acetone activation of human plasma. These inhibitory actions of PKI were stronger than Trasylol using equal kallikrein inhibitor units.

In the rat anaphylactic shock provoked by challenging with horse serum as antigen, PKI, Trasylol, soybean trypsin inhibitor and other tested substances for the inhibition of anaphylaxis did not show the definite effectiveness on the survival rate of rats.

It has been recently recognized that kallikrein-kinin system gives some effects to the clinical or experimental, pathological conditions of diseases, such as inflammation, disorders of the blood clotting system, and anaphylaxis.[3,4] For their therapy and investigations on their detailed mechanisms, naturally occurring proteinase inhibitors, for example, Trasylol (kallikrein-trypsin-inhibitor from bovine lung),[5] soybean trypsin inhibitor (SBTI), have been applied.[4]

The authors reported in the previous papers the partial purification and some properties of kallikrein inhibitors from potatoes (PKI),[1,6] and suggested that PKI had potent inhibitory action on plasma kallikrein(s),[7] which is an important factor in kallikrein-kinin system and may be related to the inflammatory process. On the other hand, it has been reported that potatoes contain various proteinase inhibitors besides PKI,[8] and Mansfeld, *et al.* studied some proteinase inhibitors from potatoes with their anti-inflammatory activities.[9] From these reasons, the authors expected anti-inflammatory effects of PKI and inhibitory action by PKI on the intrinsic activation of kallikrein-kinin system *in vitro* and *in vivo*.

1) Part II: Y. Hojima, H. Moriya and C. Moriwaki, *J. Biochem.* (Tokyo), **69**, 1027 (1971).
2) Location: *a) Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo; b) Koishikawa, Bunkyo-ku, Tokyo.*
3) R.W. Kellermeyer and B.C. Graham, *New Engl. J. Med.*, **279**, 754 (1968); *idem, ibid.*, **279**, 802 (1968); *idem, ibid.*, **279**, 859 (1968).
4) R. Vogel and G.Z.-Rüdel, "Handbuch der experimentallen Pharmakologie," Vol. 25, ed. by E.G. Erdös, Springer-Verlag, Berlin-Heidelberg-New York, 1970, pp. 550—578.
5) I. Trautschold, E. Werle and G.Z.-Rüdel, *Arzneimittel-Forsch.*, **16**, 1507 (1966).
6) *a)* Y. Hojima, H. Moriya and C. Moriwaki, *J. Biochem.* (Tokyo), **69**, 1019 (1971); *b)* H. Moriya, Y. Hojima, C. Moriwaki and T. Tajima, *Experientia*, **26**, 720 (1970).
7) M.E. Webster, *Fed. Proc.*, **27**, 84 (1968); Y. Hojima, H. Moriya and C. Moriwaki, *Arerugi* (Tokyo), **19**, 824 (1970).
8) R. Vogel, I. Trautschold and E. Werle, "Natürliche Proteinasen-Inhibitoren," Georg Thieme Verlag, Stuttgart, 1966, pp. 25—26.
9) *a)* V. Mansfeld, M. Rybák, Z. Horáková and J. Hladovec, *Hoppe-Seyler's Z. Physiol. Chem.*, **318**, 6 (1960); *b)* V. Rábek and V. Mansfeld, *Experientia*, **19**, 151 (1963).

Material and Method

Materials——The following materials were used: carrageenin (Seakem 402 type, Marine Colloids Inc.); dried egg white and α-chymotrypsin (1200 NF, u/mg) (Eisai Co.); carboxymethylcellulose (*ca.* 1000 in polymerization degree and 0.6—0.7 in etherification number, Daiichi-Pure Chem. Co.); bovine serum albumin (Armour Lab.); Trasylol (Bayer); SBTI (type I-S, Sigma Chem. Co.); phenylbutazone (Fujisawa Pharmac. Co.); mepyramine (Poulenc); dichlorodimethylsilane (Tokyo Kasei Kogyo Co.); Sephadex G-50 and G-150 (Pharmacia Co.). Other chemicals were of guaranteed reagent.

Two preparations of PKI (160 and 226 kallikrein inhibitor units (KIU)/mg, determined against hog pancreatic kallikrein) were obtained in our laboratory, and those preparations contained two, main components of inhibitor.[1,6] The former preparation was used in anti-inflammatory and anti-anaphylactic tests and the latter for the inhibition of the activation of kallikrein-kinin system *in vitro*.

Rat serum and human plasma were obtained from blood drawn from carotid and from blood (contained anti-coagulant solution in 20% v/v) purchased from Tokyo Plasma Institute, respectively. These blood were collected using polyethylene tube and siliconized vessel. Horse serum and *Bordetella pertussis* vaccine (24000×10^6 organisms/ml) were purchased from Kyokuto Pharmac. Indust. Co. and Chiba Serum Institute, respectively.

Anti-Swelling Tests of Carrageenin Edema and Egg White Edema——PKI and α-chymotrypsin were tested as anti-inflammatory drugs. Male, Wistar albino rats, 150—200 g of body weight (B.W.), were injected intraperitoneally (*i.p.*) or intramuscularly (*i.m.*) with graded doses of drugs at the volume of 0.2 ml/100 g B.W. After 30 min, 0.05 ml of 1% (w/v) carrageenin or 0.1 ml of 1% (w/v) egg white, dissolved in saline, was injected as irritant into the plantar tissue of the right hind paw of rats. On different times after the injection of irritant, the foot volume was measured by immersion into mercury to the marked level on the lateral malleolus. The mercury column was connected to a strain gauge (LPU-0.1-360-O-III, 1000 mm H_2O 10V max, Toyo Meas Tmi Ins Co.). The out put of the strain gauge was led through an amplifier (Model RP-3, Nihon Koden) to electronic polyrecorder (Model EPR-2TB, Toa Electronics). In this paper, the swelling and its inhibition were expressed with percentage from the following equations.

$$\text{Swelling } (\%) = \frac{B-A}{A} \times 100$$

A: foot volume before the irritant treatment.
B: foot volume after the irritant treatment.

$$\text{Inhibition } (\%) = \frac{Sc-Sd}{Sc} \times 100$$

Sc: swelling percentage of control group, injected with saline.
Sd: swelling percentage of drug administered group.

Anti-Exudation Test of Carboxymethylcellulose (CMC) Pouch——Male, Wistar albino rats weighing 180—220 g were injected with 25 ml of air to form pouch in the loose subcutaneous connective tissue on the dorsum. After 30 min, the rats were administered intramuscularyl with drug solution (0.2 ml/100 g B.W.). Moreover, after 30 min, the rats were injected 7 ml of 1% (w/v) CMC saline solution into the pouch. After each time, 0.3 ml of exudate fluid was picked out from pouch to determine leucocyte number (0.05 ml was used) and protein (0.2 ml). Neubauer's counting chamber and biuret reaction[10] with bovine serum albumin as standard protein were used for each determination, respectively.

Anaphylaxis in Rats——Equal numbers of male and female Wistar albino rats (B.W., 130—160 g) were sensitized with the intraperitoneal injection of 0.5 ml horse serum and 0.25 ml *Bordetella pertussis* vaccine according to the method of Dawson, *et al.*[11] Rats were challenged under light ether anesthesia at different days after sensitization with the injection of 1 ml horse serum through the femoral vein, and the mortality of rats over the next 24 hr was observed. The skin incised for the antigen injection was stitched up after challenging.

For the estimation of the inhibitory effects of some drugs on anaphylactic shock, after the intraperitoneal or intravenous injection of drug, rats were challeged as described above and the mortality over the next 24 hr was observed. All the experiments were made during the months from October to March, at the time when the sensitivity of Wistar rats to anaphylactic shock is maximal.[12]

Dog Vasodilative Activity——Vasodilative activity was used for the determination of kinin-like and kallikrein-like substances. Sample was injected into the dog femoral artery and the followed vasodilative

10) A.G. Gornall and C.S. Bardawill, *J. Biol. Chem.*, **177**, 751 (1949).
11) W. Dawson, M.S. Starr and G.B. West, *Brit. J. Pharmacol.*, **27**, 249 (1966).
12) S.I. Ankier, W. Dawson, S. Karady and G.B. West, *J. Pharm. Pharmacol.*, **17**, 187 (1965).

response was compared to that of standard hog pancreatic kallikrein, as previously described.[13] In this assay method, 2.5—3 kallikrein units (KU) have the equivalent activity with 1 μg bradykinin.

Protein Determination in Column Chromatography——The protein content of sample was estimated by measuring the absorbance at 280 mμ in a Hitachi photoelectric spectrophotometer (EPU-2A), using quartz cells with an optical path length of 10 mm.

Silicone Treatment of Glassware——To prevent the spontaneous activation of kallikrein-kinin system by glass surface, glassware used in the activation experiment of serum or plasma were siliconized with toluene solution of dichlorodimethylsilane (two times with 1% v/v solution).

Result

Inhibition of Edema

Tables I and II show the inhibitory effect of PKI on carrageenin edema, and Table III shows ineffectiveness of PKI on egg white edema. α-Chymotrypsin was used for the comparison of inhibitory effectiveness of PKI.[14] PKI administered intraperitoneally showed potent anti-inflammatory action in carrageenin edema (63% and 85% inhibitions at 2 hr after intraperitoneal administration of 2 mg/kg and 10 mg/kg, respectively), while α-chymotrypsin was not effective (Table I). In the intramuscular administration, PKI showed some anti-swelling effect as well as α-chymotrypsin, but not so effective as the intraperitoneal administration (Table II). In egg white edema, however, PKI intramuscularly administered was ineffective. On the other hand, α-chymotrypsin showed the effectiveness to some extent (Table III).

Table I. Effect of PKI *i. p.* Administered on Carrageenin Edema[a]

Drug	Time after irritation (hr)	Swelling (%) Mean±S. E.	Inhibition of edema (%)
Saline	2	23.4±6.6	—
	3	35.4±7.0	—
	4	43.0±7.9	—
PKI (2 mg/kg)	2	8.6±2.2	63[b]
	3	17.0±3.6	52[b]
	4	20.8±6.1	52[b]
PKI (10 mg/kg)	2	3.4±1.9	85[b]
	3	14.6±3.7	59[b]
	4	29.6±3.5	31[b]
α-Chymotrypsin (2 mg/kg)	2	25.2±2.9	0
	3	36.2±2.3	0
	4	47.2±4.8	0
α-Chymotrypsin (10 mg/kg)	2	25.2±5.9	0
	3	31.2±6.1	0
	4	41.0±5.2	0

a) Five rats were used for each group. *b*) Statistically significant at $p=0.05$.

Inhibition of Exudation

CMC pouch has been used as a method for testing anti-inflammatory drugs, both steroid and non-steroid.[15] By this method, the time course of the leucocyte migration and the protein exudation into the pouch fiuid can be traced on an individual animal. Table IV

13) H. Moriya, K. Yamazaki and H. Fukushima, *J. Biochem.* (Tokyo), **58**, 201 (1965).
14) G.J. Martin, R. Brendel and J.M. Beiler, *Proc. Soc. Exptl. Biol. Med.*, **86**, 636 (1954); H. Cohen, M. Graff and W. Kleinberg, *Proc. Soc. Exptl. Biol. Med.*, **88**, 517 (1955).
15) H. Ishikawa, S. Niinobe and S. Tsurufuji, *Yakugaku Zasshi*, **88**, 1472 (1968); H. Ishikawa, Y. Mori and S. Tsurufuji, *ibid.*, **88**, 1491 (1968); H. Ishikawa, Y. Mori and S. Niinobe, *Chem. Pharm. Bull.* (Tokyo), **17**, 2279, (1969).

shows the potent inhibitory effect of PKI on leucocyte migration. PKI (10 mg/kg) showed 82% inhibition 2 hr after the irritation by CMC. However, PKI hardly inhibited protein exudation (Table IV).

Table II. Effect of PKI *i. m.* Administerd on Carrageenin Edema[a)]

Drug	Time after irritation (hr)	Swelling (%) Mean±S. E.	Inhibition of edema (%)
Saline	2	73.8±6.2	—
	3	94.0±5.7	—
	4	116.0±7.5	—
PKI (2 mg/kg)	2	71.2±5.7	3
	3	92.2±6.6	2
	4	92.2±4.3	21[b)]
PKI (10 mg/kg)	2	44.6±9.3	40[b)]
	3	65.8±4.0	30[b)]
	4	66.6±6.2	43[b)]
α-Chymotrypsin (2 mg/kg)	2	66.6±6.6	9
	3	81.8±4.0	13
	4	88.8±2.8	23[b)]
α-Chymotrypsin (10 mg/kg)	2	38.0±6.3	49[b)]
	3	57.6±5.7	39[b)]
	4	62.0±4.1	47[b)]

a) Five rats were used for each group. *b*) Statistically significant at $p=0.05$.

Table III. Effect of PKI *i. m.* Administered on Egg White Edema[a)]

Drug	Time after irritation (hr)	Swelling (%) Mean±S. E.	Inhibition of edema (%)
Saline	0.25	48.7±5.1	—
	0.5	60.0±5.9	—
	1	53.7±5.1	—
	2	55.0±6.9	—
	3	49.8±7.3	—
PKI (2 mg/kg)	0.25	54.8±5.0	0
	0.5	65.7±6.3	0
	1	59.8±6.2	0
	2	56.0±4.6	0
	3	56.0±4.9	0
PKI (10 mg/kg)	0.25	47.0±2.2	3
	0.5	59.0±4.0	2
	1	52.2±5.7	3
	2	49.5±4.3	10
	3	47.8±2.3	4
α-Chymotrypsin (2 mg/kg)	0.25	44.3±4.1	9
	0.5	52.7±5.2	11
	1	42.0±5.7	22[b)]
	2	41.8±6.5	24[b)]
	3	36.8±6.0	26[b)]
α-Chymotrypsin (10 mg/kg)	0.25	40.0±4.0	18[b)]
	0.5	43.7±4.8	27[b)]
	1	37.3±5.6	31[b)]
	2	31.5±6.0	43[b)]
	3	29.2±5.6	40[b)]

a) Six rats were used for each group. *b*) Statistically significant at $p=0.05$.

Table IV. Effects of PKI *i. m.* Administered on Leucocyte Migration and Protein Exudation[a)]

Drug	Time after irritation (hr)	Leucocyte counts/mm³ Mean±S. E.	Inhibition of migration (%)	mg of protein/ml Mean±S. E.	Inhibition of exudation (%)
Saline	3	1938± 1001	—	9.10±0.75	—
	6	13338± 4690	—	13.35±1.10	—
	9	25613± 1862	—	17.95±0.57	—
	24	64788±13080	—	34.30±2.22	—
PKI (10 mg/kg)	3	338± 321	82	9.20±1.18	0
	6	9300± 2730	30	10.85±1.18	19
	9	16700± 4825	35	18.10±1.26	0
	24	45113± 8675	30	32.45±0.39	5

a) Four rats were used for each group.

Acid Activation of Rat Serum

Acidification of serum or plasma to pH 2 has been used to activate kallikrein-kinin system.[16] However in the present experiment the authors adjusted rat serum to pH 4.0 with 1N HCl and incubated at 37°, as suggested by Takagi, *et al.* for acid-induced edema.[17] A vasodilative substance was gradually released in acidified serum (Fig. 1). PKI inhibited partially or almost completely the production of this substance at the final concentration of PKI 160 KIU/ml or 800 KIU/ml, respectively. Trasylol (800 KIU/ml) did not show such inhibitory effect (Fig. 1).

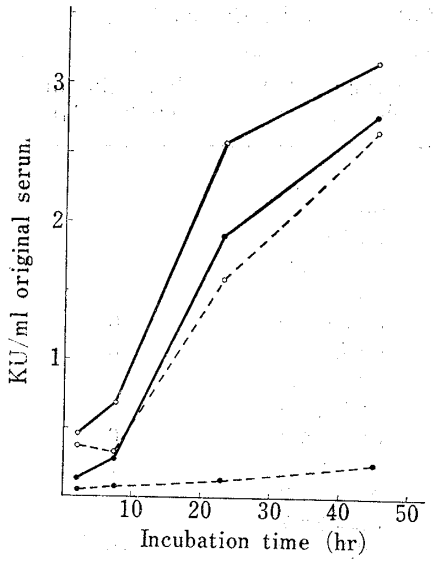

Fig. 1. Vasodilative Activity Produced from Rat Serum at pH 4.0

Rat serum (4 ml), adjusted to pH 4.0, was mixed with each 1 ml of saline, PKI (800 KIU/ml, 4000 KIU/ml) and Trasylol (4000 KIU/ml), respectively, and incubated at 37°. —○—: control, ---○---: PKI (final conc. 160 KIU /ml), ---●---: PKI (final conc. 800 KIU/ml), —●—: Trasylol.

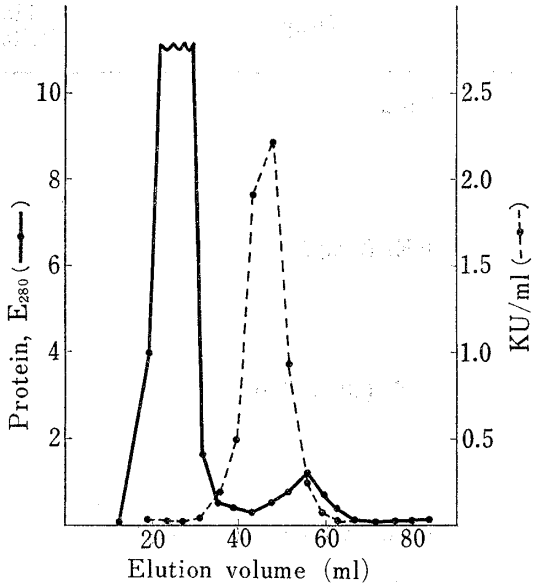

Fig. 2. Gel Filtration of Acidified Rat Serum (pH 4.0) on a Sephadex G-50 Column

Sample: 3.8 ml of rat serum incubated for 48 hr at pH 4.0 and 37°. column size: 1.5×28 cm, solvent: 0.01M CH_3COONa, pH 4.0.

16) V. Eisen, *J. Physiol.* (London), **166**, 496 (1963).
17) K. Takagi and S. Kayaoka, *Nippon Yakurigaku Zasshi*, **64**, 14§ (1968).

This vasodilative activity was also produced by acidifying serum (pH 3.5—4.5) with CH_3COOH. The active substance was shown by Sephadex G–50 gel filtration to be low in molecular weight (Fig. 2) and contracted guinea pig ileum and rat uterus in a medium containing mepyramine and atropine. This contractive activity disappeared by α-chymotrypsin treatment at neutral pH. The authors also detected bradykinin in the active fractions of gel filtration with the aid of Dansylation technique.[18] These data suggest that this vasodilative substance would be bradykinin released from serum kininogen by acid activation of kallikrein-kinin system.

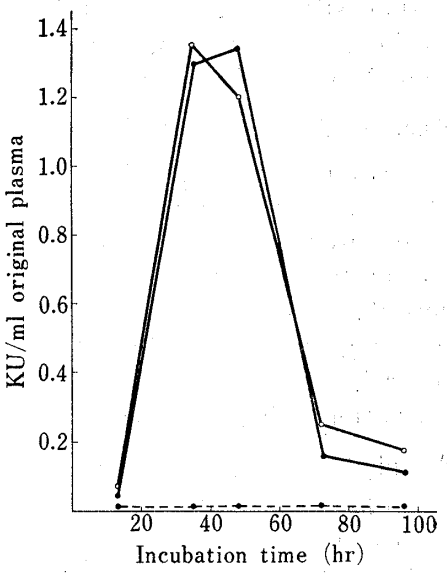

Fig. 3. Vasodilative Activity Produced from Human Plasma at pH 5.0

Human plasma (6.5 ml), adjusted to pH 5.0, was mixed with each 0.5 ml of saline, PKI (200 KIU/ml) and Trasylol (200 KIU/ml), respectively, and incubated at 37°.—○—: control, ---●---: PKI, —●—: Trasylol.

Acid Activation of Human Plasma

Saline, PKI or Trasylol was added into human plasma acidified at pH 5.0 with 3N HCl according to Frey, *et al.*[19] and then each plasma was incubated at 37°. Fig. 3 shows strong inhibitory effect of PKI on the production of vasodilative substance from acidified plasma, although Trasylol was ineffective. Trasylol was also hardly effective even if with higher final concentration (64 KIU/ml plasma).[6b] In gel filtration with a Sephadex G–150 column, the major part of produced activity was eluted in fractions of high molecular weight (Fig. 4). And the activity was inactivated after incu-

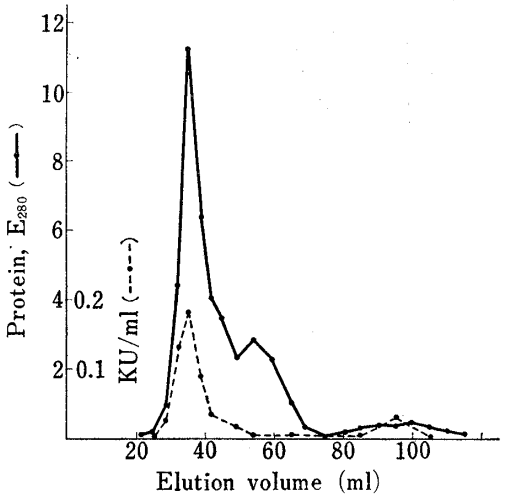

Fig. 4. Gel Filtration of Acidified Human Plasma (pH 5.0) on a Sephadex G-150 Column

Sample: 2.7 ml of human plasma incubated for 35 hr at pH 5.0 and 37°. column size: 1.3×59 cm, solvent: 0.15M NaCl adjusted to pH 5.0 with HCl

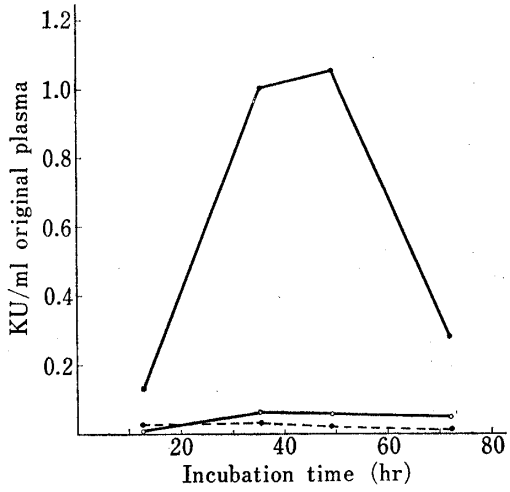

Fig. 5. Production of Vasodilative Substance from Human Plasma at Each pH Levels

Human plasma was adjusted to each pH with 3N HCl, and incubated at 37°. Activities of samples adjusted at pH 2, 3 and 7 were negligible as those at pH 4 and 6. —●—: pH 5, —○—: pH 4, ---●---: pH 6.

18) B.S. Hartley and V. Massey, *Biochim. Biophys. Acta*, **21**, 58 (1956).
19) E.K. Frey, H. Kraut and E. Werle, "Kallikrein, Padutin," Ferdinand Enke Verlag, Stuttgart, 1950, pp. 104—105.

bation with PKI. Hence this active substance would be acid-activated plasma kallikrein as Frey, *et al.* reported. This substance was produced specifically at pH 5.0 and at other pH, from 2 to 7, hardly produced (Fig. 5).

Acetone Activation of Human Plasma

Human plasma, which had contained saline as control, PKI or Trasylol, was activated at pH 7.5 standing for 3 hr at 25° and 20% (v/v) acetone concentration as previously reported.[20] Afterwards the acetone concentration of plasma was raised to 75% and acetone powder of plasma protein was prepared from the precipitate formed. This powder showed vasodilative activity in dog. The activity would be due to activated plasma kallikrein and/or other kinin-releasing enzymes.[7] Fig. 6, expressed in terms of percent inhibition comparing with the control, shows that PKI has the stronger inhibitory potency than Trasylol on the acetone activation of human plasma.

Anaphylactic Shock in Rats

Fig. 7 shows the mortality rate of rats challenged with antigen at various days after sensitization. The mortality rate in our experiment sometimes varied, especially at 10 days, and not always agreed with the result of Dawson, *et al.*[11] In their experiment the mortality over 20 days rapidly decreased as shown in Fig. 7, however the considerable mortality was observed in our experiment. The reason for this discrepancy has been inexplicable in the present stage of the investigation.

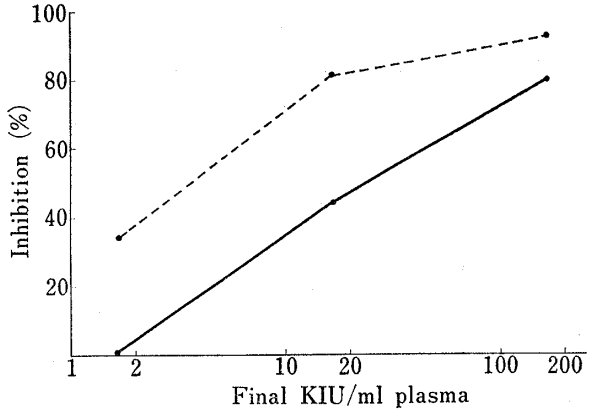

Fig. 6. Inhibitory Effects of PKI and Trasylol on Acetone Activation of Human Plasma (pH 7.5)

Human plasma (11 ml) was mixed with each 1 ml of saline, 20, 200 and 2000 KIU/ml solutions of PKI and Trasylol, respectively, and then submitted to acetone activation. Two Ku were obtained from 1 ml of control plasma. ---●---: PKI, —●—: Trasylol.

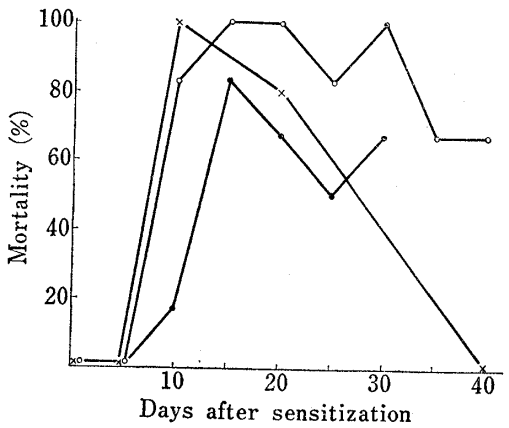

Fig. 7. Mortality of Wistar Rats Challenged by Intravenous Injection of Antigen

—○—, —●—: different results in our experiment (6 rats for each group). —×—: result of Dawson, *et al.*[11] (10 rats for each group).

Inhibition of Anaphylactic Shock

Table V shows a typical result for the inhibitory effects of various drugs on anaphylactic shock of rats. Challenging with antigen at 10 and 20 days after sensitization and the administration methods of drugs were carried out following Starr, *et al.*[21] In conclusion, together with other experimental data, any drug substance was not effective on the mortality except the somewhat inhibitory effectiveness shown by phenylbutazone. However, Starr, *et al.* reported the marked effectiveness of ascorbic acid with mepyramine, phenylbutazone and

20) H. Moriya, K. Yamazaki and H. Fukushima, *J. Biochem.* (Tokyo), **58**, 315 (1965).
21) M.S. Starr and G.B. West, *Brit. J. Pharmacol.*, **37**, 178 (1969).

SBTI.[21] In our result, PKI with two graded doses as well as SBTI seemed not effective on the survival of rats (Table V).

Table V. Effects of Some Drugs on the Mortality of Rat Anaphylaxis[a]

Drug[b]	Dose (mg/kg)	Time of administration before challenging (min)	Mortality ratio	
			10 days	20 days
Saline	1 ml	5	4/6	5/6
PKI	10	5	2/6	5/6
PKI	20	5	4/6	5/6
SBTI	20	5	4/6	5/6
Trasylol	5000 KIU	5	4/6	5/6
Phenylbutazone[c]	100	60	2/6	5/6
Ascorbic acid[c] and mepyramine[c]	200 10	15 30	4/6	4/6

a) Three male and female rats were used for each group.
b) Dissolved in saline
c) These drugs were intraperitoneally administered, and other drugs in Table intravenously.

Discussion

Mansfeld, *et al.* reported that two trypsin inhibitors from potatoes prevented kaolin induced edema formation.[9a] Their inhibitors seemed to be low in molecular weight from the result of Sephadex G-25 gel filtration,[9b] so that our PKI (molecular weight, *ca.* 25000) would be different from their inhibitors.[6b] Moreover, although various proteinase inhibitors were detected in potatoes,[8] our PKI was already ascertained to be different from those inhibitors[1,6] and the authors recently discussed the mutual relationship among potato-inhibitors.[22]

PKI showed the strong anti-inflammatory potency on carrageenin edema (Table I and II). Although the anti-inflammatory mechanism by PKI should not be imprudently mentioned since the inflammatory processes are greatly complicated, the authors should like to speculate as follows. It has been reported that carrageenin activates kallikrein-kinin system in plasma through the activation of Hageman factor, followed with kinin liberation.[23] Therefore, PKI administered into the experimental animal would be transported to the inflammatory site, unchanging its polyvalent inhibitory activity against proteinases, and then PKI block some point of the activation processes induced by carrageenin. Accordingly, PKI hardly inhibited egg white edema, which has been recognized to be mediated mainly by histamine releasing[24] (Table III). PKI also inhibited the leucocyte migration in CMC pouch method, but on the protein exudation the inhibitory effect was negligible (Table IV). Such action was reported on steroidal anti-inflammatory agents by Ishikawa, *et al.*[15]

From the acidified rat serum (pH 4.0) kinin-like substance, probably bradykinin, was released (Fig. 1), but kinin-releasing enzyme could not be detected in the high molecular fractions of Fig. 2 by the dog vasodilative assay. This would be due to the possibility that kinin-releasing enzyme from acidified rat serum could not act on dog plasma kininogen because of the species-difference between enzyme and substrate kininogen.[25] And also, the reason for the detection of kinin-like substance, although kinin is unstable in normal *in vivo* and *in vitro* condition, would be due to the non-reactivity of kininase (enzyme to inactivate

22) Y. Hojima, M. Tanaka, H. Moriya and C. Moriwaki, *Arerugi* (Tokyo), **20**, 755 (1971).
23) H.J. Schwartz and R.W. Kellermeyer, *Proc. Soc. Exptl. Biol. Med.*, **132**, 1012 (1969); M. Di Rosa and L. Sorrentito, *Brit. J. Pharmacol.*, **38**, 214 (1970).
24) J.R. Parrat and G.B. West, *Brit. J. Pharmacol.*, **13**, 65 (1958).
25) S. Jacobsen, *Brit. J. Pharmacol.*, **28**, 64 (1966).

kinin) at acidic pH.[26] For the acid activation of human plasma, activated kallikrein-like substance would be relatively unstable at pH 5.0 and 37°, therefore its activity decreased over 35—45 hr incubation time (Fig. 3 and 5). That the distinct activity of kinin-like substance was not detectable in this case (Fig. 4) would be due to the non-reactivity of the enzyme on human plasma kininogen and/or the inactivation of the substance by kininase, in this *in vitro* condition. At any rate, probably by forming the complex with enzyme or the initiator for activation of kallikrein-kinin system, PKI inhibited the activation of rat serum and human plasma by acid or acetone with stronger potency than Trasylol (Fig. 1, 3, and 6). These effects of PKI would be attributable to the inhibitory specificity of PKI onto the plasma kinin-forming system and/or stabilities of PKI in plasma and in the acidified circumstance. However, it remains unsolved on what process of the system PKI act in each activation.

On anaplylactic shock in rats, Dawson, *et al.*[11] and Starr, *et al.*[21] maintained two phases hypothesis on shock mediator: an early phase at 10 days after sensitization in which bradykinin is a main mediator and a late phase at 20 days in which bradykinin is not involved. Accordingly, SBTI (typical inhibitor of plasma kallikrein) was effective at the early phase anaphylactic shock and on the contrary, was not effective at the late phase in their experiment.[21] But, in our experiment, SBTI as well as PKI was not effective even if on anaphylaxis at 10 days (Table V). Ascorbic acid with mepyramine, reported effective by Starr, *et al.*, was also not effective. Although the authors cannot interpret these different results obtained by two groups, the authors should like to suppose that not only bradykinin produced from the activation of kallikrein-kinin system induced by antigen but also other mediators or systems, such as slow reacting substance in anaphylaxis and complement system, might be considered as combined mediators of anaphylaxis even at the early phase.

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