[Chem. Pharm. Bull. 27(10)2424—2430(1979)]

UDC 557.159-02.07 : 582.287.08

Kinin-Inactivating Enzyme from the Mushroom *Tricholoma conglobatum*. V. Its Application to Anaphylactic Shock in Rats[1]

Kazuyuki Kizuki, Hiroshi Moriya, and Chiaki Moriwaki

Laboratory of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo[2]

(Received April 28, 1979)

In order to clarify the involvement of the kallikrein-kinin system in anaphylactic shock, Shimeji kininase, which is able to block kinin action in the body due to rapid kinin destruction, was tested on anaphylactic shock in rats.

The anaphylactic shock in rats at 15 days after sensitization with egg albumin was hardly suppressed by Shimeji kininase alone or mepyramine alone, but the rats were markedly protected when this enzyme was used together with mepyramine. The rats at 30 days after sensitization were completely protected against shock by mepyramine alone and were also protected to some extent by Shimeji kininase alone. Thus, at the early phase after sensitization, both kinins and histamine appear to play important roles in anaphylaxis in rats, but in the late phase after sensitization, histamine is considered to play a more important role than kinins.

In the rats at 15 days after sensitization, the high molecular weight kininogen level in plasma did not alter on challenge with antigen, and plasma prekallikrein activation was not detected. On the other hand, a slight but significant decrease of low molecular weight kininogen level and an increase of kinin level on challenge with antigen were observed.

Keywords——kininase from the mushroom *Tricholoma conglobatum*; Shimeji kininase; kallikrein-kinin system; kininogen; anaphylactic shock; histamine; kinin blocker

Recently, there have been many reports on the properties of substances related to the kallikrein-kinin system, such as kallikreins, kinins, kininases and so on. However, the physiological and pathological significance of this system in the body is still not well understood. The roles of this system in the body could be studied by experiments in which the kinin action is specifically blocked, but at present no specific antagonist to kinins is available; only compounds with moderate antagonistic properties are known.[3]

The authors have been working on a potent kinin-inactivating enzyme, Shimeji kininase, from the mushroom *Tricholoma conglobatum* in the hope of blocking kinin action in the body.[4] This enzyme has the most potent kinin-inactivating activity ever found among plant kininases under our experimental conditions,[4a] and could block kinin action in the body for a fairly long time.[4c] Thus, this enzyme may be useful in research of the physiological and pathological significance of the kallikrein-kinin system in the body as a blocker of kinin action.

1) Part IV: K. Kizuki, H. Moriya, and C. Moriwaki, *Chem. Pharm. Bull.* (Tokyo), **27**, 1618 (1979).
2) Location: *Ichigaya Funagawaracho, Shinjuku-ku, Tokyo, 162, Japan.*
3) *a*) E.J. Walaszek, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös and A.F. Wilde, Springer-Verlag, Berlin-Heiderberg-New York, 1970, pp. 424—425; *b*) E. Schröder, *ibid.*, pp. 324—350; *c*) A. Gecse, E. Zsillinszky, and L. Szekeres, "Advances in Experimental Medicine and Biology," Vol. 70, ed. by F. Scuteri, N. Back, and G.L. Haberland, Plenum Press, New York and London, 1976, pp. 5—13.
4) *a*) K. Kizuki, C. Moriwaki, Y. Hojima, and H. Moriya, *Chem. Pharm. Bull.* (Tokyo), **24**, 1742 (1976); *b*) K. Kizuki, H. Moriya, and C. Moriwaki, *ibid.*, **27**, 654 (1979); *c*) *Idem, ibid.*, **27**, 662 (1979).

In the present investigation, the authors tested this enzyme in cases of anaphylactic shock in rats, in which the involvement of kinins would be assumed or thought to be doubtful.[5]

Materials and Methods

Materials——Egg albumin was purchased from Tokyo Kasei Kogyo Co. (Tokyo) and pertussis-diphtheria vaccine was obtained from Takeda Chemical Industries, Ltd. (Osaka). Bradykinin (BK) was a product of the Protein Research Foundation (Osaka). Trypsin (twice crystallized) was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), atropine sulfate from Iwaki Seiyaku Co. (Tokyo) and mepyramine maleate from Poulenc. Ltée-Ltd. (Montreal, Canada). Shimeji kininase was purified as described in our previous paper.[4a]

Anaphylaxis in Rats——Female Wistar rats (age 5 weeks) were sensitized both by subcutaneous injection with 2% egg albumin (0.5 ml) in saline at the thigh and by intraperitoneal injection with 0.5 ml of pertussis-diphtheria vaccine. Rats were challenged intravenously with 2% egg albumin (0.5 ml) under slight ether anesthesia at various times, and the mortality over the next 10 hr was observed.

Kininogen Contents in Plasma——The high molecular weight kininogen (HMW-K) and low molecular weight kininogen (LMW-K) contents in plasma were determined by the procedures of Katori *et al.*[6] with a slight modification.[1] The amount of kininogen was expressed as μg BK equivalent per ml of plasma.

Plasma Kininase Contents——Plasma kininase activity was assayed by the Magnus method using isolated rat uterus. An isolated rat uterus was suspended in a 10 ml bath in which De Jalon's solution containing atropine (3×10^{-8} M) and mepyramine (5×10^{-8} M) was aerated at 30° under a tension of about 2 g weight. To measure kininase activity, 5 ml of synthetic bradykinin solution (200 ng/ml) was incubated with 50 or 100 μl of plasma at 30°, then 50 μl of this mixture (9.9 or 9.8 ng bradykinin eq.) was added to the bath with a polyethylene micropipette at a selected time and the remaining kinin activity was determined. Kininase activity was calculated from the amount of inactivated bradykinin. The contractile response of bradykinin was recorded isotonically, and a standard curve was prepared using synthetic bradykinin (1 to 10 ng). The activity was expressed as the amount of enzyme that could inactivate BK (μg)/min/ml of plasma at 30°.

Kinin Contents——A polyethylene test tube containing 4 ml of ethanol was set up over a balance, and exactly 1 ml of blood was collected from the carotid artery of a rat with a polyethylene cannula. It was then heated for 10 min at 60°, and dried with a rotary evaporator under reduced pressure. The residue was reconstituted with 1 ml of saline containing 8-hydroxyquinoline (1 mg/ml) and centrifuged for 10 min at 3000 rpm. The contractile response of the supernatant was directly assayed by the Magnus method as mentioned above. Kinin content in plasma was expressed as ng of BK eq. per g of blood.

Results

Anaphylactic Shock in Rats

Figure 1 shows the mortality rates of rats challenged with antigen at various times after sensitization. After 10, 15 and 25 days sensitization, all the rats died within 10—15 min when challenged with antigen, and marked hyperemia in the small intestine was always observed. The mortality rate at 40 days, however, was 80% and the time from challenge until death had increased to 30—40 min. The rats challenged at 50 and 60 days also died, though only one rat was tested at each of these times. Marked hyperemia in the small intestine was also observed in the dead rats at 40, 50 and 60 days. The results of Dawson *et al.*[7] and Hojima *et al.*,[8] in which the rats were sensitized with horse serum, are included in Fig. 1 for comparison.

Effects of Shimeji Kininase on Anaphylactic Shock in Rats

Table I shows the inhibitory effects of Shimeji kininase on anaphylactic shock in rats at 15 days after sensitization. A single administration of 40 kininase U of Shimeji kininase (one kininase U is the amount of enzyme that can degrade 1 μg of bradykinin per min at

5) R. Voger, and G. Zickgraf-Rüdel, "Handbook of Experimental Pharmacology," Vol. XXV, ed. by E.G. Erdös and A.F. Wilde, Springer-Verlag, Berlin-Heiderberg-New York, 1970, pp. 559—563.
6) M. Katori, S. Mineshita, and T. Shigei, "Advances in Experimental Medicine and Biology," Vol. 8, ed. by F. Sicuteri, M. Rocha e Silva, and N. Back, Plenum Press, New York and London, 1970, pp. 273—282.
7) W. Dawson, M.S. Starr, and G.B. West, *Br. J. Pharmac. Chemother.*, **27**, 249 (1966).
8) Y. Hojima, H. Moriya, C. Moriwaki, and T. Tajima, *Chem. Pharm. Bull.* (Tokyo), **20**, 1312 (1972).

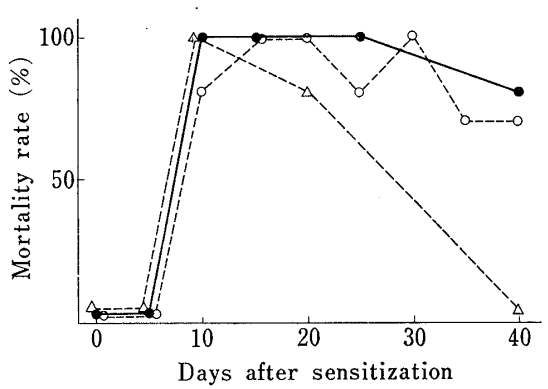

Fig. 1. Mortality Rates of Rats challenged with Antigen at Different Times after Sensitization with Egg Albumin and Pertussis-diphtheria Vaccine

●—●: our results. Five animals were employed in each group.
△---△ and ○---○: results of Dawson *et al.*[7] and Hojima *et al.*,[8] respectively.

pared with those of the control animals. The rats were completely protected from death by a single administration of mepyramine.

30°, pH 7.4 *in vitro*) did not protect the rats. The rats were also poorly protected by 120 and even 240 kininase U of Shimeji kininase; only one rat in each group survived 10 hr after challenge. An anti-histaminic agent, mepyramine, alone also had little protective effect. However, the shock was markedly suppressed when both agents were intravenously administered together; four rats in this group survived 10 hr after the challenge with antigen (the survival rate was about 67%).

On the other hand, the anaphylaxis in rats at 30 days after sensitization was significantly suppressed by Shimeji kininase alone (Table II); the survival rate at 10 hr after challenge was about 38% and the times from challenge until death of the rats that died were increased by pretreatment with Shimeji kininase com-

Table I. Effects of Shimeji Kininase and Mepyramine on the Survival Rates of Rats Challenged with Antigen at 15 Days after Sensitization

Pretreatment	Survival rate Time after challenge					
	15 min	30 min	1 hr	2 hr	10 hr	(%)
Control (saline, 0.3 ml)	1/6	1/6	0/6			(0)
Shimeji kininase 40 kininase U	0/6					(0)
Shimeji kininase 120 kininase U	1/6	1/6	1/6	1/6	1/6	(17)
Shimeji kininase 240 kininase U	5/7	3/7	2/7	1/7	1/7	(14)
Mepyramine 1 mg	4/8	2/8	2/8	1/8	1/8	(13)
Shimeji kininase 40 kininase U + mepyramine 1 mg	6/6	4/6	4/6	4/6	4/6	(67)

The survival rates (number of surviving rats/number of rats employed in each group) were observed for 10 hr after challenge with antigen. Shimeji kininase and mepyramine were intravenously administered to the rats 15 days after sensitization, 1 min before the challenge with antigen. The survival rates at 10 hr after challenge are shown in parentheses, expressed as percentages.

Sensitivity of the Sensitized Rats to Bradykinin and Histamine

As anaphylaxis in rats at 15 days after sensitization was markedly suppressed when Shimeji kininase and mepyramine were administered together (Table I), bradykinin and histamine instead of the antigen were intravenously injected into the rats at 15 days after sensitization. The doses of bradykinin used were extremely large; the total kininogen content in plasma (HMW-K plus LMW-K) was less than 2 μg of BK eq./ml in our experiments (see Fig. 2), so that if kinins were liberated from kininogen in the body, the amount of liberated kinins would be much less than the highest dose of bradykinin used. The histamine content

Table II. Effects of Shimeji Kininase and Mepyramine on the Survival Rates of Rats Challenged with Antigen at 30 Days after Sensitization

Pretreatment	Survival rate Time after challenge					
	15 min	30 min	1 hr	2 hr	10 hr	(%)
Control (saline, 0.3 ml)	4/7	2/7	2/7	1/7	0/7	(0)
Shimeji kininase 100 kininase U	13/13	10/13	9/13	6/13	5/13	(38)
Mepyramine 1 mg	7/7	7/7	7/7	7/7	7/7	(100)

See the footnote to Table I.

Table III. Sensitivity of Rats at 15 Days after Sensitization with Egg Albumin and Pertussis-diphtheria Vaccine

Samples (μg/100 g rat body weight)	Mortality rate
Bradykinin 14 μg	0/5
Histamine 20 μg	0/5
Bradykinin 14 μg + histamine 20 μg	0/5
Bradykinin 30 μg + histamine 40 μg	0/5
Bradykinin 80 μg + histamine 100 μg	0/5

Bradykinin and histamine dissolved in saline were intravenously administered to the rats 15 days after sensitization, and the mortality rates of rats (number of dead rats/number of rats employed in each group) were observed for 10 hr.

in the rat whole body is not known, but the dose can be assumed to be relatively large. As shown in Table III, none of the rats died after intravenous injection of bradykinin or histamine, or even after receiving a mixture of high doses of both agents. Rats which were not sensitized, of course, did not die after the intravenous injection of these agents.

Effects of Sensitization and Challenge on Plasma Kininogen, Kinin and Kininase Contents

Figure 2 shows the contents of HMW-K and LMW-K in plasma before and after challenge with antigen at various times after sensitization. The open symbols in Fig. 2 are the results obtained before challenge. The control levels of HMW-K and LMW-K (0 day in Fig. 2) were 0.88 ± 0.05 and 0.43 ± 0.09 μg BK eq./ml, respectively. After sensitization with egg albumin and pertussis-diphtheria vaccine, the HMW-K level did not change from the level of the control rats. In contrast, the LMW-K level increased significantly at 10 days after sensitization and reached about 1.7 times the control level ($p<0.01$). After that time it gradually decreased to the control level. At 10 and 20 days, when all the rats died on challenge, a slight but significant decrease of LMW-K level on challenge (closed squares in Fig. 2) was observed ($p<0.01$ and $p<0.025$, respectively), but the HMW-K level was unaltered by challenge with antigen (closed circles in Fig. 2). At 5 days, when the rats did not die on challenge, the HMW-K and LMW-K levels did not change compared to those of non-challenged rats.

Figure 3 shows the plasma kinin levels obtained before and after challenge with antigen. The control level of kinin (day 0 in Fig. 3) was 7.6 ± 1.8 ng of BK eq./g of blood. The level increased significantly at 10 days after sensitization and reached about twice the control value ($p<0.01$). After that time, the kinin level recovered more or less to the normal level.

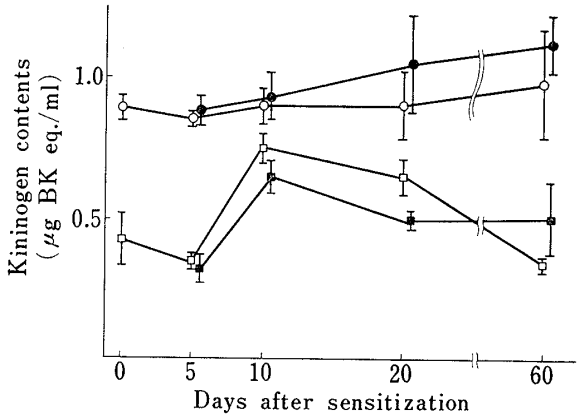

Fig. 2. HMW-K and LMW-K Levels in Plasma before and after Challenge with Antigen at Different Times after Sensitization with Egg Albumin and Pertussis-diphtheria Vaccine

○—○ and ●—●: HMW-K levels in plasma before and 5 min after challenge with antigen, respectively.
□—□ and ■—■: LMW-K levels in plasma before and 5 min after challenge with antigen, respectively. The rats at day 0 were not sensitized. The vertical ranges for each point show the standard error. Five to nine rats were employed for each point.

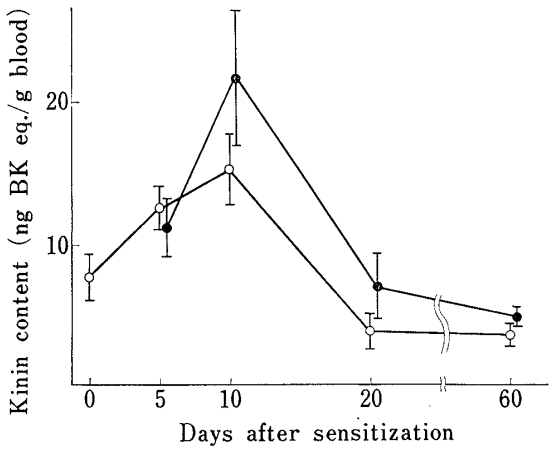

Fig. 3. Kinin Levels in Plasma before and after Challenge with Antigen at Different Times after Sensitization with Egg Albumin and Pertussis-diphtheria Vaccine

○—○ and ●—●: Kinin levels in plasma before and 5 min after challenge with antigen, respectively. The rats at day 0 were not sensitized. The vertical ranges at each point indicate the standard error. Five to eight animals were employed for each point.

On comparison before and after challenge at 10 and 20 days, the kinin levels at 10 and 20 days increased 1.4 and 1.9-fold ($p<0.25$ and $p<0.05$, respectively) on challenge with antigen, respectively, but at 5 days the kinin level did not alter on challenge with antigen.

The kininase level in plasma was virtually constant until 60 days, with a slight increase at 5 days after sensitization (Fig. 4).

Failure of Kinin Liberation on Treatment of Sensitized Rat Plasma with Antigen

Egg albumin was added to fresh rat plasma collected from rats at 15 days after sensitiza-

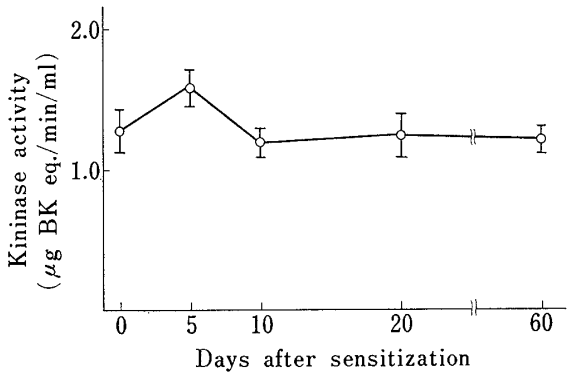

Fig. 4. Kininase Activity in Plasma at Different Times after Sensitization with Egg Albumin and Pertussis-diphtheria Vaccine

The rats at day 0 were not sensitized. Seven to nine animals were employed for each point. The vertical ranges for each point show standard errors.

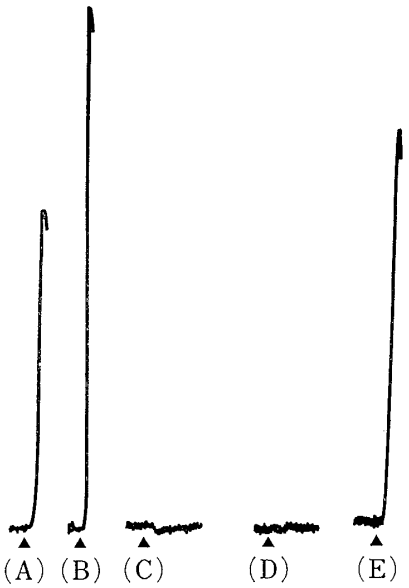

Fig. 5. Failure of Kinin Liberation on Addition of Antigen to the Plasma Collected from Sensitized Rats

(A) and (B): bradykinin 2 and 5 ng, respectively.
(C) and (D): 10 μl of egg albumin solution (100 mg/ml in saline) was added to fresh rat plasma (1 ml) collected from rats at 15 days after sensitization and incubated in the presence of 8-hydroxyquinoline (1 mg/ml) at 30°. After 10 and 30 min, 50 μl aliquots of this mixture were added to the organ bath.
(E): Glass beads were added to the plasma used in (C) and (D) (1: 1, w/v) and incubated in the presence of 8-hydroxyquinoline at 30°. After 10 min, 5 μl was assayed.

tion (1 mg/ml). Kinin liberation in this plasma was checked by the Magnus method using isolated rat uterus. As shown in Fig. 5-C and -D, no kinin activity was observed in the plasma even after incubation with antigen for 30 min. On the other hand, when the same plasma was treated with glass beads, it contracted guinea pig ileum as the result of plasma prekallikrein activation (Fig. 5-E). Failure of kinin liberation by the antigen can account for the unchanged HMW-K level on challenge, as shown in Fig. 2.

These observations suggest that plasma prekallikrein activation may not be involved in anaphylaxis in rats as a primary reaction.

Discussion

During anaphylactic shock, changes in the levels of substances related to the kallikrein-kinin system in the plasma were observed and the involvement of this system in anaphylactic shock has attracted considerable interest.[5]

In the present investigation, the authors tested Shimeji kininase in cases of anaphylactic shock in rats. This enzyme could block kinin action in the body due to rapid destruction of kinin, but did not suppress the actions of acetylcholine, histamine and serotonin.[4c]

At the beginning of the present study, the authors investigated the mortality rate of rats challenged with antigen at various times after sensitization. As shown in Fig. 1, the maximum sensitivity of rats to specific antigen was observed at 10 to 25 days after sensitization, then the sensitivity gradually decreased, in accord with the observations of Dawson *et al.*[7] and Hojima *et al.*[8]

Shimeji kininase markedly protected the rats at 15 days after sensitization when used together with mepyramine, but Shimeji kininase alone or mepyramine alone hardly protected the rats at all (Table I). The absence of protection by mepyramine alone is in good accord with the results of Dawson *et al.*[7] and Starr *et al.*[9] On the other hand, at 30 days the rats were significantly protected by Shimeji kininase alone, and completely protected by mepyramine alone (Table II). These findings indicate that both kinins and histamine play important roles in anaphylactic shock in rats at 15 days after sensitization. At 30 days, when the sensitivity to antigen had fallen compared to that at 15 days, histamine may play a more important role than kinins. Starr *et al.*[9] and Dawson *et al.*[7] reported that there are two phases in anaphylactic shock in rats, *i.e.*, an early phase at 10 days after sensitization, in which kinin is the main mediator rather than histamine, and a late phase at 20 days in which kinin is not involved. However, Hojima *et al.* reported that soybean trypsin inhibitor, which inhibits plasma kallikrein but does not inhibit glandular kallikrein, and potato kallikrein inhibitor and Trasylol, which inhibit both kallikreins, did not protect the rats at 10 or 20 days after sensitization.[8] This observation seemed to rule out a role of kinin in the early phase, but the failure of these inhibitors to protect rats can now be explained by our present observations. Namely, at the early phase, in which the sensitivity to specific antigen is nearly maximum, both kinins and histamine probably play important roles in anaphylaxis in rats. Thus, the inhibition of kallikrein alone by these inhibitors would not be sufficient to protect the rats.

On the other hand, the complete protection of rats by mepyramine in the period after maximum sensitivity to specific antigen suggested that histamine played a more important role than kinins. This consideration is consistent with the results of Starr *et al.* and Dawson *et al.* at the late phase, but does not rule out the involvement of kinins in anaphylaxis at this time, because Shimeji kininase alone suppressed the shock to some extent (Table II). Kinins may also play an important role even at this time.

In addition to the protection of rats against shock by kinin blocking agents such as Shimeji kininase, the fall of the kininogen level and rise of the kinin level in plasma confirm

9) M.S. Starr and G.B. West, *Br. J. Pharmac.*, 37, 178 (1969).

the involvement of the kallikrein-kinin system in anaphylactic shock. Some investigators have already reported such a fall of the kininogen and rise of the kinin level.[5] However, these findings were not obtained consistently in all laboratories. In fact, even an increase of kininogen level was reported.[5,7] In the present investigation, the authors did not observe marked kininogen diminution induced by challenge with antigen, but at 10 and 20 days after sensitization, a slight decrease of LMW–K level and increase of kinin level on challenge with antigen were observed. The HMW–K level did not change on challenge. On the other hand, the addition of antigen to the plasma collected from the sensitized rats did not cause plasma prekallikrein activation (Fig. 5). Therefore, we can hypothesize that the glandular kallikreins rather than the plasma kallikrein were activated first, although the unchanged HMW–K level remains unexplained, because the plasma kallikrein generally acts on HMW–K specifically, while the glandular kallikreins act on both HMW–K and LMW–K. In rats that die in shock, damage to the small intestine is always observed, so that there is a possibility that the intestinal kallikrein plays an important role in anaphylactic shock in rats. Recently, the rat intestinal kallikrein was partially purified in our laboratory and it was shown that this enzyme has the general properties of a glandular kallikrein.[10] The possibility of involvement of the intestinal kallikrein in shock has been discussed by some investigators but no consensus has been emerged.[9,11]

On the other hand, damage to the small intestine or other organs would cause changes in the tissue surface. The plasma kallikrein is activated by contact of the plasma with a negatively charged surface. Thus, the change of the tissue surface may secondarily cause plasma prekallikrein activation and a reduction of HMW–K. Therefore, the time of blood collection after challenge might greatly affect the observed kininogen contents after challenge with antigen. This may be one reason why the reduction of kininogen level on challenge has not been consistently observed in all laboratories. In this work, it was necessary to collect the blood 5 min after challenge with antigen, because the rats generally died within 10—15 min, and some animals died within 6—9 min. Thus, if the blood could be collected at a time later than 5 min, different results would be obtained. The controls are also very important. Namely, the levels of substances related to the kallikrein-kinin system in plasma varied from the levels at day 0 with the passage of time after sensitization; the LMW–K level at 10 and 20 days and kinin level at 10 days were significantly increased after sensitization with egg albumin (Figs. 2 and 3). The kininase level at 5 days was also slightly increased (Fig. 4). Thus, if the values at day 0 are taken as a control value, even an increase of kininogen level on challenge would be obtained, as in the results of Dawson *et al.*[7] Namely, at 10 days after sensitization, they observed a 50% increase of the total kininogen level (HMW–K plus LMW–K) after challenge with antigen. However, this increase is not due to the challenge with antigen, judging from our present observations, *i.e.*, the kininogen level at this time was significantly increased by sensitization, so that if very strong kinin liberation due to the challenge did not take place rapidly, the decrease of kininogen level might not be observed. The marked increase of LMW–K level can not yet be interpreted.

Rats at 15 days after sensitization did not die on intravenous injection of bradykinin, histamine, or even a mixture of high doses of both agents. This observation might appear to conflict with the observed protection of rats by a mixture of Shimeji kininase and mepyramine, and seems not to support the involvement of kinins and histamine in anaphylaxis. However, this is not considered to rule out the involvement of kinins and histamine in anaphylaxis in rats because there is a big difference between the physiological significance of endogeneously generated substances and exogeneously administered ones with regard to their local concentrations and other factors.

10) C. Moriwaki, H. Fujimori, and Y. Yoyono, "Kininogenases," Vol. 4, ed. by G.L. Haberland, J.W. Rohen, and T. Suzuki, F.K. Schattauer-Verlag, Stuttgart-New York, 1977, pp. 283—290.
11) E. Werle, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **245**, 254 (1963).