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# ANTI-INFLAMMATORY EFFECT OF PROTEINASE INHIBITORS ON CARRAGEENIN-INDUCED INFLAMMATION IN RATS

HIDEO NAKAGAWA,* KAZUYOSHI WATANABE, KATSURO SHUTO and SUSUMU TSURUFUJI
Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Sendai 980, Japan

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**Abstract**—Proteinase inhibitors were evaluated for their anti-inflammatory actions on carrageenin-induced inflammation in rats. The development of granulation tissue and the exudate were markedly suppressed by a single injection of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) into the carrageenin-air-pouch immediately after carrageenin injection, whereas a single injection of TPCK at 12 or 24 hr after carrageenin injection was less effective or slightly effective respectively. These results suggest that proteinase inhibitors exert their anti-inflammatory actions by interfering with the initial inflammatory reactions after carrageenin injection. When the wet weight of granulation tissue and the weight of exudate were measured on day 4 after the simultaneous injection of carrageenin and inhibitors, a single injection of serine- and thiol-proteinase inhibitors including TPCK, leupeptin, antipain, chymostatin and cystamine suppressed the development of granulation tissue, though EDTA and *o*-phenanthroline, metallo-proteinase inhibitors, were also effective at a high dose. Exudate was reduced by treatment with TPCK in a dose-dependent manner, while EDTA and *o*-phenanthroline were effective only at a high dose. On the other hand, the migration of polymorphonuclear leukocytes into the carrageenin-air-pouch (the inflammatory lesion) was markedly suppressed by TPCK and leupeptin, while a high dose of cystamine and *o*-phenanthroline was slightly effective, and antipain, chymostatin, pepstatin, elastatinal, EDTA, *trans*-1-aminomethylcyclohexane 4-carboxylic acid and aprotinin were without effect.

It has been proposed that proteinases may be involved in a variety of pathological processes related to inflammation [1, 2]. Proteinases play a role in the generation of chemical mediators such as kinins [3] and of chemotactic factors [4, 5] and also in the direct destruction of structural components of tissues. It would be expected, therefore, that inflammatory processes mediated by proteinases would be susceptible to inhibition by proteinase inhibitors. A thiol-proteinase activity increases in parallel with the development of the cutaneous Arthus-type inflammation, and a specific inhibitor responsible for the cessation of the inflammation is present in the healing skin site of Arthus-type inflammation in rabbits [6].

Hyman and Vischer [7] demonstrated that proteinase inhibitors such as aprotinin, ovomucoid, soybean trypsin inhibitor and *t*-AMCHA† decreased the vascular permeability in a reverse passive Arthus reaction in the rat skin. Janoff *et al.* [8] and Troll *et al.* [9] demonstrated that proteinase inhibitors including TPCK inhibited vascular permeability and PMN infiltration in mouse ears treated with phorbol myristate acetate, a tumor promoter. It has been also demonstrated that proteinase inhibitors such as aprotinin [10], soybean trypsin inhibitor [11] and endogenous proteinase inhibitors [12] suppress experimental inflammation induced by urate crystals, kaoline and adjuvant respectively. Superoxide anion radicals have been regarded as being mediators of inflammation and of damage to biomembranes, and proteinase inhibitors block the formation of superoxide anion radicals in activated human PMNs and monocytes [13, 14].

In a previous paper [15], we demonstrated that proteinase inhibitors including serine-proteinase inhibitors suppressed carrageenin-induced inflammation in rats. The present studies are a further investigation of the effects of a number of proteinase inhibitors on the PMN migration into inflammatory lesions, the increase in exudate, and the development of granulation tissue in carrageenin-induced inflammation in rats.

## MATERIALS AND METHODS

*Inhibitors*. Leupeptin, antipain, chymostatin, pepstatin and elastatinal were purchased from the Peptide Institute, Osaka, Japan. TPCK and aprotinin (isolated from bovine lung; 17 trypsin inhibitor units/ml of 0.9% benzyl alcohol–0.9% NaCl) were obtained from the Sigma Chemical Co., St. Louis, MO., U.S.A. All of the other inhibitors and chemicals were commercially available reagent grade.

* Author to whom all correspondence should be sent.
† Abbreviations: *t*-AMCHA, *trans*-1-aminomethylcyclohexane 4-carboxylic acid; PMN(s), polymorphonuclear leukocyte(s); and TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

Leupeptin (20–140 mg/ml), antipain (70 mg/ml), *t*-AMCHA (40 or 120 mg/ml), cystamine (20, 50 or 100 mg/ml) and EDTA (42 or 70 mg/ml) were dissolved in 0.9% NaCl. TPCK (7–50 mg/ml) and chymostatin (70 mg/ml) were dissolved in ethanol. *o*-Phenanthroline (20 or 40 mg/ml) was dissolved in 25% (v/v) ethanol–0.9% NaCl, and elastatinal (70 mg/ml) in dimethylsulfoxide–0.9% NaCl (1:1, v/v). The injected volume of the inhibitors except for aprotinin was 1 ml/kg of body wt. Control rats received 1 ml of the respective solvent/kg of body wt. The injected volume of aprotinin was 5.9 ml/kg of body wt (100 trypsin inhibitor units/kg; approx. $9 \times 10^4$ kallikrein inhibitor units/kg). All of the inhibitors were dissolved in the solvents just before the injection.

*Treatment with inhibitors.* Male rats (Donryu and Sprague–Dawley strains) weighing 150–170 g were used in the present studies. Inflammation was induced by subcutaneous injection of 4 ml of a 2% (w/v) solution of carrageenin (Seakem 202, Marine Colloid Inc., Springfield, NJ, U.S.A.) into a preformed air-pouch on the back of rats [16]. Proteinase inhibitors were injected into the air-pouch immediately after the injection of carrageenin solution. Control rats were given the vehicle.

The anti-inflammatory action of inhibitors was estimated by measuring the wet weight of granulation tissue and the weight of exudate on day 4 after the injection of carrageenin and the inhibitor, because a quantitative collection of granulation tissue is difficult before day 4. As another index of anti-inflammatory action of inhibitors, the number of PMNs that had migrated into the carrageenin-air-pouch was counted at 3, 6 and 9 hr after the injection of carrageenin and the inhibitor; 0.1 ml of the pouch fluid was collected and diluted 20–100 times with 0.1% (w/v) trypan blue dissolved in phosphate-buffered saline, and then the number of the total cells was counted microscopically. The viability of the cells was checked simultaneously with cell counting and no inhibitor markedly reduced the viability of the cells that migrated into the carrageenin-air-pouch. The PMN percentage of the total cells in the pouch fluid was about 95% 3–9 hr after carrageenin injection.

## RESULTS AND DISCUSSION

TPCK is an active-site histidine alkylating agent and a specific inhibitor of chymotrypsin [17, 18]. In addition, TPCK inhibits papain, a thiol proteinase, by forming a covalent component with the essential sulfhydryl group [19]. To investigate the most effective time during carrageenin-induced inflammation to inject proteinase inhibitors, a single dose of TPCK was injected into the carrageenin-air-pouch at 0, 12 or 24 hr after carrageenin injection. The results are summarized in Fig. 1. The development of granulation tissue and the weight of exudate were markedly suppressed by a single injection of TPCK into the carrageenin-air-pouch immediately after carrageenin injection (0 hr), whereas a single injection of the inhibitor at 12 or 24 hr after carrageenin injection was less effective or slightly effective respectively. These results are consistent with those reported in

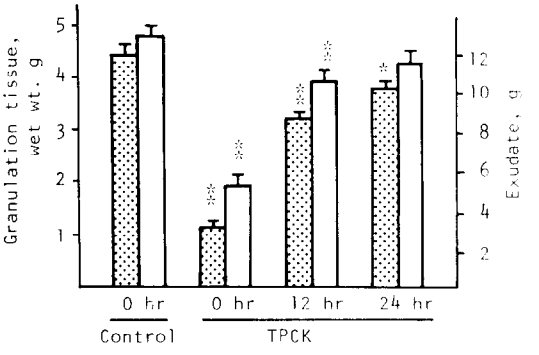


Fig. 1. Effect of a single injection of TPCK on carrageenin-induced inflammation in rats. TPCK (20 mg/ml, 1 ml/kg body wt) was injected into the carrageenin-air-pouch 0, 12 or 24 hr after carrageenin injection. Control rats were given vehicle (ethanol, 1 ml/kg) immediately (0 hr) after carrageenin injection. Wet weight of granulation tissue (▩) and weight of exudate (□) were measured on day 4 after carrageenin injection. Each group included seven rats. Each column and bracket represent the mean ± S.E.M. Values significantly different from the control: * $P < 0.05$; and ** $P < 0.01$.

the previous paper [15] that ε-amino-*n*-caproic acid *n*-hexyl ester, an inhibitor of trypsin and plasmin [20], markedly suppressed the development of granulation tissue by a single injection of the inhibitor into the carrageenin-air-pouch immediately after carrageenin injection, while repeated injections of the inhibitor starting at 12, 24 and 48 hr after carrageenin injection were less effective, slightly effective and ineffective respectively. Our data indicate that proteinase inhibitors exert their anti-inflammatory actions by interfering with the initial inflammatory reactions. In the present studies, therefore, effects of proteinase inhibitors on the migration of PMNs, the development of granulation tissue, and

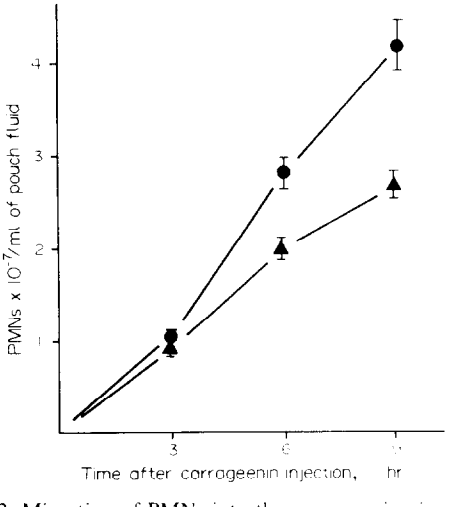


Fig. 2. Migration of PMNs into the carrageenin-air-pouch in rats. Ethanol [1 ml/kg body wt (▲)] and 0.9% NaCl [1 ml/kg (●)] were injected into the carrageenin-air-pouch immediately after carrageenin injection. Each group included eight rats. Each point represents the mean ± S.E.M.

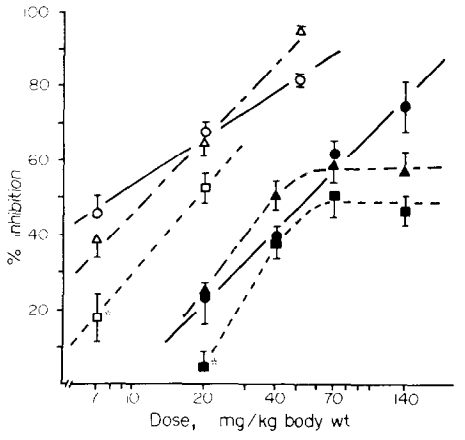


Fig. 3. Effect of a single injection of TPCK and leupeptin on the migration of PMNs into the carrageenin-air-pouch in rats. Various doses of TPCK (○, △, □) and leupeptin (●, ▲, ■) were injected into the carrageenin-air-pouch immediately after carrageenin injection. The number of PMNs that had migrated into the pouch was counted at 3 hr (○, ●), 6 hr (△, ▲) and 9 hr (□, ■) after the injection of carrageenin and the inhibitors. Each group included seven or eight rats. The results are expressed as percent inhibition, and each point represents the mean ± S.E.M. A typical change in the number of PMNs in control groups is shown in Fig. 2. Key: ★ values are statistically insignificant; all other values are significant versus corresponding controls ($P < 0.01$).

the increase in exudate were studied by a single injection of proteinase inhibitors into the carrageenin-air-pouch immediately after carrageenin injection.

*Inhibitory effects of TPCK and leupeptin.* A typical change in the number of PMNs in the carrageenin-air-pouch is shown in Fig. 2; the number of PMNs

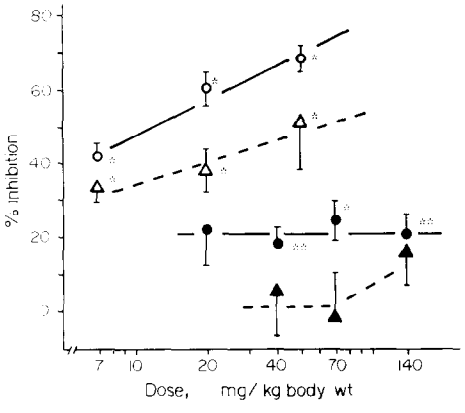


Fig. 4. Effect of a single injection of TPCK and leupeptin on the exudate and the development of granulation tissue induced by carrageenin in rats. Various doses of TPCK (○, △) and leupeptin (●, ▲) were injected into the carrageenin-air-pouch immediately after carrageenin injection. Wet weight of granulation tissue (●, ○) and weight of exudate (▲, △) were measured on day 4 after the injection of carrageenin and the inhibitors. Each group included seven or eight rats. The results are expressed as percent inhibition, and each point represents the mean ± S.E.M. Values are statistically significant versus corresponding controls: * $P < 0.01$; and ** $P < 0.025$.

increased steadily, and $4.2 \times 10^7$ cells/ml of pouch fluid were present at 9 hr after carrageenin injection. Ethanol, at the dose (1 ml/kg) used to dissolve proteinase inhibitors, caused a significant decrease in the migration of PMNs into the carrageenin-air-pouch (Fig. 2). Dimethylsulfoxide–0.9% NaCl (1:1, v/v; 1 ml/kg) also suppressed the PMN migration, while 0.9% benzyl alcohol–0.9% NaCl (5.9 ml/kg) was without effect (data not shown).

TPCK and leupeptin inhibited the migration of PMNs into the carrageenin-air-pouch. Both the inhibitors inhibited the PMN migration in a dose-dependent manner at 3 hr after the injection of carrageenin and the inhibitors (Fig. 3). At 6 or 9 hr after the injection of carrageenin and the inhibitors, TPCK still showed a dose-dependent inhibition of PMN migration, whereas the inhibition by leupeptin was less than 60% inhibition and dose dependence disappeared at higher doses (> 40 mg/kg) of leupeptin (Fig. 3). This insufficient inhibition by leupeptin may be accounted for by the finding that leupeptin is a reversible (competitive) inhibitor of trypsin-like serine proteinases and thiol proteinases, whereas TPCK is an irreversible inhibitor of chymotrypsin-like serine proteinases and thiol proteinases. In addition, TPCK is an alkylating agent and may interfere with biochemical processes *in vivo*, resulting in a marked suppression of the inflammation; e.g. TPCK has strong inhibitory effects on vascular permeability in mouse ears treated with phorbol myristate acetate [8] and on $O_2^-$ production by human PMNs and monocytes [14].

TPCK suppressed both the development of granulation tissue and the exudate in a dose-dependent manner (Fig. 4). On the other hand, leupeptin slightly suppressed the wet weight of granulation tissue and had no effect on exudate (Fig. 4).

*Effects of other inhibitors.* The time course of the effect of proteinase inhibitors on the migration of PMNs into the carrageenin-air-pouch is shown in Table 1. Cystamine, an inhibitor of thiol-proteinases [21], and *o*-phenanthroline, an inhibitor of metallo-proteinases, inhibited PMN migration at a high dose (100 mg and 40 mg/kg respectively), but their inhibitory activities rapidly decreased with time (Table 1). *t*-AMCHA, an inhibitor of plasminogen activator [22], did not suppress the PMN migration. Antipain, chymostatin, pepstatin and elastatinal had no inhibitory effect on PMN migration at a dose of 70 mg/kg, while leupeptin caused a marked suppression at the same dose. Antipain is an inhibitor of thiol-proteinases including papain and cathepsins, though trypsin and plasmin are also inhibited [23]. Chymostatin inhibits chymotrypsin, papain and cathepsins, but does not inhibit plasmin, trypsin and kallikrein [23]. Elastatinal is a specific inhibitor of elastase, and pepstatin is an inhibitor of carboxyl proteinases including renin [24]. Thus, our data suggest that the proteinases which are inhibited by antipain, chymostatin, pepstatin, elastatinal, EDTA and *t*-AMCHA do not contribute to PMN migration, though extrapolation from *in vitro* experiments to the activities of proteinases *in vivo* is speculative.

Antipain and leupeptin have similar inhibitory activities toward proteinases as described above, but the activity of antipain in inhibiting plasmin and

Table 1. Effect of a single injection of proteinase inhibitors on carrageenin-induced inflammation in rats*

| Inhibitors | Dose (mg/kg) | Maximum concn in the pouch† (mM) | PMNs in pouch fluid (% of control) 3 hr | 6 hr | 9 hr | Granulation tissue (% of control) day 4 | Exudate (% of control) day 4 |
|---|---|---|---|---|---|---|---|
| Antipain | 70 | 4.9 | 85 ± 11 | 89 ± 6 | 89 ± 6 | 70 ± 6‡ | 91 ± 11 |
| Chymostatin | 70 | 4.9 | 82 ± 12 | 98 ± 11 | 121 ± 7 | 75 ± 5‡ | 77 ± 10 |
| Pepstatin | 70 | 4.3 | 102 ± 9 | 92 ± 9 | 94 ± 3 | 107 ± 5 | 97 ± 8 |
| Elastatinal | 70 | 6.0 | 113 ± 6 | 99 ± 6 | 116 ± 7 | 98 ± 9 | 81 ± 9 |
| Cystamine | 20 | 3.8 | 131 ± 9§ | 118 ± 8 | 104 ± 10 | 82 ± 5‖ | 84 ± 13 |
| | 50 | 9.4 | 84 ± 5‡ | 97 ± 5 | 103 ± 6 | 69 ± 4‡ | 77 ± 9 |
| | 100 | 18.9 | 67 ± 5‡ | 80 ± 7‖ | 86 ± 3‡ | 61 ± 7‡ | 109 ± 22 |
| EDTA | 42 | 4.0 | 117 ± 11 | 102 ± 5 | 102 ± 5 | 112 ± 8 | 78 ± 4 |
| | 70 | 6.6 | 106 ± 6 | 94 ± 5 | 87 ± 5 | 77 ± 9‖ | 63 ± 10§ |
| *o*-Phenanthroline | 20 | 4.3 | 94 ± 11 | 89 ± 4 | 98 ± 6 | 79 ± 7 | 69 ± 14 |
| | 40 | 8.6 | 67 ± 6§ | 61 ± 3‡ | 89 ± 5 | 49 ± 3‡ | 38 ± 7‡ |
| *t*-AMCHA | 120 | 32.4 | 97 ± 7 | 97 ± 4 | 103 ± 8 | 90 ± 7 | 106 ± 14 |
| Aprotinin | ¶ | ¶ | 92 ± 5 | 114 ± 10 | 104 ± 8 | 98 ± 7 | 84 ± 13 |

* Proteinase inhibitors were injected into the carrageenin-air-pouch immediately after carrageenin injection. The number of PMNs that migrated into the carrageenin-air-pouch was counted at 3, 6 and 9 hr after the injection of carrageenin and the inhibitors. Wet weight of granulation tissue and weight of exudate were measured on day 4 after carrageenin injection. Each group included seven or eight rats. Data are shown as percentages of control (means ± S.E.M.)

† Values indicate the calculated concentration of the injected inhibitor which was diluted with 4 ml of 2% carrageenin solution.

‡ Statistically significant difference from control, $P < 0.01$.

§ Statistically significant difference from control, $P < 0.025$.

‖ Statistically significant difference from control, $P < 0.05$.

¶ The dose of aprotinin was 100 trypsin inhibitor units (approx. $9 \times 10^3$ kallikrein inhibitor units)/kg body wt.

kallikrein is much weaker than that of leupeptin [23, 25]. This difference in the inhibitory activities may account for the lack of inhibitory effect of antipain on PMN migration. Wiggins *et al.* [5] demonstrated that rabbit plasma kallikrein was capable of producing chemotactic activity toward rabbit PMNs from rabbit C5. However, aprotinin, an inhibitor of trypsin-like proteinases including kallikrein, had no effect on PMN migration (Table 1), suggesting that kallikrein was not a proteinase responsible for producing chemotactic activity toward PMNs in the present *in vivo* experiments. On the other hand, Hyman and Vischer [7] and Spilberg and Osterland [10] demonstrated that aprotinin significantly suppressed PMN infiltration in the intrapleural reverse passive Arthus reaction in rats [7] and in acute gouty arthritis induced by urate crystal in rabbits [10]. The discrepancy between these results and our results may be accounted for by differences in animal inflammation models used and in doses of aprotinin.

Simpson and Ross [26] demonstrated that neutrophil depletion induced by daily injections of antineutrophil sera caused no differences between control and neutropenic wounds with respect to extent of repair, including connective tissue formation. Our data also do not support the notion that development of granulation tissue depends on PMN infiltration; antipain, chymostatin and EDTA had no effect on PMN migration but significantly suppressed the development of granulation tissue (Table 1). The development of granulation tissue was suppressed by a single injection of cystamine in a dose-dependent manner, while EDTA and *o*-phenanthroline were effective at a high dose only (Table 1).

In conclusion, the development of granulation tissue was suppressed by a single injection of serine- and thiol-proteinase inhibitors including TPCK, leupeptin, antipain, chymostatin and cystamine, though metalloproteinase inhibitors were also effective at a high dose. On the other hand, the relative potencies of the inhibitory effect of proteinase inhibitors on the migration of PMNs into the inflammatory lession were TPCK > leupeptin >> cystamine, *o*-phenanthroline. Further study will be required to identify the proteinases which are important in carrageenin-induced inflammation in rats.

## REFERENCES

1. C. G. Cochrane and A. Janoff, in *The Inflammatory Process* (Eds. B. W. Zweifach, L. Grant and R. T. McCluskey), 2nd Edn, Vol. 3, p. 85. Academic Press, New York (1974).
2. S. Honig, S. Hoffstein and G. Weissmann, *Pathobiol. A.* **8**, 315 (1978).
3. S. Wasi, H. Z. Movat, E. Pass and J. Y. C. Chan, in *Neutral Proteases of Human Polymorphonuclear Leukocytes* (Eds. K. Havemann and A. Janoff), p. 245. Urban & Schwarzenberg, Baltimore (1978).
4. P. Venge, in *Neutral Proteases of Human Polymorphonuclear Leukocytes* (Eds. K. Havemann and A. Janoff), p. 264. Urban & Schwarzenberg, Baltimore (1978).
5. R. C. Wiggins, P. C. Giclas and P. M. Henson, *J. exp. Med.* **153**, 1391 (1981).
6. K. Udaka and H. Hayashi, *Biochim. biophys. Acta* **97**, 251 (1965).

7. Y. G. Hyman and T. L. Vischer, *Agents Actions* **8**, 532 (1978).
8. A. Janoff, A. Klassen and W. Troll, *Cancer Res.* **30**, 2568 (1970).
9. W. Troll, A. Klassen and A. Janoff, *Science* **169**, 1211 (1970).
10. I. Spilberg and C. K. Osterland, *J. Lab clin. Med.* **76**, 472 (1970).
11. J. Hladovec, V. Mansfeld and Z. Horakova, *Experientia* **14**, 146 (1958).
12. D. A. Lewis, R. B. Capstick and R. Best, *Biochem. Pharmac.* **25**, 1435 (1976).
13. B. D. Goldstein, W. Witz, M. Amoruso and W. Troll, *Biochem. biophys. Res. Commun.* **88**, 854 (1979).
14. S. Kitagawa, F. Takaku and S. Sakamoto, *J. clin. Invest.* **65**, 74 (1980).
15. H. Nakagawa, K. Shuto, M. Isaji, K. Watanabe and S. Tsurufuji, *J. Pharmacobis-Dynamics* **4**, 429 (1981).
16. M. Fukuhara and S. Tsurufuji, *Biochem. Pharmac.* **18**, 475 (1969).
17. G. Schoellmann and E. Shaw, *Biochemistry* **2**, 252 (1963).
18. J. C. Powers, in *Methods in Enzymology* (Eds. W. B. Jakoby and M. Wilchek), Vol. XLVI, p. 197. Academic Press, New York (1977).
19. J. R. Whitaker and J. Perez-Villaseñor, *Archs Biochem. Biophys.* **124**, 70 (1968).
20. M. Muramatu and S. Fujii, *J. Biochem., Tokyo* **65**, 17 (1969).
21. T. Sanner and A. Pihl, *J. biol. Chem.* **238**, 165 (1963).
22. S. Okamoto, S. Oshiba, H. Mihara and U. Okamoto, *Ann. N.Y. Acad. Sci.* **146**, 414 (1968).
23. H. Suda, T. Aoyagi, M. Hamada, T. Takeuchi and H. Umezawa, *J. Antibiot., Tokyo* **25**, 263 (1972).
24. H. Umezawa and T. Aoyagi, in *Proteinases in Mammalian Cells and Tissues* (Ed. A. J. Barrett), p. 637. North-Holland Publishing, Amsterdam (1977).
25. H. Fritz, B. Forg-Brey and H. Umezawa, *Hoppe-Seyler's Z. physiol. Chem.* **354**, 1304 (1973).
26. D. M. Simpson and R. Ross, *J. clin. Invest.* **51**, 2009 (1972).