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PROTEINASE INHIBITORS SUPPRESS THE FORMATION OF GRANULATION TISSUE IN THE CARRAGEENIN-INDUCED INFLAMMATION IN RATS*

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Effect of proteinase inhibitors on the carrageenin-induced inflammation was studied. The formation of granulation tissue was markedly inhibited by a single injection of ε-amino-*n*-caproic acid *n*-hexyl ester (EACA hexyl ester, 300 mg/kg) into the carrageenin-air-pouch immediately after carrageenin injection, whereas repeated injections of the inhibitor starting at 12 hr, 24 hr and 48 hr after carrageenin injection were less effective, slightly effective and ineffective, respectively. A dose-dependent inhibition of both the formation of granulation tissue and the migration of polymorphonuclear leukocytes (PMNs) into the inflammatory locus was found by a single injection of EACA hexyl ester into the carrageenin-air-pouch immediately after carrageenin injection. Similarly, a single injection of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK, 50 mg/kg) and N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK, 30 mg/kg) inhibited both the formation of granulation tissue and the migration of PMNs into the inflammatory locus.

These results suggest that serine proteinase inhibitors such as EACA hexyl ester, TPCK and TLCK exert their anti-inflammatory actions by interfering with the initial inflammatory reactions including the migration of PMNs into inflammatory locus after carrageenin injection.

Keywords—inflammation; anti-inflammatory agents; PMN migration; granulation tissue; proteinase inhibitors; TPCK; TLCK; ε-amino-*n*-caproic acid *n*-hexyl ester

INTRODUCTION

One of the important cellular responses to injury is the infiltration of PMNs into the inflamed tissues. During phagocytosis and on the death of the cells, PMNs release lysosomal enzymes into the inflammatory locus. PMNs, thus, are one of the main sources of proteinases responsible for tissue damage in inflammatory processes.[1] Therefore, the inhibition of the migration of PMNs into the inflammatory locus and the inhibition of proteinases released from PMNs are important to reduce PMN-mediated inflammatory processes. It has been demonstrated that administration of proteinase inhibitors such as soybean trypsin inhibitor (SBTI),[2] aprotinin[3] and endogenous proteinase inhibitors[4] suppressed experimental inflammation induced by kaolin, urate crystals and adjuvant, respectively. The work reported here demonstrates that serine proteinase inhibitors such as EACA hexyl ester, TPCK and TLCK inhibit the migration of PMNs into the inflammatory locus and suppress the formation of granulation tissue in the carrageenin-induced inflammation in rats.

* The abbreviations used are: EACA, ε-amino-*n*-caproic acid; EACA hexyl ester, ε-amino-*n*-caproic acid *n*-hexyl ester *p*-toluenesulfonate; SBTI, soybean trypsin inhibitor; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, N-α-*p*-tosyl-L-lysine chloromethyl ketone; DMSO, dimethyl sulfoxide; PMN(s), polymorphonuclear leukocyte(s).

MATERIALS AND METHODS

Inhibitors —EACA and EACA hexyl ester were purchased from Tokyo Kasei Kogyo Co., Ltd., SBTI (type 1-S), TLCK and TPCK were from Sigma Chemical Co., *o*-phenanthroline was from Wako Pure Chemical Industries, Ltd., leupeptin, chymostatin and pepstatin were from Peptide Institue, Osaka. EACA (300 mg/ml), EACA hexyl ester (300 mg/ml), SBTI (100 mg/ml) and leupeptin (40 mg/ml) were dissolved in 0.9% NaCl solution. *o*-Phenanthroline (30 mg/ml) was dissolved in ethyl alcohol. TPCK (50 mg/ml) and TLCK (30 mg/ml) were dissolved in DMSO. The injected volume of the inhibitors described above was 1 ml/kg body weight. Chymostatin (20 mg/ml) and pepstatin (20 mg/ml) were dissolved in 0.9% NaCl-DMSO (1:1, v/v) and 2 ml/kg body weight was injected. Control rats received an equivalent volume of the respective solvent (1 or 2 ml/kg body weight). All of the inhibitors were dissolved in the solvents just before the injection.

Treatment with Inhibitors —An inflammation in the form of carrageenin-air-pouch was induced on the back of male Donryu rats according to the procedure of Tsurufuji *et al.*[5]; in rats weighing 140—170 g, 4 ml of 2% (w/v) solution of Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N.J., U.S.A.) was injected into the air-pouch already formed. Proteinase inhibitors were injected into the air-pouch immediately after the injection of carrageenin solution (in case of a single injection) and the injection was repeated every 12 hr until day 3 (in case of repeated injections). Control rats were given the vehicle. On day 4 after carrageenin injection, the granulation tissue was harvested and the wet weight of the tissue was used as an index of anti-inflammatory action of inhibitors.

The number of PMNs migrated into the carrageenin-air-pouch 2—7 hr after injection of carrageenin and inhibitors was estimated; 0.1 ml of the pouch fluid containing PMNs was diluted 2—10 times with phosphate-buffered saline and an aliquot of the diluted solution was mixed with the equal volume of 0.2% (w/v) trypan blue dissolved in phosphate-buffered saline, and the number of the total cells was counted microscopically. The percentage of PMNs to the total cells in the pouch fluid was about 95% 2—7 hr after carrageenin injection.

RESULTS

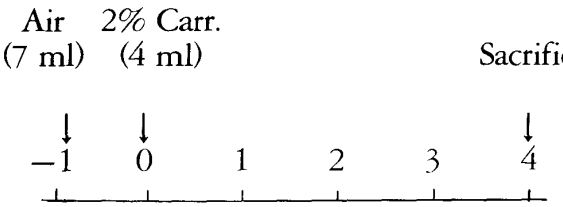
EACA hexyl ester, an inhibitor of plasmin and trypsin,[6] markedly inhibited the formation of granulation tissue, when the inhibitor (300 mg/kg) was injected into the carrageenin-air-pouch immediately after carrageenin injection and was repeatedly injected every 12 hr until day 3 (Table I). *p*-Toluenesulfonic acid sodium salt (150 mg/kg body weight, dissolved in 1 ml of 0.9% NaCl) was injected as another vehicle, because EACA hexyl ester was the salt of *p*-toluenesulfonic acid. Table I shows that *p*-toluenesulfonic acid sodium salt has no effect on the carrageenin-induced inflammation. Conse-

TABLE I. *Effect of EACA Hexyl Ester on the Formation of Granulation Tissue in Rats*

	No. of rats	Net body wt. (g)	Pouch fluid (g)	Granulation tissue, wet wt. (g)
Saline (0.9% NaCl)	10	168±4	8.49±0.85	4.84±0.19
p-Toluenesulfonic acid sodium salt (150 mg/kg)	10	169±2	8.25±1.30	4.75±0.22
EACA hexyl ester (300 mg/kg)	10	166±4	8.60±0.31	2.85±0.11*

EACA hexyl ester (300 mg/kg) was injected into the carrageenin-air-pouch immediately after carrageenin injection and the injection was repeated every 12 hr until day 3 (8 injections in total). Data are shown as means ± S.E. Values are significantly different from the control; * $p < 0.001$.

TABLE II. *Effect of Various Doses of EACA Hexyl Ester on the Formation of Granulation Tissue in Rats*



	Granulation tissue, wet wt. (g)	Percent inhibition
Expt. 1;		
Control:	4.37±0.19	
Group 1-A:	1.82±0.09**	58%
Group 2-B:	4.36±0.24	0%
Expt. 2;		
Control:	4.26±0.26	
Group 1-A:	1.79±0.20**	58%
Group 1-B:	3.32±0.17*	22%
Group 2-A:	2.12±0.22**	50%
Group 2-B:	2.85±0.20**	33%

EACA hexyl ester (300 mg/kg; ■) or 0.9% NaCl (1 ml/kg; □) was injected into the carrageenin-air-pouch immediately after carrageenin injection and was repeatedly injected every 12 hr thereafter (the experimental schedule is shown in the left column). Wet weight of granulation tissue and weight of exudate were measured on day 4 after carrageenin injection. Data are shown as means ± S.E. Each group in experiments 1 and 2 included 10 and 8 rats, respectively. Values are significantly different from the control; * $p < 0.01$, ** $p < 0.001$.*

TABLE III. *Effect of a Single Injection of EACA Hexyl Ester (300 mg/kg) on the Amounts of Collagen and Noncollagen Protein in Granulation Tissue (Day 4)*

	Control	EACA hexyl ester (300 mg/kg)
Net body wt. (g)	153±2	155±4
Granulation tissue, wet wt. (g)	4.26±0.26	2.12±0.22**
Collagen;		
Hyp (mg) in whole tissue	3.06±0.23	2.13±0.30*
Hyp (mg)/g wet wt.	0.74±0.08	0.99±0.08*
Noncollagen protein;		
Protein (mg) in whole tissue	59.2±5.3	12.0±2.5**
Protein (mg)/g wet wt.	13.7±0.5	5.3±0.6**

The granulation tissues from the group 2-A and the control group in Expt. 2 shown in Table II were used for the measurement of the amounts of both proteins. Each group included 8 rats. Data are shown as means ± S.E. Values are significantly different from the control; * $p < 0.05$, ** $p < 0.001$.*

quently, 0.9% NaCl solution was injected to control rats in all of the following experiments using EACA hexyl ester.

To investigate the most effective phase of EACA hexyl ester in the carrageenin-induced inflammation, the injection of the inhibitor into the carrageenin-air-pouch was started at various times after carrageenin injection. The results are summarized in Table II. Wet weight of granulation tissue was significantly inhibited by the treatment with EACA hexyl ester (Table II), whereas the amount of exudate and the net body weight were not affected by the treatment with EACA hexyl ester in all of the groups in Expts. 1 and 2 in Table II (data not shown). A single injection of EACA hexyl ester markedly inhibited the formation of granulation tissue (group 2-A in Expt. 2, Table II) and the inhibitory effect of the inhibitor was much stronger in the A group than in the corresponding B group (Table II). These results suggest that the inhibitor exerts its inhibitory action within 12 hr after carrageenin injection.

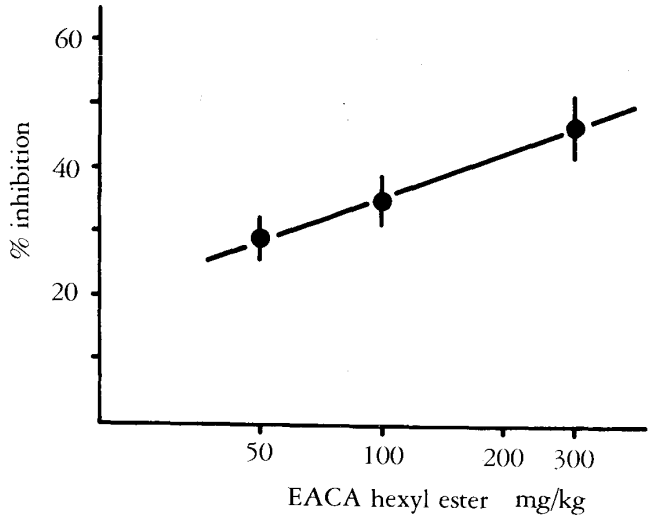


FIG. 1. *Dose-dependent Inhibition of the Formation of Granulation Tissue by a Single Injection of EACA Hexyl Ester*

EACA hexyl ester was injected into the carrageenin-air-pouch immediately after carrageenin injection. Wet weight of granulation tissue was measured on day 4 after carrageenin injection. Each point represents the mean ± S.E. of 6 determinations.

PMNs are the main cell type (more than 90% of total cells) in the pouch fluid during the first 12 hr after carrageenin injection. Therefore, the inhibitory effects of proteinase inhibitors on the formation of granulation tissue and the migration of PMNs were studied by a single injection of proteinase inhibitors into the carrageenin-air-pouch immediately after carrageenin injection.

The amounts of collagen and noncollagen protein in granulation tissues from rats treated with a

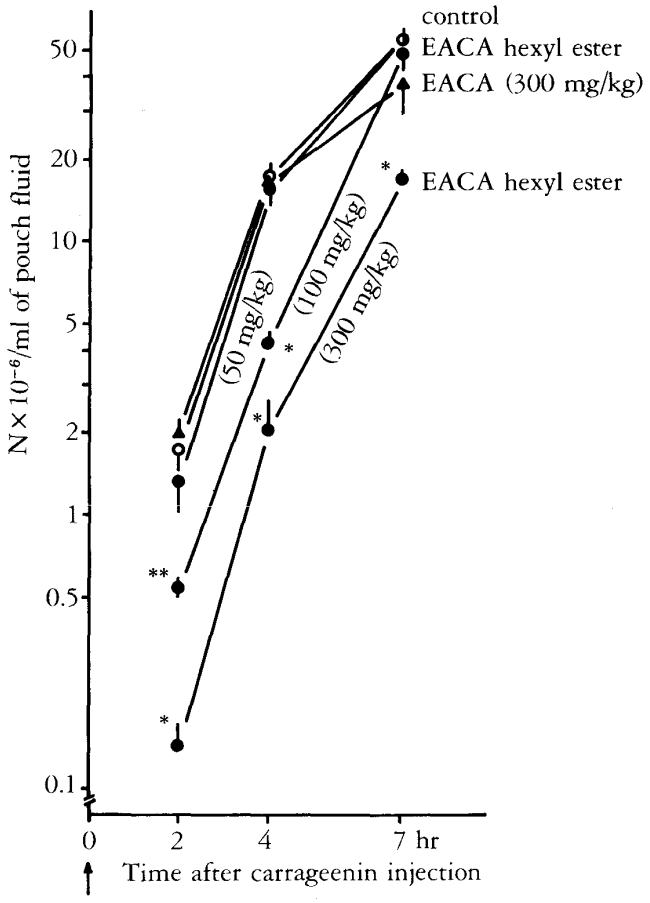


FIG. 2. *Effect of EACA and EACA Hexyl Ester on the Migration of PMNs into the Carrageenin-air-pouch in Rats*

*EACA (300 mg/kg; ▲ — ▲) and EACA hexyl ester (50, 100 and 300 mg/kg; ● — ●) were injected into the carrageenin-air-pouch immediately after carrageenin injection (denoted by an arrow). Each point represents the mean ± S.E. of 5 or 6 determinations. Statistically significant difference against control is shown by the following marks; * $p < 0.001$, ** $p < 0.01$.*

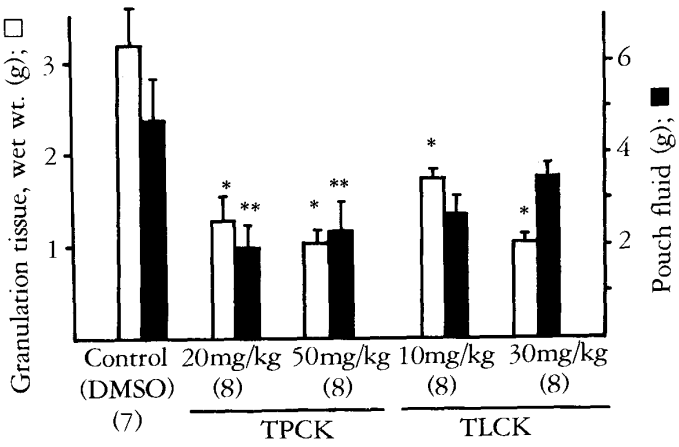


FIG. 3. *Effect of a Single Injection of TPCK and TLCK on the Carrageenin-induced Inflammation in Rats*

*TPCK (20 and 50 mg/kg) and TLCK (10 and 30 mg/kg) were injected into the carrageenin-air-pouch immediately after carrageenin injection. Wet weight of granulation tissue (□) and weight of the pouch fluid (■) were measured on day 4 after carrageenin injection. Each column and bracket is the mean ± S.E. of 7 or 8 determinations. The number of rats used are shown in parentheses. Statistically significant difference against control is shown by the following marks; * $p < 0.01$, ** $p < 0.025$.*

TABLE IV. *Effect of a Single Injection of Proteinase Inhibitors on the Formation of Granulation Tissue in the Carrageenin-induced Inflammation in Rats*

	Granulation tissue, wet wt. (g)
Expt. 1;	
Control (0.9% NaCl, 1 ml/kg)	4.33±0.25
Leupeptin (40 mg/kg)	3.47±0.29*
Chymostatin (40 mg/kg)	3.73±0.21
Pepstatin (40 mg/kg)	3.92±0.27
SBTI (100 mg/kg)	4.06±0.20
o-Phenanthroline (30 mg/kg)	3.45±0.15*
Expt. 2;	
Control (0.9% NaCl, 1 ml/kg)	3.60±0.30
EACA (300 mg/kg)	3.78±0.28
DMSO (2 ml/kg)	3.70±0.30

*Data are shown as means ± S.E. Each group in Expts. 1 and 2 included 7 and 8 rats, respectively. Values are significantly different from the control; * $p < 0.05$.*

single injection of EACA hexyl ester (300 mg/kg; group 2-A in Expt. 2, Table II) were measured according to the procedure described in the previous paper.[7] Table III shows that formation of collagen and noncollagen protein in the whole granulation tissue was inhibited by 30% and 80%, respectively.

A dose-dependent inhibition of the formation of granulation tissue was found by a single injection of EACA hexyl ester into the carrageenin-air-pouch immediately after carrageenin injection (Fig. 1). In addition, a dose-dependent inhibition of the migration of PMNs into the carrageenin-air-pouch was observed by a single injection of EACA hexyl ester (Fig. 2). On the other hand, a single injection of EACA (300 mg/kg) had no

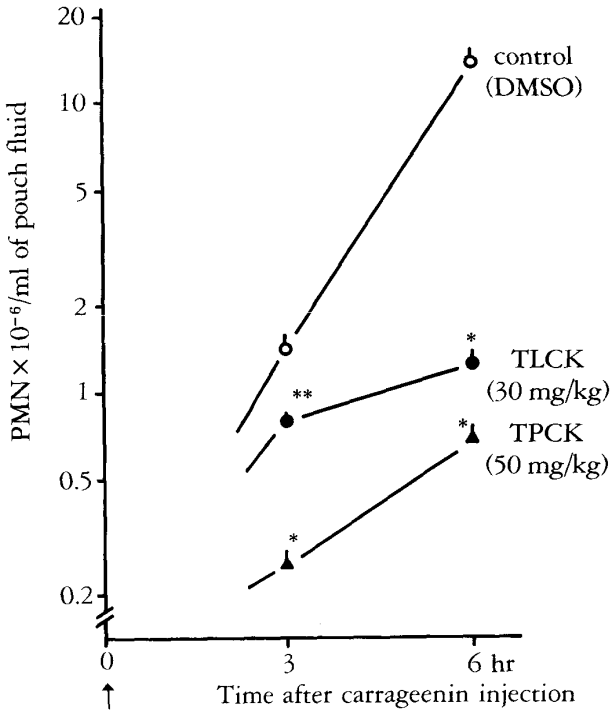


FIG. 4. *Effect of TPCK and TLCK on the Migration of PMNs into the Carrageenin-air-pouch in Rats*

*TPCK (50 mg/kg, ▲ – ▲) and TLCK (30 mg/kg, ● – ●) were injected into the carrageenin-air-pouch immediately after carrageenin injection (denoted by an arrow). Each point represents the mean ± S.E. of 7 or 8 determinations. Statistically significant difference against control is shown by the following marks; * $p < 0.001$, ** $p < 0.005$.*

effect on the migration of PMNs (Fig. 2).

Effects of other proteinase inhibitors on the carrageenin-induced inflammation were studied by a single injection of the inhibitors immediately after carrageenin injection. The results are summarized in Table IV and Fig. 3. Leupeptin (40 mg/kg) and *o*-phenanthroline (30 mg/kg) significantly inhibited, and TPCK (20 mg and 50 mg/kg) and TLCK (10 mg and 30 mg/kg) markedly inhibited the formation of granulation tissue (Table IV and Fig. 3). In addition, TPCK (50 mg/kg) and TLCK (30 mg/kg) markedly inhibited the migration of PMNs into the carrageenin-air-pouch at least until 6 hr after carrageenin injection (Fig. 4). On the other hand, all of the inhibitors had no effect on the amount of exudate and net body weight (data not shown) except that TPCK suppressed the amount of exudate (Fig. 3).

DISCUSSION

EACA hexyl ester, a serine proteinase inhibitor, inhibited the formation 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 hr, 24 hr and 48 hr after carrageenin injection were less effective, slightly effective and ineffective, respectively (Table II). These results suggest that the inhibitory effect of the inhibitor on the formation of granulation tissue is mainly exerted during the first 12 hr after carrageenin injection.

In the early phase of inflammation the first cell type to appear in the inflammatory locus is PMN which is one of the main sources of enzymes responsible for tissue damage in inflammatory processes.[1,8] Therefore, the effect of a single injection of EACA hexyl ester on the migration of PMNs into inflammatory locus was studied. EACA hexyl ester exerted a dose-dependent inhibition on the migration of PMNs into the carrageenin-air-pouch 2—7 hr after carrageenin injection (Fig. 2). The results suggest that EACA hexyl ester reduces PMN-mediated tissue injury by inhibiting the accumulation of PMNs which are the main source of proteinases released from cells in the early phase of inflammation.

EACA, in contrast to EACA hexyl ester, had no effect on the migration of PMNs and the formation of granulation tissue (Fig. 2 and Table IV), though both EACA and EACA hexyl ester are known as anti-fibrinolytic agents inhibiting the degradation of polymerized fibrin by plasmin. This discrepancy may be accounted for by the findings that EACA hexyl ester inhibits fibrinolysis through the inhibition of plasmin, while EACA inhibits fibrinolysis through the interaction with fibrin, protecting it against proteolysis by plasmin.[6,9-11] The results suggest that serine proteinases, which are important in the early phase of inflammation, are inhibited by EACA hexyl ester, but not by EACA.

In analogy with EACA hexyl ester, TPCK and TLCK, which are the histidine-specific serine proteinase inhibitors,[12] inhibited both the formation of granulation tissue and the migration of PMNs into the inflammatory locus by a single injection of the inhibitors into the carrageenin-air-pouch immediately after carrageenin injection (Figs. 3 and 4). The results are consistent with those of the *in vitro* experiments[13] that preincubation of human neutrophils with either TPCK or TLCK suppressed chemotaxis of the neutrophils when assessed in Boyden chambers *in vitro*. Leupeptin and *o*-phenanthroline also inhibited the formation of granulation tissue (Table IV). Leupeptin inhibits serine proteinases such as plasmin and trypsin, though it inhibits most strongly cathepsin B, a thiol proteinase.[14] *o*-Phenanthroline, a chelating agent, inhibits metalloproteinases, but it also inhibits many other enzymes, whose activity depends on the presence of more or less tightly bound divalent cations.

It is suggested that collagen, elastin and vascular basement membrane can become targets of neutral proteinases from PMNs *in vivo*.[1] In addition, nonstructural components of connective tissue, namely, serum soluble constituents such as kininogens and complement factors may serve as important substrates for proteinases activated during inflammation including those originating

in PMNs.[1)] Furthermore, neutral proteinases of PMNs cause a rheumatoid arthritic-like syndrome including articular cartilage matrix dissolution and pannus formation when injected into normal rat knee joints.[15)] Therefore, the inhibitory effect of serine proteinase inhibitors on the formation of granulation tissue may be attributed to the inhibitory action of the inhibitors on (1) the accumulation of PMNs in the inflammatory locus and (2) neutral proteinases of PMNs. The present study demonstrates that serine proteinase inhibitors suppress the accumulation of PMNs in the inflammatory locus, though the effect of the inhibitors on the proteinases of rat PMNs is unclear and is currently under investigation.

REFERENCES

1) C.G.Cochrane and A.Janoff, "The Inflammatory Process," 2nd ed., Vol. 3, eds. by B.W.Zweifach, L.Grant and R.T.McCluskey, Academic Press, Inc., New York, 1974, pp. 85–162.
2) J.Hladovec, V.Mansfeld and Z.Horakova: Inhibitory action of trypsin and trypsin-inhibitors on experimental inflammation in rats, *Experimentia*, **14**, 146–147 (1958).
3) I.Spilberg and C.K.Osterland: Anti-inflammatory effect of the trypsin-kallikrein inhibitor in acute arthritis induced by urate crystals in rabbits, *J. Lab. Clin. Med.*, **76**, 472–479 (1970).
4) D.A.Lewis, R.B.Capstick and R.Best: The control of rheumatic disease by endogenous protein, *Biochem. Pharmacol.*, **25**, 1435–1436 (1976).
5) S.Tsurufuji, H.Sato, K.R.Min and K.Ohuchi: Difference in the antiinflammatory effect of indomethacin between acute and chronic stages of carrageenin-induced inflammation, *J. Pharm. Dyn.*, **1**, 8–14 (1978).
6) M.Muramatu and S.Fujii: Inhibitory effects of ω-amino acid esters on the activities of trypsin, plasmin and thrombin, *J. Biochem.*, **65**, 17–26 (1969).
7) H.Nakagawa, M.Fukuhara and S.Tsurufuji: Effect of a single injection of betamethasone disodium phosphate on the synthesis of collagen and noncollagen protein of carrageenin granuloma in rats, *Biochem. Pharmacol.*, **20**, 2253–2261 (1971).
8) R.Vinegar, J.F.Truax and J.L.Selph: Quantitative studies of the pathway to acute carrageenin inflammation, *Fed. Proc.*, **35**, 2447–2454 (1976).
9) M.Muramatu, T.Onishi, S.Makino, S.Fujii and Y.Yamamura: Inhibition of caseinolytic activity of plasmin by various synthetic inhibitors, *J. Biochem.*, **57**, 402–406 (1965).
10) S.Okamoto, S.Oshiba, H. Mihara and U.Okamoto: Synthetic inhibitors of fibrinolysis: *in vitro* and *in vivo* mode of action, *Ann. N.Y. Acad. Sci.*, **146**, 414–429 (1968).
11) C.M.Ambrus, J.L.Ambrus, H.B.Lassman and I.B.Mink: Studies on the mechanism of action of inhibitors of the fibrinolysin system, *Ann. N.Y. Acad. Sci.*, **146**, 430–447 (1968).
12) J.C.Powers, "Method in Enzymology," Vol. 46, eds. by W.B.Jakoby and M.Wilchek, Academic Press, Inc., New York, 1977, pp. 197–208.
13) E.J.Goetzl: Modulation of human neutrophil polymorphonuclear leucocyte migration by human plasma alpha-globulin inhibitors and synthetic esterase inhibitors, *Immunol.*, **29**, 163–174 (1975).
14) T.Aoyagi, S.Miyata, M.Nanbo, F.Kojima, M.Matsuzaki, M.Ishizuka, T.Takeuchi and H.Umezawa: Biological activities of leupeptins, *J. Antibiotics*, **22**, 558–568 (1969).
15) A.L.Oronsky and C.W.Buermann, "Neutral Proteases of Human Polymorphonuclear Leukocytes," eds. by K.Havemann and A.Janoff, Urban & Schwarzenberg, Inc., Baltimore, 1978, pp. 361–372.