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Studies on Anti-Plasminic Agents

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It was shown that dilauroyl- and dimyristoyl-L-lysine were more powerful inhibitors than the other fattyacyl derivatives of L-lysine in the proteolytic system of plasmin. In the case of carbon atom number of fattyacyl radical, upon increasing of carbon atom number the inhibitory activity was enhanced to maximum at the C-12 acyl radical, however, higher homologs decreased the inhibitory activity gradually.

The inhibitory effect of dilauroyl-L-lysine given to rabbits intravenously was more remarkable than that of ε-aminocaproic acid when the effect was examined by the streptokinase activation test of blood samples taken at various intervals.

Reversal by dilauroyl-L-lysine of the accelerated fibrinolysis in circulatroy rabbit blood produced by streptokinase was demonstrated.

Dicarbobenzoxy-L-lysine and dilauroyl-L-lysine were shown to be potent anti-inflammatory compounds. These compounds were intravenously active as the inhibitor of extravasation of trypan blue in a localized skin area of the rabbit, where the lung extract was injected intracutaneously.

Studies on synthetic inhibitors of plasmin and plasminogen activation have resulted that basic amino acids,[2] their ploymers[3] and esters,[4] and ω-amino acids[5–8] have been effective as plasmin inhibitors.

Mirsky, *et al.* reported that plasmin hydrolyzed the peptide linkages including L-lysine or L-arginine such as L-lysyl-L-lysine or L-arginyl-L-tryptophan in the polypeptide chain.[9,10] Their observations prompted us to study the inhibitions of plasmin by the derivatives of L-lysine and L-arginine. The previous publication from this laboratory has detailed the effects of synthetic derivatives of L-lysine, L-arginine and ε-aminocaproic acid on plasmin activity.[11] In the previous work, the authors have found that N,N′-disubstituted L-lysine, which is protected by *p*-toluenesulfonyl or carbobenzoxyl group, was more powerful in the inhibition of proteolysis by plasmin than the other L-lysine derivatives, however, this compound showed weak inhibitory activity in fibrinolysis by plasmin. In 1951, Astrup, *et al.* found that several detergents had the inhibitory effects on the fibrinolysis by plasmin.[12]

Thus it seemed to be nearly accepted that long chain fattyacyl derivatives of L-lysine have the property as detergent and are available to inhibit both proteolysis and fibrinolysis of plasmin. The present paper deals with the first step of our studies in evaluating the anti-

1) Location: *a) Nanakuma, Fukuoka. b) Dozimahamadori, Fukushimaku, Osaka.*
2) S. Mullertz, *Proc. Soc. Exptl. Biol. & Med.*, **85**, 326 (1954).
3) I. Ginsburg, A. de Vries, and E. Katchalski, *Science*, **116**, 15 (1952).
4) W. Troll, S. Sherry, and J. Wachman, *J. Biol. Chem.*, **208**, 85 (1954).
5) F.B. Ablondi, J.J. Hagen, M. Phillips, and E.C. de Renzo, *Arch. Biochem. Biophys.*, **82**, 153 (1959).
6) N. Alkjaersig, A.P. Fletcher, and S. Sherry, *J. Biol. Chem.*, **234**, 832 (1959).
7) M. Yokoi, *J. Japanese Physiol. Soc.*, **22**, 1103 (1960).
8) M. Mangyo, *J. Japanese Biochem. Soc.*, **36**, 735 (1964).
9) I.A. Mirsky, G. Perisutti, and N.G. Davis, *J. Clin. Invest.*, **38**, 14 (1959).
10) I.A. Mirsky, G. Perisutti, and N.G. Davis, *Endocrinology*, **64**, 992 (1959).
11) A. Nagamatsu, T. Okuma, M. Watanabe, and Y. Yamamura, *J. Biochem.*, **54**, 491 (1963).
12) T. Astrup and N. Alkjaersig, *Nature*, **165**, 565 (1951).

plasminic effects of fattyacyl derivatives of L-lysine in comparison with the other anti-plasminic agents, and also suggests that the some compounds among these L-lysine derivatives have an anti-inflammatory activity.

Experimental

Materials——

Inhibitors: ε-Aminocaproic acid and 1-aminomethylcyclohexane-4-carboxylic acid were obtained through the courtesy of Daiichi Seiyaku Co., Ltd. The other inhibitors were synthesized in this laboratory by us and tested for purity, subjected to elementary analyses. All inhibitors were dissolved in buffered saline or equivalent amount of sodium hydroxide solution and the solutions were adjusted to pH 7.4.

Plasminogen: Euglobulin solutions were used as plasminogen preparation. The solutions were prepared from human bank blood or rabbit blood by the method of Lewis.[13]

Activator: Commercial varidase (Lederle), one vial of which consisted to 100000 units of streptokinase and 25000 units of streptodornase, was diluted in buffered saline.

Thrombin: Commercial thrombin (Mochida) was diluted to 100 units per milliliter in buffered saline.

Fibrinogen: One per cent fibrinogen solution was prepared from commercial fibrinogen (Nutritional Biochemicals Corporation).

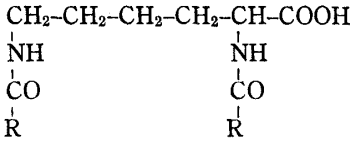
Casein: After purification of commercial product, 4 per cent solution was prepared by the method of Norman[14] and adjusted to pH 7.4 with sodium hydroxide.

Permeability enhancing substance: Rabbit lung extract was used as permeability enhancing substance according to Moon, *et al.*[15] The fresh lung obtained from killed rabbit was homogenized with 4 volumes of 0.9 per cent sodium chloride, after standing for 2 hours at room temperature, the solution was centrifuged for 10 minutes at 3000 rpm and the supernatant was supplied.

Syntheses of Fattyacyl-L-lysines:

A. Fatty acids were converted to corresponding acid chlorides by the treatment with thionyl chloride. Acid chlorides were coupled with L-lysine dihydrochloride in alkaline aqueous solution, and the fatty-acyl-L-lysines were obtained upon the neutralization of alkali salts. After the washing with warm ether, the products were recrystallized from hot ethyl acetate and petroleum ether.

Table I. Structures and Properties of N,N'-Difattyacyl-L-lysines



R	mp (°C) in lit.	mp (°C)	Formula	Analysis (%) Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
CH_3		not crystallized	$C_{10}H_{18}O_4N_2$	52.16	7.88	12.17	52.20	7.75	12.21
C_3H_7		not crystallized	$C_{14}H_{26}O_4N_2$	58.72	9.15	9.78	58.32	9.06	9.64
C_5H_{11}		94—96	$C_{18}H_{34}O_4N_2$	63.13	10.01	8.18	62.98	9.93	8.14
C_7H_{15}		96—97	$C_{22}H_{42}O_4N_2$	66.29	10.62	7.03	66.12	10.03	6.98
C_9H_{19}		106—110	$C_{26}H_{50}O_4N_2$	68.68	11.08	6.16	66.35	10.95	6.02
$C_{11}H_{23}$		114—116	$C_{30}H_{58}O_4N_2$	70.54	11.45	5.48	70.25	11.31	5.24
$C_{13}H_{27}$	115—116[a]	114—115	$C_{34}H_{66}O_4N_2$	72.03	11.73	4.94	71.96	11.53	4.93
$C_{15}H_{31}$	121—122[b]	116—117.5	$C_{38}H_{74}O_4N_2$	73.26	11.97	4.50	73.20	11.81	4.34
$C_{17}H_{35}$	110—113[c]	117—118	$C_{42}H_{82}O_4N_2$	74.28	12.17	4.13	73.96	12.01	4.13

a) T. Ueda, S. Koto, and S. Toyoshima (to Dai-Nippon Drug Manufg. Co.), Japan. 620 ('58), Feb. 5 [*C. A.*, **53**, P 1172 *h* (1959)].
b) T. Tsubone, *Seikagaku*, **35**, 67 (1963).
c) B. W. Town (to Schwarz Laboratories, Inc.), U. S. Patent 2850387, Sept. 2, 1958 [*C. A.*, **53**, P 608*d* (1959)].

13) J.H. Lewis and J.H. Ferguson, *J. Clin. Invest.*, **29**, 486 (1950).
14) P.S. Norman, *J. Exptl. Med.*, **106**, 423 (1957).
15) V.H. Moon and G.A. Tershakovec, *Arch. Pathol.*, **55**, 384 (1953).

B. The solutions of fatty acids (0.02 mole) and triethylamine (0.02 mole) in chloroform were cooled to −5° and ethyl chloroformate (0.02 mole) was added thereto. After 30 minutes, a second precooled solution of L-lysine ethyl ester ditosylate (0.01 mole) and triethylamine (0.02 mole) in chloroform were added and the reaction mixtures were allowed to stand overnight at room temperature. The mixtures were washed successively with water, 3 per cent bicarbonate solution and water, and finally dried over anhydrous sodium sulfate. The filtrates were condensed *in vacuo* to oily syrup, the residues were saponified in ethyl alcohol and alkaline solutions were acidified with concentrated hydrochloric acid. The precipitates were filtered and recrystallized from ethyl acetate and petroleum ether.

Structures and properties of fattyacyl-L-lysines are listed in Table I.

Surface Active Effects of Fattyacyl-L-lysines: The surface active effects of fattyacyl-L-lysines were determined with the surface tensions of their sodium salt solutions by using surface tensionmeter (Du Noüy).

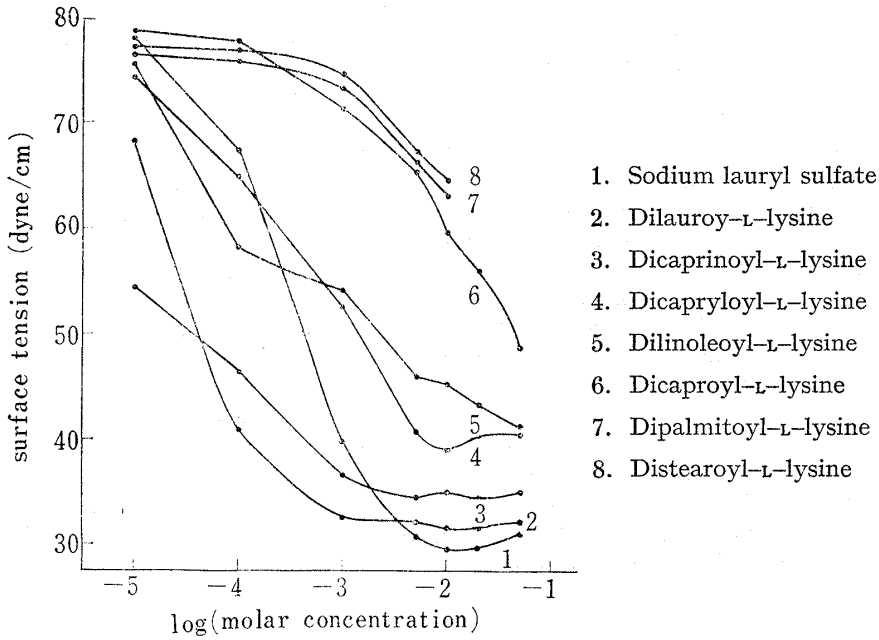


Fig. 1. The Surface Tension of N,N'-Difattyacyl-L-lysine

The results in Fig. 1 indicated that dilauroyl-L-lysine and dicaprinoyl-L-lysine had significant surface active effect comparing with sodium laurylsulfate which was very potent detergent. It might be noteworthy that the extension of carbon atom numbers of fattyacyl radical was required for the remarkable increase of the surface tension of their solutions. However, lauroyl radical showed maximum effect while higher homologs showed weaker effect gradually.

Table II. Inhibitory Effects of N,N'-Difattyacyl-L-lysine on Caseinolysis by Plasmin

Inhibitor	Molar concentration (%)		
	2×10^{-2} M	1×10^{-2} M	1.5×10^{-2} M
Diacetyl-L-lysine	4.4		
Dibutyroyl-L-lysine	0		
Dicaproyl-L-lysine	0		
Dicapryloyl-L-lysine	41.4	26.6	14.6
Dicaprinoyl-L-lysine	60.6	43.7	34.8
Dilauroyl-L-lysine	56.7	50.4	36.0
Dimyristoyl-L-lysine	56.1	48.8	39.4
Dipalmitoyl-L-lysine		16.3	13.2
Distearoyl-L-lysine		16.1	8.2
Dicarbobenzoxy-L-lysine	51.8	32.2	25.6
ε-Aminocaproic acid	11.1		

Inhibitions of Proteolytic Activity: Inhibitory effects of the fattyacyl derivatives of L-lysine on the proteolytic activity of plasmin were determined by a modification of the Norman's assay.[14]

Reaction mixture of 1 ml of euglobulin solution, 1 ml of 4 per cent casein solution and 1 ml of inhibitor solution was incubated at 37° for several minutes, and then 0.1 ml of varidase solution (250 units) was added. As a control, reaction mixture containing 1 ml of buffer solution instead of inhibitor solution was run parallel. After half minute and 30 minutes of incubation at 37°, 3 ml of 10 per cent trichloroacetic acid was added to the reaction mixture. After one hour's standing at room temperature, the resulting precipitate was removed by filtration with Toyo Filter Paper No. 2, and then the filtrate was treated with Folin-Ciocalteu reagent according to the method of Mounter, *et al.*[16] The optical density of the resulting blue color solution from 30 minutes specimen was read in a spectrophotometer at the wave length 660 mμ, with the half minute specimen as blank value. The rate of inhibition was calculated by comparison with optical density obtained from control.

Table II illustrated the inhibitory effects of the fattyacyl derivatives of L-lysine on the proteolytic activity in purified casein solution. It can be clearly seen that dicaprinoyl-, dilauroyl- and dimyristoyl-L-lysine have more powerful inhibitory effect than the other fattyacyl compounds. Particularly, dilauroyl derivative shows most powerful inhibition in all instances. In this experiment, the extension of carbon atom numbers of fattyacyl radical was required for the increase of the inhibitroy effect on proteolysis by plasmin.

Inhibitory Effects of Some Synthetic Compounds on Plasmin Activity *in Vivo*: Two hundreds mg of ε-aminocaproic acid, 100 mg of dicarbobenzoxy-L-lysine and 10 mg of dilauroyl-L-lysine were respectively given intravenously to rabbits weighing about 2.0 kg. Blood samples were drawn by venopuncture at the times mentioned in Table III, and the actions of compounds were measured by the streptokinase activation test of the fibrinolysis. An assay system was devised consisting of the following reagents, 0.2 ml of fibrinogen solution, 0.2 ml of euglobulin solution, 0.1 ml of thrombin solution and 0.1 ml of varidase solution. The time required to lyse the clot thus formed was measured starting with the addition of thrombin as zero time. All tests were run in a 37° water bath using test tubes of 10 mm internal diameter. The results obtained were shown in Table III. When 200 mg of ε-aminocaproic acid was given intravenously to rabbits, a very marked retardation of the lysis time in the streptokinase activation test of fibrinolysis was observed 1 hr after the injection. The action of dicarbobenzoxy-L-lysine, however, was different from that of ε-aminocaproic acid and the intravenous administration of 100 mg of this agent produced no significant retardation of lysis time. The inhibitory effect of dilauroyl-L-lysine was obviously stronger than that of ε-aminocaproic acid. In the rabbits to which 10 mg of dilauroyl-L-lysine were given intravenously, the retardation of lysis time was obviously confirmed in each blood sample which was taken at 1 hr to 3 hr after the injection.

Table III. The Effects of the Intravenous Administration of Anti-plasminic Agents to Rabbits

Substance	Dosage (mg)	Lysis time (sec)				
		Before injection	After injection			
			1 hr	2 hr	3 hr	4 hr
ε-Aminocaproic acid	200	227	315			
ε-Aminocaproic acid	200	330	606			
Dicarbobenzoxy-L-lysine	100	475	450			
Dicarbobenzoxy-L-lysine	100	345	318			
Dilauroyl-L-lysine	10	277	458	420		
Dilauroyl-L-lysine	10	230	435	397	331	320
Dilauroyl-L-lysine	10	291	470	430	376	288

The results indicated that smaller amount of dilauroyl-L-lysine than ε-aminocaproic acid could reveal its potent anti-fibrinolytic effect on the circulatory blood *in vivo*.

Reversal and Inhibition by Dilauroyl-L-lysine of Lytic System in Blood Stream produced in Rabbits: It has been reported by Okamoto, *et al.*[17] and Miller, *et al.*[18] that the intravenous administration of ε-aminocaproic acid or 1-aminomethylcyclohexane-4-carboxylic acid was effective to reverse the activated

16) L.A. Mounter and B.A. Shipley, *J. Biol. Chem.*, **231**, 855 (1958).
17) S. Okamoto and U. Okamoto, *Keio J. Med.*, **11**, 105 (1962).
18) J.M. Miller and D.A. Robinson, *A.M.A. Arch. Surgery*, **78**, 33 (1959).

fibrinolysis of the circulatory blood in animal experiments. The same effect was examined in the following experiments using dilauroyl-L-lysine.

In order to activate the lytic system in the circulatory blood of rabbits, 2 ml of human serum and 25000 units of streptokinase were given intravenously to rabbits. The activation of the lytic system in blood was easily demonstrated by estimating the time required for the complete lysis of the formed clots by euglobulin samples. Fig. 2a illustrated that the remarkable activation of lytic system in blood lasted for more than 90 minutes after the injection of streptokinase. Results shown in Fig. 2b, however, indicated that shortened clot lysis time by the activation procedure was turned normal by the injection of 10 mg of dilauroyl-L-lysine to the rabbits. It was also indicated as shown in Fig. 2c that the administration of dilauroyl-L-lysine prior to the activation procedure prevented the appearance of the rapid clot lysis in circulatory blood.

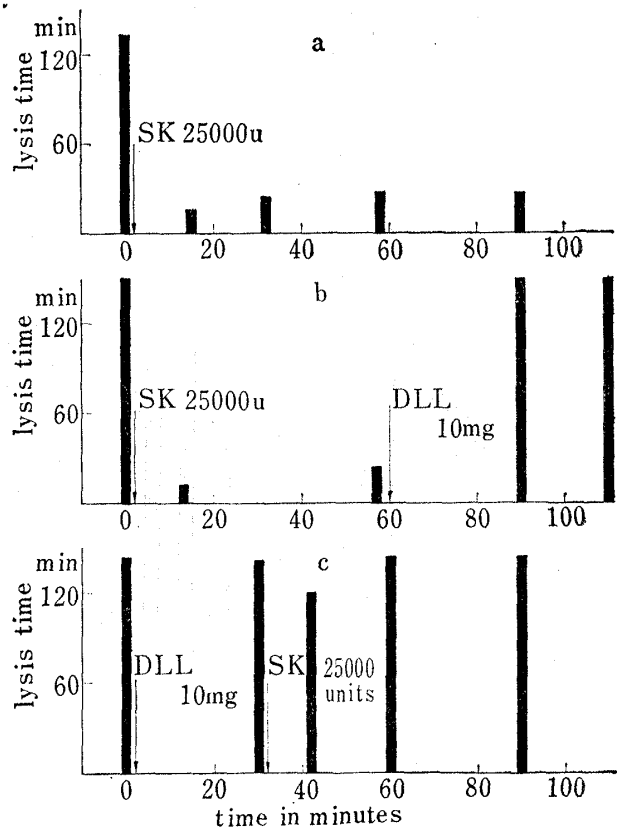


Fig. 2. Reversal or Inhibition of the Activation of Lytic System in Blood of Rabbits by DLL

Effects of Some Anti-plasminic Agents on Experimental Inflammation: The effects of dilauroyl-L-lysine, dicarbobenzoxy-L-lysine and 1-aminomethylcyclohexane-4-carboxylic acid on the experimental inflammation induced by lung extract in the rabbit skin were investigated.

In order to induce the change of capillary permeability in the rabbit skin, from 0.1 to 0.2 ml of lung extract (permeability enhancing substance) was injected intracutaneously, and 0.1 ml of Mamushi venon (0.1%) was injected simultaneously as control. At 10 or 15 minutes after the injection, 10 ml of 1 per cent trypan blue solution were injected intravenously. Anti-inflammatory effects of agents were determined by the inhibitory activities of the agents on extravasation of trypan blue in the injected site of skin of the rabbit.

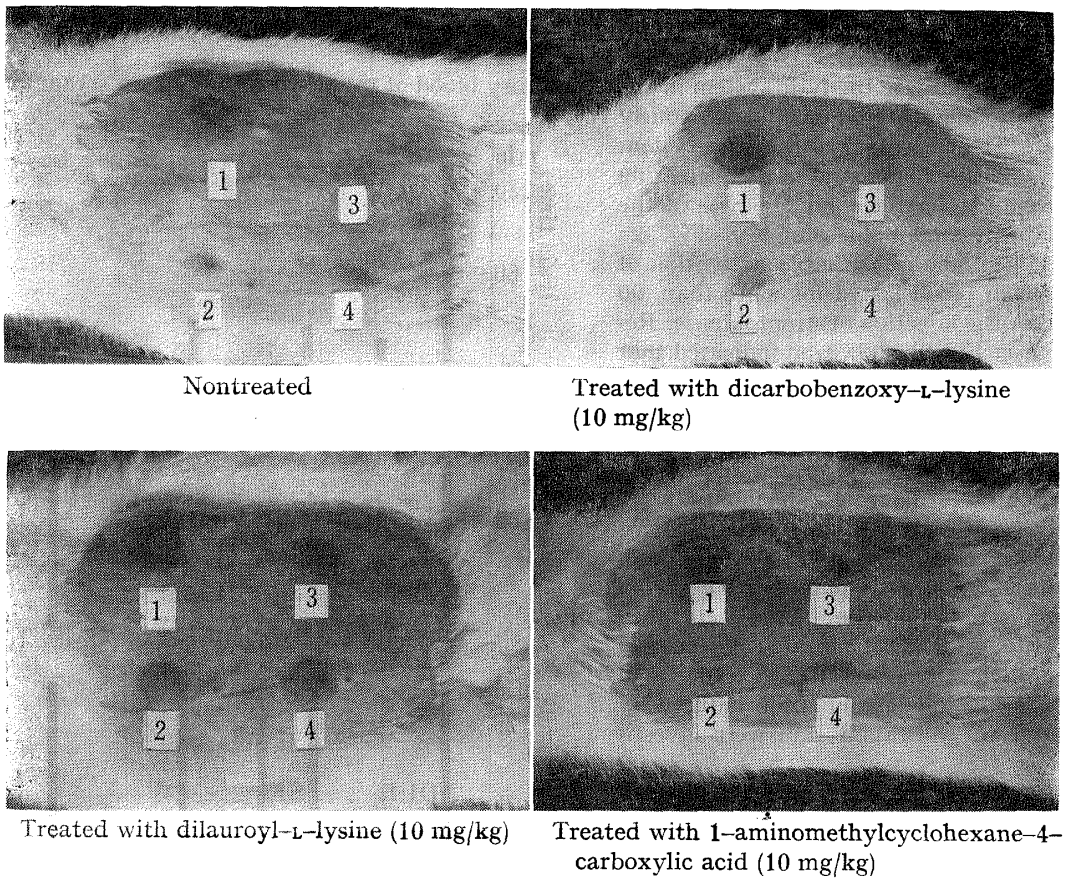
Fig. 3 shows the anti-inflammatory activities of some anti-plasminic agents to the enhancement of permeability in the rabbit skin. It was quite evident that dilauroyl-L-lysine and dicarbobenzoxy-L-lysine markedly inhibited extravasation of the dye when 10 mg per kg of agents were administered intravenously. 1-Aminomethylcyclohexane-4-carboxylic acid was unable to prevent when the same amount of agent was administered.

Discussion

In recent years, the synthetic amino acid, ε-aminocaproic acid, has been shown to be an effective agent for inhibiting fibrinolytic activity, and this amino acid has been broadly applied to ameliorate the fibrinolytic hemorrhage or the allied syndromes. However, it was shown that a large amount of this agent must be given to patient, in order to suppress the extensively accelerated fibrinolysis of blood.

These observations prompted us to study on a more potent inhibitor to plasmin than ε-aminocaproic acid. Okamoto, *et al.*[17] have reported that 1-aminomethylcyclohexane-4-carboxylic acid was a new potent inhibitor of the fibrinolytic system and the inhibitory effect of this compound was far more potent than that of ε-aminocaproic acid when examined by the fibrinolytic system *in vitro* and also *in vivo*.

In 1963, the authors have found that dicarbobenzoxy-L-lysine was more powerful inhibitor to proteolysis of plasmin than ε-aminocaproic acid, however, this compound showed weak inhibitory effect on fibrinolysis.[11]



Nontreated

Treated with dicarbobenzoxy-L-lysine (10 mg/kg)

Treated with dilauroyl-L-lysine (10 mg/kg)

Treated with 1-aminomethylcyclohexane-4-carboxylic acid (10 mg/kg)

Fig. 3. Effects of Some Anti-plasminic Agents on Experimental Inflammation Conditions of Intracutaneous Injections

1. 0.1 ml of Mamushi venon (0.1%)
2. 0.1 ml of lung extract
3. 0.15 ml of lung extract
4. 0.2 ml of lung extract

From the data presented in this paper it is readily seen that dilauroyl-L-lysine, dimyristoyl-L-lysine are potent inhibitor of proteolysis. The activity of dilauroyl-L-lysine given to rabbits intravenously was examined by administration of ε-aminocaproic acid, dicarbobenzoxy-L-lysine and dilauroyl-L-lysine and estimating the inhibitory effect on streptokinase activation of blood samples taken from the rabbits at various intervals. The resulting data illustrated that the most potent effect of dilauroyl-L-lysine among the tested three agents was clearly demonstrated. Results shown in Table III, for instance, indicated that the inhibition of the streptokinase activation was markedly observed in the blood samples taken from rabbits after the intravenous administration of 10 mg of dilauroyl-L-lysine, while the inhibition was not observed at all blood samples taken at one hour after the injection of 100mg of dicarbobenzoxy-L-lysine. Results shown in Fig. 2 demonstrated reversal or inhibition by dilauroyl-L-lysine of lytic system in blood stream produced in rabbits by streptokinase.

The prevention of enhancement of permeability has been widely used as a test for anti-inflammatory agents. The present investigation suggested that N,N'-disubstituted L-lysine had potent anti-inflammatory effect. However, it is necessary to make it clear that inflammation is not one event, but a series of events occurring in orderly sequence, though not necessarily dependent on each other for their development. In fact, no known anti-inflammatory drug is equally effective in suppressing all parts of the inflammatory response, and many act only on one component of the reaction or even on a single phase of one component. Thus the authors are now studying the other aspect of the mode of action of N,N'-disubstituted L-lysine to the inflammation.