Immunology 1978 **34** 509

Inhibition of the classical and alternative pathways by amino acids and their derivatives

YUMIKO TAKADA, Y. ARIMOTO, H. MINEDA & A. TAKADA *Department of Physiology, Hamamatsu University School of Medicine, 3600 Handacho, Hamamatsu-shi, Shizuoka* 431-31, *Japan*

Received 20 *June* 1977; *accepted for publication* 4 *July* 1977

Summary. Effects of various aminoacids and their derivatives on the classical pathway and alternative pathway of the complement were studied. Leupeptin, acetyl-leucyl-leucyl-arginal, inhibited CH_{50} and C1-esterase, but did not inhibit the alternative pathway. When aminoacids of carbon chains of the order of seven were used, arginine and lysine had stronger effects than trans-aminomethyl cyclohexane carboxylic acid (t-AMCHA), cis-aminomethyl cyclohexane carboxylic acid (cis-AMCHA) and epsilon aminocaproic acid (EACA). SH-compounds, cysteine, homocysteine and glutathione, had the strongest inhibitory effects among these aminoacids on both classical and alternative pathways. When effects on C1 esterase were compared, arginine, lysine, t-AMCHA, cis-AMCHA and EACA had weak inhibition while SH-compounds showed strong inhibition. Poly-L-lysine, which had extremely strong inhibition of CH_{50}, had no inhibition on C1 esterase. The inhibitory effects of antifibrinolytic agents, EACA and t-AMCHA, were weak but when effects on early parts of the classical pathway, $C(1,4,2)H_{50}$ were tested, some inhibitory activities were recognized. Thus inhibitory effects of these agents were due to their activities on the early parts of the classical pathway.

Correspondence: Dr Y. Takada, Department of Physiology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan.

INTRODUCTION

Plasmin, the fibrinolytic enzyme, has been shown to convert C1s to C1s̄ (Ratnoff & Naff, 1967), to function as a substitute for Factor D of the properdin system (Brade, Nicholson, Bitter-Suermann & Hadding, 1974), to function as a substitute for C1s̄ to activate EAC42 to EAC$\overline{42}$ (Loos, 1977), to possess the capacity to cleave C3 directly (Ward, 1967), and to destroy the functional capacity of the C1 inhibitor (Harpel, 1970). Therefore the influence of antifibrinolytic agents on the complement system via an action on plasmin are considerable. Soter, Austen & Gigli (1975) showed that an antifibrinolytic agent, epsilon aminocaproic acid (EACA), inhibited the intrinsic activation of C1 without inhibiting the already active molecule. Earlier studies of Taylor & Fudenberg (1964) also showed that the principal action of EACA is to limit the activation of C1 to C$\overline{1}$. Other effects of 1 M EACA on the whole complement system are: (1) to facilitate the generation of anaphylatoxin by spontaneous C3 cleavage, ascribed either to inhibition of the anaphylatoxin inactivator (Vallota & Müller-Eberhard, 1973) or inhibition of the C3b inactivator (C3bINA) (Vogt, Schmidt, Lynen & Dieminger, 1975), and (2) to increase by more than 50% the amount of C5 cleaved with maximum recovery of C5a anaphylatoxin activity (Vallota, 1977). However, lower

concentrations of EACA inhibit C3 cleavage by interfering with the interaction of the properdin factors and their action on C3 (Vogt *et al.*, 1975). Since EACA had so many effects on the complement system, it seemed probable that another fibrinolytic agent, trans-aminomethyl cyclohexane carboxylic acid (t-AMCHA) might also inhibit the complement system. Preliminary experiments showed that t-AMCHA inhibited the complement system a little stronger than EACA, and cis-AMCHA, which is the stereoisomer of t-AMCHA and has little anti-fibrinolytic activity (Okamoto, Sato, Takada & Okamoto, 1963), had almost the same inhibition of the complement system as t-AMCHA.

The present study describes the inhibitory effects of EACA and structurally related compounds on the complement system.

MATERIALS AND METHODS

Buffers

Veronal-buffered saline, pH 7·5, 0·15 M, containing 0·1% gelatin, 0·00015 M Ca^{++} and 0·0005 M Mg^{++} (GVB^{++}); veronal buffered saline containing 0·01 M ethylenediamine tetraacetate and 0·1% gelatin (EDTA-GVB) and veronal buffered saline-glucose with gelatin, Ca^{++} and Mg^{++} (gl-GVB^{++}) were prepared by standard methods (Rapp & Borsos, 1970). Veronal-buffered saline, pH 7·5, containing 0·02 M Mg^{++} and 0·008 M ethylene glycol bis-amino tetraacetic acid (EGTA-VB) was prepared by the method of Platts-Mills & Ishizaka (1974).

Complement

Whole blood was collected from normal human volunteers and guinea-pigs. The sera were prepared by keeping blood for 1 h at room temperature, separating the serum, and storing it at −70°.

Compounds tested

Leupeptin was kindly donated by Dr T. Aoyagi, Institute of Microbial Chemistry, Tokyo. EACA, t-AMCHA, cis-AMCHA, glutathione and γ-amino-butyric acid were obtained from Daiichi Seiyaku Co. Ltd., Tokyo. L-lysine-HCl, L-arginine-HCl, DL-norleucine, DL-ornithine-HCl, L-citrulline and β-alanine were purchased from Wako pure chemical industries, Ltd., Osaka. L-cysteine, DL-homocysteine and 7-aminoheptanoic acid were purchased from Nakarai chemicals, Ltd., Kyoto. Poly-L-lysine-HBr of 477,500 mol. wt was purchased from Miles Labs., Inc., Elkhart, Indiana.

Sheep red blood cells (SRBC) and rabbit red blood cells (RRBC)

SRBC were purchased from Nihon Biotest Laboratory, Tokyo. RRBC were taken from a rabbit and stored in Alsever's solution.

Haemolysin

Adult rabbits were immunized by intravenous injection of 6·5 ml of 20 vol. % SRBC. Rabbits were bled on the 7th day after injection. Sera were taken and heated for 30 min at 56° and stored at −20°.

Assay on inhibitory effect on CH_{50}

CH_{50} was performed by the method of Mayer (1961), but the volume was modified to 1/5 (Okada, 1973). CH_{50} value was the same as Mayer's unit.

The assay method for inhibitors was as follows. The mixture of 0·5 ml of diluted human serum and 0·5 ml of inhibitor was incubated with 0·5 ml of sensitized SRBC (EA) of 2×10^8/ml at 37° for 60 min, then 8·5 ml of saline was added, and centrifuged at 2000 rev/min, for 10 min. The optical density of the supernatant was measured at 414 nm. The per cent inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = 100 - \frac{CH_{50} \text{ with inhibitor}}{CH_{50} \text{ without inhibitor}} \times 100$$

Assay of inhibitory effect on $C(1,4,2)H_{50}$

$C(1,4,2)H_{50}$ was performed by the method of Okada (1973). The inhibitory effect of inhibitors was measured as follows: the mixture of 0·5 ml of diluted human serum and 0·5 ml of each inhibitor was incubated with 0·5 ml of EA of 2×10^8/ml at 30° for 15 min, then 0·5 ml of EDTA-GVB and 0·5 ml of guinea-pig serum diluted to 12·5 times with EDTA-GVB were added and incubated at 37° for 60 min. After 60 min, 7·5 ml of EDTA-GVB was added and centrifuged at 2000 rev/min for 10 min, and the optical density of the supernatant was measured at 414 nm.

Esterolysis by C1 esterase

C1 esterase was obtained from out-dated human blood by dilution and acidification as described by Haines & Lepow (1964). The haemolytic activity of C1 esterase thus obtained was measured by the method of Borfos & Rapp (1963) and it was 10^{13}–10^{14}

SFU/ml. The esterolytic activity of C1 esterase was measured using N-acetyl-L-tyrosine ethyl ester (ATEe) as substrate. Since preliminary experiments showed that no preincubation was needed, 0·1 ml of C1 esterase (10^{13} SFU/ml) was mixed with 0·4 ml of each inhibitor and 0·5 ml of ATEe (20 μM/ml) simultaneously. After 30 min incubation at 37°, the ester remaining was determined by Hesterin's method as modified by Roberts (1958).

Assay of inhibition for alternative pathway

Activity of alternative pathway was measured by using RRBC (Platts-Mills & Ishizaka, 1974). Inhibitory activity was measured as follows: the RRBC were washed three times in EGTA-VB and they were adjusted to $1{\cdot}5 \times 10^8$/ml. Serum was diluted in EGTA-VB. The mixture of 0·2 ml of diluted serum and 0·2 ml of inhibitor was mixed with 0·2 ml of RRBC at 0° and the mixture was incubated at 37° for 30 min. After incubation 4·4 ml of cold EDTA-GVB were added, the tubes were centrifuged at 2000 rev/min for 10 min and the optical densities of the supernatants were measured at 412 nm. Per cent inhibition was calculated in the same way as in the case of CH_{50}.

RESULTS

Effects of leupeptin on complement system

Leupeptin is one of protease inhibitors from actinomycete fermentation (Aoyagi, Miyata, Namba, Kojima, Matsuzaki, Ishizuka, Takeuchi & Umezawa, 1969). It inhibits plasmin, kallikrein and C1 (Aoyagi *et al.*, 1969; Takahashi, Tamoto & Koyama, 1976). Effects of leupeptin on the complement system were studied by our methods. The results are shown in Fig. 1. The strong inhibition of hydrolysis of ATEe indicates that leupeptin inhibits C1s. The inhibition for $C(1,4,2)H_{50}$ is explained by the inhibition by C1s. Leupeptin did not inhibit the alternative pathway, which also indicates that leupeptin does not inhibit C3 to C9.

Effects of various substances of haemolysis expressed by CH_{50}

Structures of compounds tested are shown in Fig. 2a and 2b. Fig. 2a shows the structures of EACA and related compounds and Fig. 2b shows the structures of SH compounds.

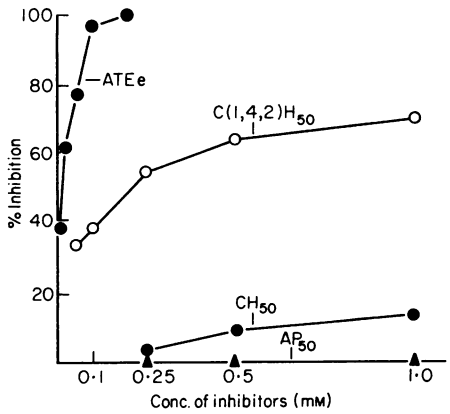

Figure 1. Effects of leupeptin on the complement system.

Fig. 3 shows the inhibitory effect of these substances on CH_{50}. SH-compounds (cysteine, glutathione and homocysteine) had the strongest inhibitory effects among these substances. Arginine, lysine, norleucine, ornithine and 7-aminoheptanoic acid had stronger effects than t-AMCHA, cis-AMCHA and EACA. Citrulline showed 2·9% inhibition at 30 mM. γ-amino butyric acid (GABA) and β-alanine had no effects. Thioglycollic acid and thioacetic acid were cytotoxic to EA, so that their effects could not be studied. Poly-L-lysine inhibited haemolysis completely even at a concentration of 10^{-19} M.

Effects of various substances on haemolysis expressed by $C(1,4,2)H_{50}$

Fig. 4 shows the inhibitory effects of various substances on $C(1,4,2)H_{50}$. As shown here, inhibitory effects of various substances on $C(1,4,2)H_{50}$ were different from the effects on CH_{50}. Although inhibition on CH_{50} of arginine (20·9%) and lysine (13·8%) at 20 mM was lower than that of cysteine (90·8%) and glutathione (63·4%), inhibitory effects on $C(1,4,2)H_{50}$ of arginine (58·7%) and lysine (69·6%) at 20 mM was comparable with that of cysteine (69·9%) and glutathione (77·1%). Also on CH_{50} even at 30 mM t-AMCHA, cis-AMCHA and EACA showed only 5·7, 5·7 and 1·6% inhibition, respectively, but on $C(1,4,2)H_{50}$ at 30 mM they showed 37·5, 28·1 and 31·2% inhibition, respectively.

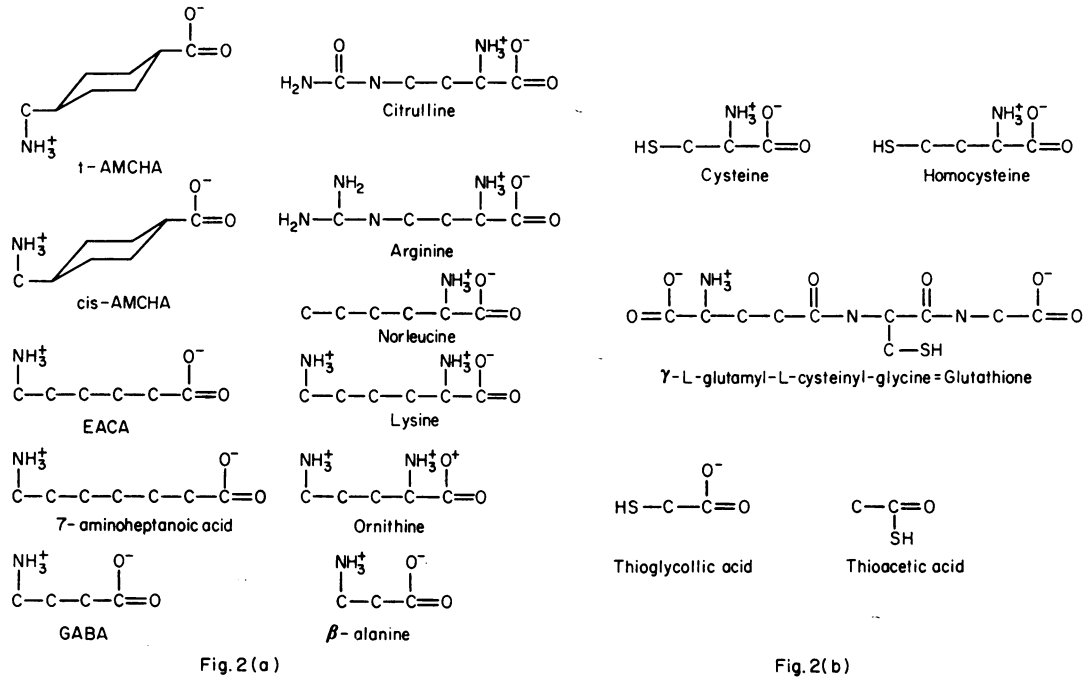

Figure 2. (a) Structures of EACA and related compounds. (b) Structures of SH compounds.

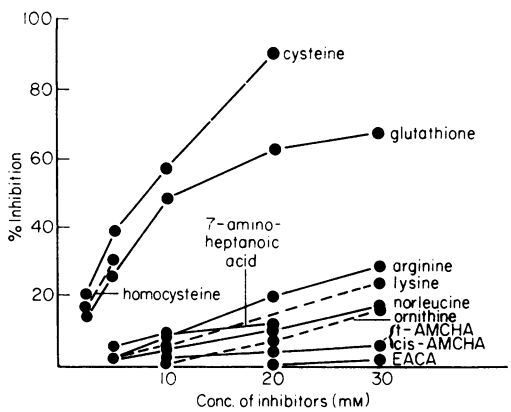

Figure 3. Effects of various substances on haemolysis expressed by CH_{50}.

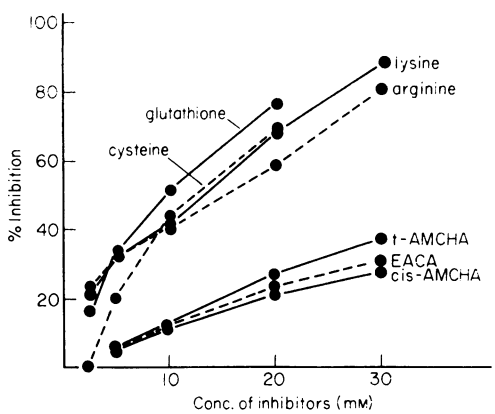

Figure 4. Effects of various substances on haemolysis expressed by $C(1,4,2)H_{50}$.

Effects of various substances on hydrolysis of ATEe

Since C1 esterase hydrolyses ATEe, the effects of various substances on hydrolysis of ATEe was studied.

Fig. 5 shows the effects of various substances on hydrolysis of ATEe. Here again while SH-compounds showed strong inhibition, t-AMCHA, cis-AMCHA, EACA, arginine and lysine showed weak inhibition. Norleucine and ornithine showed no effects. Poly-L-lysine had no effect below 10^{-7} M.

Effects of various substances on the alternative pathway

Inhibitory effects of various substances on the alternative pathway are shown in Fig. 6. Glutathione

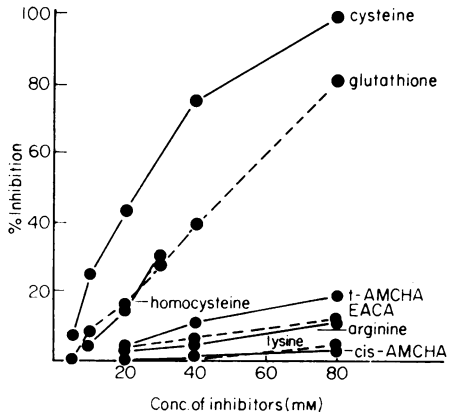

Figure 5. Effects of various substances on hydrolysis of ATEe.

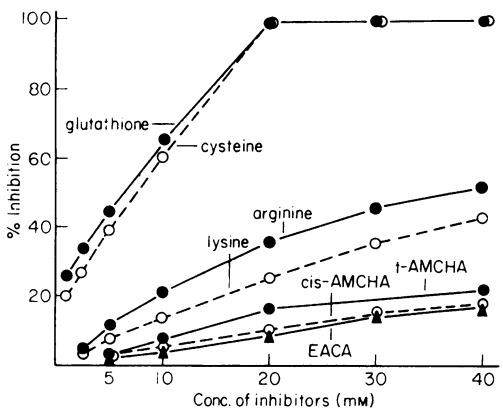

Figure 6. Effects of various substances on the alternative pathway.

and cysteine had strong inhibitory effects. Inhibitory effects of arginine and lysine were stronger than those of t-AMCHA, cis-AMCHA and EACA.

DISCUSSION

One of proteinase inhibitors obtained from actinomycete fermentation, leupeptin, inhibits plasmin, kallikrein and trypsin (Aoyagi *et al.*, 1969). Since its chemical structure is acetyl-leucyl-leucyl-arginal, a derivative of tripeptide, we were interested in its effects on the complement system. As shown in Fig. 1, leupeptin inhibited C1 esterase; however, in contrast to other compounds tested in the present study it did not inhibit the alternative pathway.

Secondly, inhibitory effects of EACA and related compounds on the complement system were studied. Loos, Volankis & Stroud (1976) demonstrated that the interaction of the complement components was influenced by electric charge; that is, negatively charged polyanions, e.g. dextran sulphate and heparin, inhibited the consumption of C4 and C2 by C1$\bar{s}$, because of the interference of these substances with the binding of C4 and C2 to C1$\bar{s}$. The results obtained by us suggest that the positively charged amino groups and chain length of carbon atoms have an important role in inhibition by interfering with formation of the electrostatic bond between various components of the complement system. Although SH-compounds had strong inhibition, compounds devoid of amino groups (e.g. thioglycollic acid and thioacetic acid) lacked inhibitory effect. Diamino-amino carboxylic acids produced greater inhibition than mono-amino carboxylic acids of similar chain lengths (e.g. lysine and EACA). The polycation, poly-L-lysine, had strong inhibitory effect on CH_{50}. Those aminoacid with chain lengths shorter than five carbon atoms (e.g. GABA and β-alanine) lacked inhibitory activity.

Concerning the effects of SH compounds (cysteine, glutathione and homocysteine) the early components of the classical pathway, especially C1 esterase (Figs 4 and 5) and the alternative pathway (Fig. 6) were inhibited. The inhibition of C1 esterase might partly be due to the reducing activity of the SH group. Vallota & Müller-Eberhard (1973) reported that cysteine inhibited anaphylatoxin (AT) formation by preventing the cleavage of C3. Thus both the classical and alternative pathway should be inhibited by SH-containing aminoacids and our results indicate that it is the case. Other SH-compounds glutathione and homocysteine, had almost the same inhibitory activity, and they might also inhibit C3 cleavage.

The inhibitory activity of antifibrinolytic agents (EACA and t-AMCHA) is partly due to inhibition of plasmin. Plasmin possesses the capacity to cleave C3 directly (Ward, 1967), activates C1s to C1$\bar{s}$ (Ratnoff & Naff, 1967) and works as a substitute for C1$\bar{s}$ to activate EAC42 to EAC$\overline{42}$ (Loos, 1977). In fact Soter *et al.* (1975) demonstrated that there was selective inhibition of C1 by EACA without effect on the levels of C4, C2, C3 and C9. Earlier studies on the elaboration of esterolytic activity also cited its action on activation of C1 to C$\bar{1}$ (Taylor & Fudenberg, 1964). Our experiments showed that

EACA, t-AMCHA inhibited $C(1,4,2)H_{50}$ (Fig. 4), but had little inhibition on C1 esterase (Fig. 5), so it is considered that they inhibited the activation of C1 to $C\bar{1}$, and/or the formation of the C3 convertase. Since cis-AMCHA, which had no antifibrinolytic activity, had the same degree of inhibition on the complement system, it appears that the mechanisms of inhibition of these antifibrinolytic agents to the complement system are different from their effects on fibrinolysis.

Although the effects of 1 M EACA on the whole complement system are to facilitate the generation of anaphylatoxin by spontaneous C3 cleavage (Vallota & Müller-Eberhard, 1973), and to increase by more than 50% the amount of C5 cleaved with maximum recovery of C5a anaphylatoxin activity (Vallota, 1977), lower concentration (below 0·5 M) of EACA inhibited the spontaneous cleavage of C3 by interfering with the interaction of the properdin factors and their action of C3 (Vogt *et al.*, 1975). Since the concentration of EACA we used was below 40 mM, the inhibition of alternative pathway may be explained by the inhibition of C3 cleavage. The mechanisms of inhibition of t-AMCHA and cis-AMCHA may be the same as EACA, because the same degree of inhibition was observed among these 3 compounds.

In the case of lysine and arginine, the inhibition of the alternative pathway might be the same as EACA, since they also inhibited C3 cleavage (Vallota & Müller-Eberhard, 1973).

Considering the effects of these compounds on later components of the complement system, Soter *et al.* (1975) reported that EACA had no influence on the titration of C3 and C9. Since EACA and related compounds inhibited $C(1,4,2)H_{50}$ more than CH_{50} in our experiments, inhibition by these substances of later stages of the complement system is unlikely.

Iwamoto (1975) reported that t-AMCHA inhibited digestion of fibrin by plasmin, but did not inhibit degradation of casein or hydrolysis of tosyl arginine methyl ester (TAMe) by plasmin. He concluded that the action of EACA or t-AMCHA is very specific for fibrinolysis. According to his hypothesis plasmin had specific receptors for t-AMCHA, and binding of t-AMCHA to these receptors prevented interaction of plasmin to fibrin, but this binding did not inhibit caseinolysis or TAMe-esterolysis. It seems unlikely that components of complement (for instance C3-convertase) have the same kind of receptor sites for t-AMCHA as plasmin; thus we assume that inhibition of $C(1,4,2)H_{50}$ by t-AMCHA is by a mechanism which is different from its inhibition of plasmin. We are currently studying the inhibition of the complement system by synthetic substances of which the structures are similar to EACA or t-AMCHA.

REFERENCES

AOYAGI T., MIYATA S., NAMBA M., KOJIMA F., MATSUZAKI M., ISHIZUKA M., TAKEUCHI T. & UMEZAWA H. (1969) Biological activity of leupeptins. *J. Antibiotics*, **22**, 558.

BORSOS T. & RAPP H.J. (1963) Chromatographic separation of the first component of complement and its assay on a molecular basis. *J. Immunol.* **91**, 851.

BRADE V., NICHOLSON A., BITTER-SUERMANN D. & HADDING U. (1974) Formation of the C3-cleaving properdin enzyme on zymosan. Demonstration that Factor D is replaceable by proteolytic enzymes. *J. Immunol.* **113**, 1735.

HAINES A.L. & LEPOW I.H. (1964) Studies on humanC'1 esterase. I. Purification and enzymatic properties. *J. Immunol.* **92**, 456.

HARPEL P.C. (1970) C1 inactivator inhibition by plasmin. *J. Clin. Invest.* **49**, 568.

IWAMOTO M. (1975) Plasminogen-plasmin system. IX. Specific binding of tranexamic acid to plasmin. *Thrombos. Diathes. haemorrh.* **33**, 573.

LOOS M., VOLANKIS J.E. & STROUD R.M. (1976) Mode of interaction of different polyanions with the first (C1, $C\bar{1}$), the second (C2) and the fourth (C4) component of complement. III. Inhibition of C4 and C2 binding site(s) on $C1\bar{s}$ by polyanions. *Immunochemistry*, **13**, 789.

LOOS M. (1977) Generation of $EAC\bar{4}\bar{2}$ (C3 convertase) by proteolytic enzymes. *Fed. Proc.* **36**, 1209.

MAYER M.M. (1961) *Experimental Immunochemistry*, 2nd edn, p. 133. Charles C. Thomas, Springfield, Ill.

OKADA H. (1973) Complement assay methods for clinical laboratories. *Rinsyo Men-eki*, **5**, 941 (in Japanese).

OKAMOTO S., SATO S., TAKADA Y. & OKAMOTO U. (1964) An active stereo-isomer (trans-form) of AMCHA and its antifibrinolytic (antiplasminic) action *in vitro* and *in vivo*. *Keio J. Med.* **13**, 177.

PLATTS-MILLS T.A.E. & ISHIZAKA K. (1974) Activation of the alternative pathway of human complement by rabbit cells. *J. Immunol.* **113**, 348.

RAPP H.J. & BORSOS T. (1970) *Molecular Basis of Complement Action*, p. 75. Appleton Century Crofts, New York.

RATNOFF O.D. & NAFF G.B. (1967) The conversion of C'1s to C'1 esterase by plasmin and trypsin. *J. exp. Med.* **125**, 337.

ROBERTS R.S. (1958) Measurement of the rate of plasmin action on synthetic substrates. *J. Biol. Chem.* **232**, 285.

SOTER N.A., AUSTEN K.F. & GIGLI I. (1975) Inhibition by ε-amino-caproic acid of the activation of the first component of complement system. *J. Immunol.* **114**, 928.

TAKAHASHI K., TAMOTO K. & KOYAMA J. (1976) Effects of

protease inhibitors of actinomycetes on the first component of human complement. *J. Antibiotics*, **29**, 983.

TAYLOR F.B. JR & FUDENBERG H. (1964) Inhibition of the C_1 component of complement by amino acids. *Immunology*, **7**, 319.

VALLOTA E.H. & MÜLLER-EBERHARD H.J. (1973) Formation of C3a and C5a anaphylatoxins in whole human serum after inhibition of the anaphylatoxin inactivator. *J. exp. Med.* **137**, 1109.

VALLOTA E.H. (1977) Inhibition of C5 convertase by epsilon aminocaproic acid (EACA): A limiting factor in the generation of C5a anaphylatoxin (AT). *Fed. Proc.* **36**, 1245.

VOGT W., SCHMIDT G., LYNEN R. & DIEMINGER L. (1975) Cleavage of the third complement component (C3) and generation of the spasmogenic peptide, C3a, in human serum via the properdin pathway: Demonstration of inhibitory as well as enhancing effects of epsilon-aminocaproic acid. *J. Immunol.* **114**, 671.

WARD P.A. (1967) A plasmin-split fragment of C3 as a new chemotactic factor. *J. exp. Med.* **126**, 189.