Br. J. Pharmac. (1972), **46**, 157–166.

Activation of arginine and tyrosine esterase in serum from patients with hereditary angio-oedema

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Summary

1. The role of clotting factor XII in the activation of the complement subunit C1s to C1 esterase was examined.

2. In sera from patients with hereditary angio-oedema who lack the α_2-glycoglobulin C1 inhibitor, silicates and other potent activators of clotting factor XII induced far less C1 esterase activity than did the weaker factor XII activators, carrageenin and cellulose sulphate. In contrast, the intensity of the induced plasma kallikrein activity corresponded more closely to the clot-promoting effect of the factor XII activators.

3. Spontaneous generation of C1 esterase activity was only slightly delayed in hereditary angio-oedema sera previously depleted of factor XII. In normal sera, C1 esterase did not develop spontaneously and could not be induced.

4. Experiments with inhibitors suggested that the spontaneous activation of C1s may consist of two phases: factor XII and other plasma proteases first activate small amounts of C1s; the resulting C1 esterase then activates the bulk of C1s. The observed spontaneous activation suggests that when fully activated, the C1s present in 1 ml of human serum will hydrolyse 1–2 μmol of ATEe/minute.

Introduction

C1 inhibitor, an α_2-neuramino-glycoglobulin, acts by combining stoicheiometrically with C1 esterase, the active enzymic form of the C1s subunit of the first component of complement (Levy & Lepow, 1959); other proteolytic serum enzymes such as C1r, plasmin, kallikrein, permeability factor, and clotting factors XII and XI are also inhibited by this inhibitor (Kagan & Becker, 1963; Ratnoff, Pensky, Ogston & Naff, 1969; Forbes, Pensky & Ratnoff, 1970). In quiescent conditions, all these proteolytic enzymes circulate as preactive precursors. They are readily activated by a wide variety of physiological and pathological stimuli. In patients with hereditary angio-oedema, the active enzymes are not neutralized because the patients' plasma lacks C1 inhibitor (Landermann, Webster, Becker & Ratcliffe, 1962; Donaldson & Evans, 1963). The unchecked activities of some of the enzymes, notably C1 esterase and plasma kallikrein, lead to the episodes of localized oedema characteristic of hereditary angio-oedema. Attacks are provoked by trauma, emotion, exercise, and other forms of stress; involvement of immune mechanisms has only rarely been demonstrated (Donaldson & Rosen, 1966; Schur

& Austen, 1968). The biochemical pathways by which the precursors of kallikrein and C1 esterase are activated in hereditary angio-oedema attacks are not fully understood. Most workers think that proteases present in plasma itself play an important role. Plasma prekallikrein is activated by clotting factor XII or some of its molecular subunits (Margolis, 1958a; Kaplan & Austen, 1970; Movat, Poon & Takeuchi, 1971). Other known plasma activators of prekallikrein are permeability factor and plasmin. The activation of C1 esterase by antigen-antibody complexes requires C1q and the enzyme C1r (Naff & Ratnoff, 1968; Lachmann, 1969). Activation by plasmin (Ratnoff & Naff, 1967) has also been reported. Donaldson (1968a) found that C1 esterase activity was induced in hereditary angio-oedema serum, but not in normal serum, when purified factor XII was added in concentrations much higher than those normally present. Ellagic acid, a specific activator of factor XII, induced in hereditary angio-oedema serum transient C1 esterase activity after 48 h incubation, but not after 2 hours. Experiments with inhibitors suggested that factor XII may achieve this not directly, but by activating prekallikrein; the resulting kallikrein would then activate the pre-esterase C1s (Donaldson, 1968a; Gigli, Mason, Colman & Austen, 1968). Factor XII may thus link complement with the other three defensive blood reactions which it initiates, viz. intrinsic clotting, fibrinolysis, and plasma kinin formation. The biological importance of factor XII lies in the ease with which it is activated by abnormal endogenous surfaces and by many exogenous surfaces.

To clarify the possible role of clotting factor XII in triggering attacks of hereditary angio-oedema, several soluble and insoluble activators of this factor were compared for their potency in inducing clot-promoting, C1 esterase, and plasma kallikrein activities in normal and in hereditary angio-oedema sera. Furthermore, the activation of C1s was studied in the presence of agents which inhibit factor XII and associated enzymes.

Methods

N-Acetyl-L-tyrosine ethyl ester monohydrate (ATEe), N-benzoyl-L-arginine ethyl ester hydrochloride (BAEe) and dextran sulphate were obtained from British Drug Houses; Alton-Bell extract from Diagnostic Reagents Ltd., chondroitin sulphate from Nutritional Biochemicals Corp., ellagic acid from Aldrich Chemical Co., hexadimethrine bromide (Polybrene) from Abbott Laboratories ; sodium polyanetholsulphonate (Liquoid) from Roche Products; ϵ-amino caproic acid (ϵ-ACA) and soya bean trypsin inhibitor (SBTI) from Sigma Co. Carrageenin was kindly given by Dr. B. O. Hughes, Beecham Research Laboratories; aprotinin (Trasylol) by Bayer Products, Germany; trans-aminomethyl cyclohexane carboxylic acid (AMCHA) by Kabi Pharmaceuticals. Cellulose sulphate was prepared by the method of Astrup, Galsmar & Volkert (1944). A human serum fraction rich in C1 esterase was separated and activated by the method of Nelson (1965). Preparations containing 60 to 110 units/ml were obtained (1 C1 esterase unit splits 33·3 nmol of ATEe per min; Levy & Lepow, 1959). Microcrystals of monosodium urate were prepared as described by Seegmiller, Howell & Malawista (1962).

Sera from patients with hereditary angio-oedema were kindly supplied by Professor M. D. Milne, The Westminster Hospital, London, S.W.1, and Dr. R. Bennett, The Royal Postgraduate Medical School, London, W.12. No C1 inhibitor was detected in one serum and 1·1 U/ml in the other (normal=18—35 U/ml).

Assay of C1 esterase and of its inhibitor

Esterolytic activities were measured in a Radiometer titrator. Activating agents and inhibitors dissolved in 0·9% NaCl solution were brought to pH 7·2 before being added to the assay system. Occasionally, the complete system had to be adjusted, before the assay, to pH 7·2 or 7·4 with a few μl of 50 mM NaOH or HCl. The substrate ATEe was added as a 0·5 M stock solution in 2-ethoxy-ethanol. The hydrolysis of 25 mM ATEe by 50 μl of serum in a total volume of 2·0 ml was followed by continuous automatic titration with 5 or 10 mM NaOH at 37° C and pH 7·2. Activating agents were added during titration after recording the spontaneous esterolysis for 2 minutes.

Induced and spontaneous activation of C1s was studied only with serum samples which hydrolysed before the experiment not more than (0·2 μmol ATEe/ml serum)/min and not more than (0·5 μmol BAEe/ml serum)/minute. To suppress the activation of esterolytic activities which occurs in hereditary angio-oedema serum spontaneously at 37° C or room temperature (Lundh, Laurell, Wetterqvist, White & Granerus, 1968 ; Donaldson, 1968b), stored samples (−60° C) were kept below 4° C during thawing and until used. Experiments were then performed as promptly as possible.

When the rate of C1 esterase generation was compared in several examples of the same serum, 50 μl of cold serum was added to prewarmed (37° C) incubation mixtures in a fixed order at 2 min intervals. The activity in the samples was measured in the same order and intervals. Between readings, samples were kept at 37° C.

C1 inhibitor was measured by a modification of the method of Levy & Lepow (1959). The hydrolysis of 25 mM ATEe by a standard dose of C1 esterase (4–5 units) was established. Ten μl of test serum was then incubated for 3 min with the same dose of C1 esterase in a Radiometer titration vessel. Saline and ATEe were then added. The hydrolysis of 25 mM ATEe was measured as described for C1 esterase, and the esterolytic activity in the two tests compared. The protein concentration in these systems was less than 1 mg/ml; no buffer was added. Normal sera contained 18–35 inhibitor units/ml (1 unit inhibits 10 esterase units, that is the hydrolysis of 333 nmol of ATEe per min; Levy & Lepow, 1959).

Assay of plasma kallikrein

The hydrolysis of 10 mM BAEe by 50 or 100 μl of serum in a total volume of 2 ml was followed by continuous titration with 5 mM NaOH at 37° C and pH 7·4, and in the presence of heparin (500 U/ml serum), AMCHA (6 μmol/ml serum) and edetate (3 μmol/ml serum). These inhibitors prevented activation of other plasma enzymes which split BAEe.

Removal of clotting factor XII

The method of Nossel (1964) was slightly modified. Plasma or serum was mixed with celite (30 mg/ml) for 40 min at 4° C. The procedure removed the bulk of factor XII, since in treated plasma kaolin did not accelerate clotting. Control serum or plasma was kept at 4° C for 40 min without celite.

Assay of clot-promoting effects

The 'kaolin clotting time' method (Margolis, 1958b) was adapted to measure the effects of kaolin and other activating materials. Results were expressed as a 'clot-promoting index'

$$\left(= \frac{\text{clotting time in sec without activating material}}{\text{clotting time in sec with activating material}}\right).$$

Results

Figure 1 shows the C1 esterase and plasma kallikrein measured as ATEe and BAEe hydrolysis which were induced in a normal and in an HAO serum by substances known to activate factor XII. Several compounds related to the

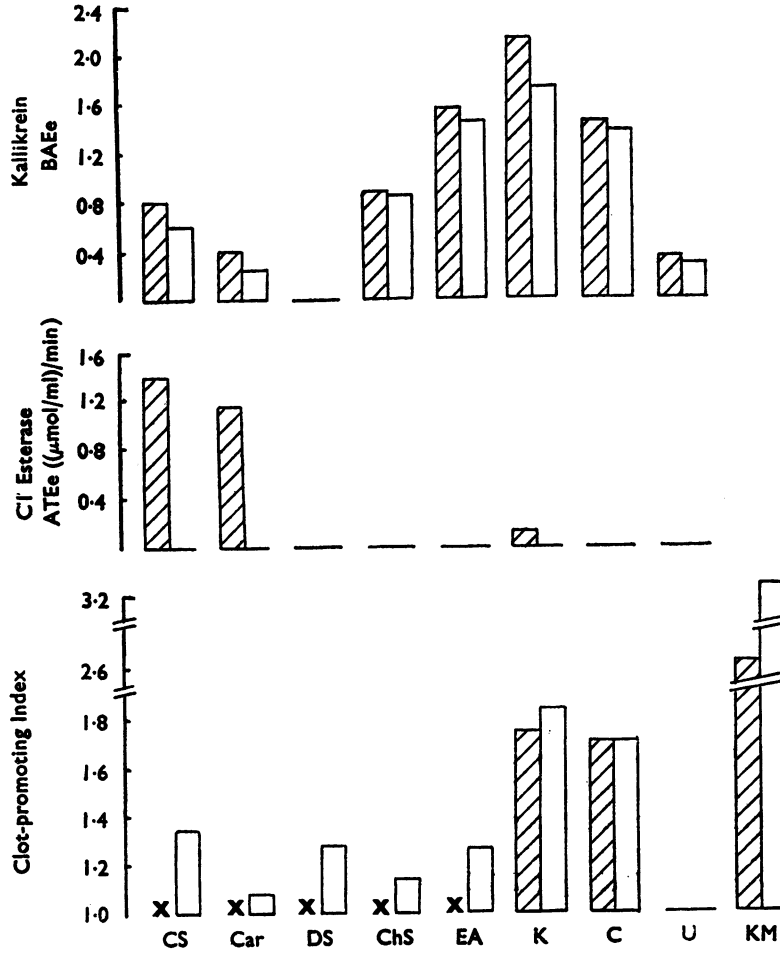

FIG. 1. Activation of esterolytic and clot-promoting activities. The basal hydrolysis of BAEe by plasma kallikrein and of ATEe by C1 esterase was recorded for 2 minutes. One μmol of ellagic acid (EA) or 2 mg of cellulose sulphate (CS), carrageenin (Car), dextran sulphate (DS), chondroitin sulphate (ChS), kaolin (K), celite (C) or urate (U) per ml of hereditary angio-oedema serum (shaded columns) or normal serum (clear columns) were then added without interrupting the recording. The height of the columns shows induced minus spontaneous activity. Clotting tests were carried out with 400 times smaller concentrations of activating materials. KM=maximum clot promotion by excess of kaolin (10 mg/ml). X=not tested.

activating substances were also tested. In these assays, 1 ml of serum was activated with 1 μmol of ellagic acid, or with 2 mg of the other activating substances. In this range, the responses varied with the amounts of activating agents applied, showing that these concentrations did not mobilize the full esterolytic potential of the sera. It was therefore possible to compare the relative efficacy of the various activating materials.

The clot-promoting effect was measured mainly in normal plasma, because only limited amounts of hereditary angio-oedema plasma were available. Cellulose sulphate and carrageenin accelerated clotting optimally at a concentration of 5 μg/ml; 10–15 μg/ml already delayed clotting as described by Astrup *et al.* (1944) and Kellermeyer & Kellermeyer (1969). The clot-promoting action of all substances was therefore tested in concentrations 400 times lower than those used in esterolytic assays. In this way, the relative clot-promoting efficacy of the activating materials could be related to their effects on esterolysis. Only a small part of the clotting potential of plasma was engaged in these tests and the intrinsic clotting system was not the limiting factor of the reaction. This was evident from the finding that full mobilization of this system by an excess of kaolin (10 mg/ml plasma) produced much faster clotting. The clotting tests with hereditary angio-oedema plasma suggested that the lack of C1 inhibitor did not lead to significant consumption and loss of factor XII, since the clot-promoting effect of kaolin and celite was of normal magnitude.

ATEe hydrolysis developed spontaneously, and could be enhanced by activating agents, only in hereditary angio-oedema sera, and not in normal sera. In contrast, BAEe was slowly hydrolysed both by normal and by hereditary angio-oedema sera even before activation. The rates of this spontaneous BAEe splitting were usually higher in hereditary angio-oedema sera. However, the enhancement of BAEe hydrolysis induced by any one activating substance was similar in both types of sera.

The most intense hydrolysis of ATEe was induced by the sulphated polysaccharides cellulose sulphate and carrageenin, which promoted clotting and BAEe hydrolysis only moderately. Potent activators of clotting factor XII, such as kaolin, celite, or ellagic acid, induced only little hydrolysis of ATEe. This clearly differed from the hydrolysis of BAEe, which was most potently activated by those substances which also produced the greatest clot-promoting effect.

The time course of the induced esterolytic activities was also different. ATEe hydrolysis was enhanced within a few seconds of adding an activating agent, and remained constant after that. Increased BAEe hydrolysis developed after a lag period of 30 to 120 s, and the final rate was attained after only 3–4 minutes. Figure 1 shows the rate of BAEe hydrolysis at 4 min after activation.

Further studies on the role of factor XII were carried out using both the activation of C1s by polysaccharides and the spontaneous generation of C1 esterase. In the experimental conditions (37° C, pH 7·2, 50 μl of serum), detectable spontaneous hydrolysis of ATEe developed within 10–30 min, and attained maximum levels at 40–80 min (Figs. 2–5). At room temperature the development was slower.

Effect of previous removal of factor XII

The extensive treatment of sera with celite, required for the removal of factor XII by consumption and adsorption, did not reduce the subsequent activation of ATEe esterase in hereditary angio-oedema serum by cellulose sulphate or carrageenin (Fig. 2). Spontaneous development of ATEe esterase was slightly delayed in hereditary angio-oedema serum depleted of factor XII, but eventually attained similar intensity as in control samples.

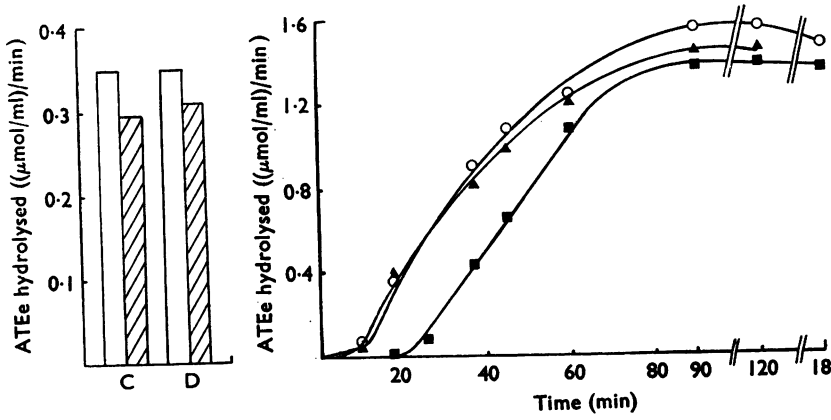

FIG. 2. Effect of exposure to silicates. Serum was activated by 2 mg/ml of carrageenin (clear columns) or of cellulose sulphate (shaded columns). C=control hereditary angio-oedema serum; D=same serum depleted of clotting factor XII. Spontaneous generation of C1 esterase was similar in glass (○) and in plastic (▲) vessels, but its early phase was delayed in factor XII depleted serum (■).

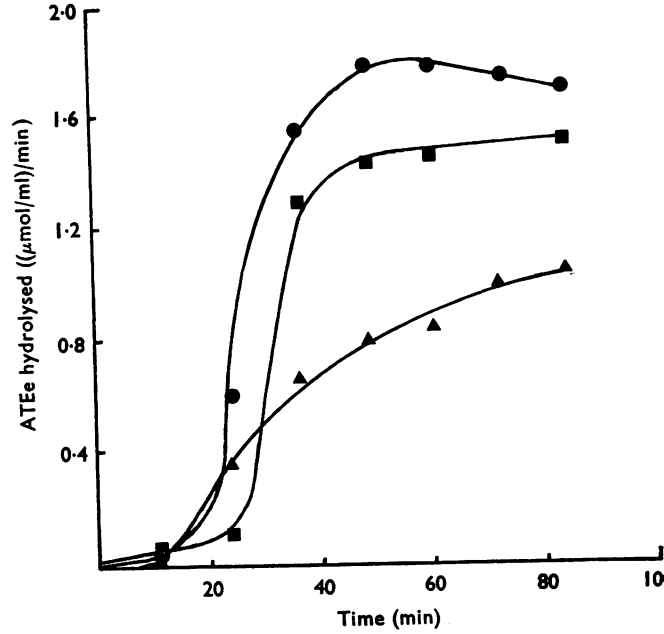

FIG. 3. Spontaneous development of C1 esterase in hereditary angio-oedema serum (●). Effect of aspirin 1 μmol/ml serum (▲) and of SBTI 10 mg/ml serum (■). Control (●).

The results suggest that clotting factor XII is not involved in the activation of C1 esterase by polysaccharides, but may accelerate the spontaneous activation of this esterase. However, the influence of factor XII was not sufficient to increase the rate at which C1 esterase developed spontaneously in glass vessels whose walls activate factor XII, above the rate occurring in non-activating plastic vessels (Fig. 2).

Inhibition of C1s activation

Activation of C1s occurring spontaneously or induced by polysaccharides in hereditary angio-oedema serum was not inhibited by preincubation for 5 min with

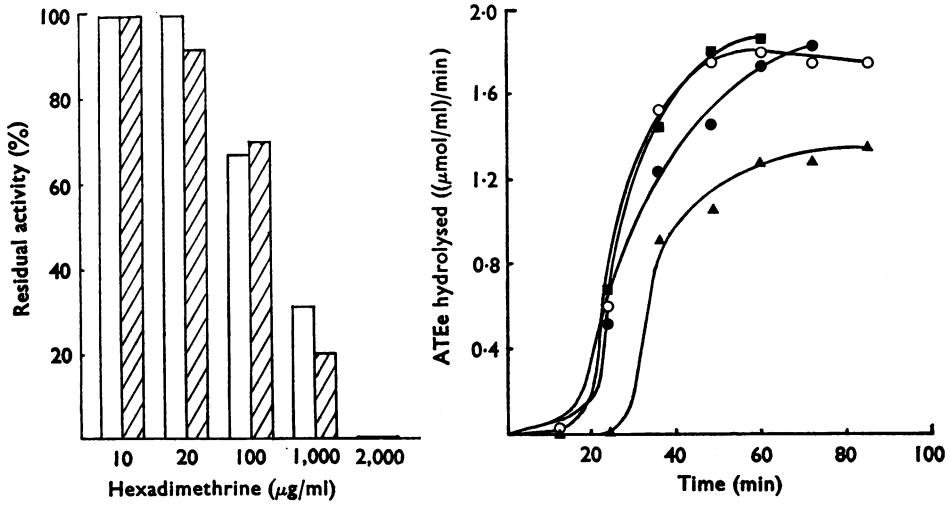

FIG. 4. Inhibition by hexadimethrine bromide. Carrageenin and cellulose sulphate were applied as in Fig. 2. Spontaneous activation in HAO serum (○) was depressed by hexadimethrine 100 μg/ml (▲), but not by 20 μg/ml (●) or 1 μg/ml (■).

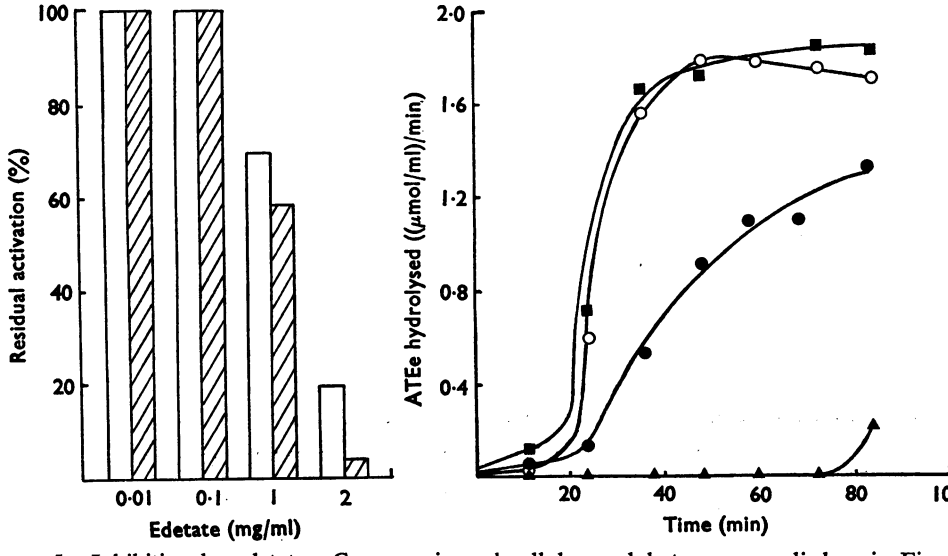

FIG. 5. Inhibition by edetate. Carrageenin and cellulose sulphate were applied as in Fig. 2. Spontaneous activation in HAO serum (○) was depressed by edetate 2 mg/ml (▲) and 1 mg/ml (●), but not by 100 μg/ml (■).

the following substances (figures in parentheses give the highest tested inhibitor concentration per ml serum; final concentrations in the assay system were 40 times lower): heparin (500 U); protamine sulphate (1 mg); dextran sulphate (1 mg).

Several inhibitors had no consistent effect on the activation by carrageenin and cellulose sulphate, but depressed the spontaneous generation of C1 esterase in hereditary angio-oedema serum. SBTI slowed down the spontaneous activation, but reduced only slightly the final level of hydrolysis attained (Fig. 3). Similar, but smaller and less consistent delays were produced by sodium polyanetholsulphonate (0·1 mg/ml serum), ϵ-ACA (10 μmol/ml), AMCHA (16 μmol/ml) and aprotinin (5,000 U/ml). Only in the presence of acetylsalicylate (1 μmol/ml) were the final levels of C1 esterase activity significantly reduced (Fig. 3).

The antiheparin compound hexadimethrine bromide and the chelating agent sodium edetate inhibited both the activation by carrageenin and cellulose sulphate and the spontaneous development of C1 esterase. Both drugs were effective only in fairly high concentrations (Figs. 4 and 5). Neither drug inhibited active C1 esterase.

Discussion

The observed hydrolysis of ATEe was attributed to C1 esterase, and that of BAEe to plasma kallikrein, on the following considerations. C1 esterase is the only intrinsic ATEe-splitting enzyme found in the fluid phase of human plasma or serum. The ATEe hydrolysis generated on the surface of sensitized erythrocytes when C3 is fixed on to these cells, does not occur in solution (Lachmann, 1969; Muller-Eberhard, 1969).

BAEe is potently hydrolysed by numerous plasma enzymes in addition to kallikrein. These are clotting factors, fibrinolytic enzymes and C1 esterase; their activation can be prevented by heparin, AMCHA and sodium edetate. Since BAEe hydrolysis was measured in the presence of these three inhibitors, the observed activity could be attributed to plasma kallikrein.

Our findings suggest that clotting factor XII is not involved in the activation of C1s in hereditary angio-oedema sera by cellulose sulphate and carrageenin: the activation was seen even after removal of factor XII; other, more potent activators of the clot-promoting action of factor XII had little or no effect on C1s activation. The different patterns of the induced clotting and ATEe-splitting activities could possibly be attributed to the fact that large molecular units of active factor XII promote clotting, and small units activate prekallikrein and C1s (Kaplan & Austen, 1970; Movat *et al.*, 1971). However, this interpretation seems unlikely, because the induced BAEe hydrolysis (attributable to plasma kallikrein) showed a similar pattern to the clot-promoting effects, and the findings reflect the accepted view that factor XII activates plasma prekallikrein.

The finding that hexadimethrine bromide inhibited the activation of C1s by cellulose sulphate and carrageenin, does not necessarily prove involvement of factor XII. Although this compound does inhibit factor XII (Ratnoff & Miles, 1964; Eisen, 1964), it may have suppressed the activating effect of the two polysaccharides cellulose sulphate and carrageenin by combining with their sulphate radicals, in the same way as it combines with heparin. The failure of the other inhibitors argues against the involvement of several other known or claimed

enzymic activators of C1s. Thus C1r was excluded by the failure of polyanethol-sulphonate (Ratnoff & Naff, 1967) to inhibit the activation of C1s by polysaccharides; permeability factor and plasma kallikrein were excluded by the failure of SBTI and aprotinin; plasmin by the failure of SBTI and aprotinin and by the failure of the inhibitors of plasminogen activation, ε-ACA and AMCHA.

Some, or possibly all of these enzymes may, however, participate in the 'spontaneous' generation of C1 esterase in hereditary angio-oedema sera. This activation was delayed by most of the tested inhibitors. The inhibition was particularly noticeable in the early phase of activation and tended to disappear later. The findings suggest that the observed spontaneous activation may consist of two phases: first, C1r, or factor XII and its associated proteases convert small amounts of C1s to C1 esterase; second, this C1 esterase then autocatalytically activates the bulk of C1s. An autocatalytic phase was also suggested by the very pronounced S-shaped curve of the spontaneous C1 esterase generation.

The present findings are compatible with the recent suggestion by Hadjiyannaki & Lachmann (1971) that in hereditary angio-oedema plasma C1 esterase may be generated autocatalytically, once all available C1 esterase inhibitor is bound and digested by one or several of the serum proteases which it inhibits. The autocatalytic activation of C1s is now being studied in more detail.

The observed effects of SBTI, ε-ACA and aprotinin on the spontaneous activation of C1s are in partial agreement with the report of Donaldson (1968b) that this activation was delayed and occasionally reduced by SBTI; delayed and eventually enhanced by aprotinin; and not consistently influenced by ε-ACA. The activation of C1s recorded by Donaldson (1968b) was much slower than that found in our experiments. The reasons for this difference are not clear.

Hexadimethrine bromide depressed both the rate and the eventual level of spontaneous C1 esterase generation, but only in concentrations higher than those effective against clotting factor XII. The mechanism of this inhibition remains to be established.

Finally, the inhibition by sodium edetate of spontaneous and polysaccharide-induced C1s activation, suggests that these, like other modes of activation, require calcium ions.

The mechanism by which aspirin inhibits the spontaneous generation is not known. We were unable to confirm reports that 0·4 mM aspirin reduces total haemolytic complement titres (Van Oss, Friedmann & Fontaine, 1961).

It is not known whether the mechanism of the observed spontaneous development of C1 esterase is related to the *in vivo* activation of C1s which leads to attacks in patients with hereditary angio-oedema. Reports that in some patients these attacks can be prevented or suppressed by the inhibitors of plasminogen activation ε-ACA and AMCHA (Lundh *et al.*, 1968) suggest that in clinical attacks fibrinolytic factors are of greater importance.

Note on units. The esterolytic units of C1 esterase defined by Levy & Lepow (1959) are widely used (1 unit=hydrolysis of 0·5 μmol of ATEe in 15 min or 33·3 nmol per min). It may be more convenient to use units conforming with the definition of international units (1 unit=hydrolysis of 1 μmol of ATEe per min).

From the present measurements of the spontaneously developing activity in two hereditary angio-oedema sera it appears that the esterolytic potential of C1s in 1 ml of human serum is of the order of 1–2 international units (Figs. 2–5).

We are grateful to Professor C. A. Keele for valuable comments and advice.

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(*Received November* 4, 1971)