Br. J. Pharmac. (1973), **49**, 678–687.

Effects of suramin on complement, blood clotting, fibrinolysis and kinin formation

V. EISEN AND C. LOVEDAY

Rheumatology Research Department, The Middlesex Hospital Medical School, London W1P 9PG

Summary

1. The inhibition by suramin of complement components, and of blood clotting, fibrinolytic, and plasma kinin forming factors depended on the conditions of the assay and on the substrates used.

2. Haemolysis by complement was more effectively inhibited in red blood cell suspensions, than in agarose gel plates. In esterolytic tests, the activation of component 1 (C1) to C1 esterase was significantly inhibited by 0·1–0·3 mM suramin, and the activity of C1 esterase by 0·5 mM suramin.

3. Part of the anticoagulant effect of suramin is due to inhibition of the action of thrombin on fibrinogen.

4. Suramin did not inhibit fibrin degradation by the fibrinolytic system in plasma. In esterolytic tests, the activation of plasminogen was more potently inhibited than the activity of plasmin.

5. Activation of plasma kallikrein, measured either by kinin formation or by esterolysis, was inhibited by 0·1–0·3 mM suramin. Active plasma kallikrein was inhibited by 0·3–0·5 mM suramin. Pancreatic kallikrein was weakly inhibited, and urinary kallikrein not at all.

Introduction

The 'classical' reaction sequence of complement is initiated by activation of component 1 (C1), generating C1 esterase. Activation of C1 results in the activation of C4 and then C2. A C142 complex is formed which activates C3. Sequential activation of C5, 6, 7, 8, 9 completes the haemolytic reaction. C1 esterase and probably several other activated components are proteases.

An alternative pathway in which properdin directly activates C3 has recently been described (Lachmann & Nicol, 1973).

Suramin, the symmetrical 3″-urea of the sodium salt of 8-(3-benzamido-4-methylbenzamido) napthalene-1, 3, 5-trisulphonic acid (mol. wt. 1429·2) used in the treatment of trypanosomiasis and onchocerciasis, inhibits numerous enzymes (Wills & Wormall, 1950), amongst them proteases like trypsin, chymotrypsin and papain. Fong & Good (1972) found that haemolytic complement is inhibited at the stages of the interaction of sensitized red blood cells with C1, and the interaction of activated C1 with C4 and then C2. In addition they reported inhibition in the sequence of steps involving C3–9. The intermediate reactions of the haemolytic sequence were studied with complement components bound on to sensitized red cells.

This paper is concerned with the effects of suramin on haemolytic and on non-haemolytic actions of complement. Effects on three other processes involving plasma proteases were also investigated. These were: blood clotting, fibrinolysis, and plasma kinin formation.

Some of the present results have been reported to the IX Symposium of the Collegium Internationale Allergologicum, London, September 1972 (Eisen & Loveday, 1973).

Methods

Serum complement. Total haemolytic complement was measured as 50% haemolytic units (CH 50) by the method of Osler, Strauss & Mayer (1952), using smaller (one fifth) volumes.

Complement assays on plates. Glass slides were primed by coating with 1% agarose in CFD, which was allowed to dry. Sensitized cells (EA) were obtained by incubating sheep red blood cells (E) with horse anti-sheep haemolytic serum (Osler *et al.*, 1952). Suramin was added either to the test serum or to the agarose gel.

Assay of C4 (Lachmann, Hobart & Aston, 1973). Guinea-pig serum was depleted of C4 by exposure to 0·7% ammonia for 45 minutes. A portion (0·15 ml) of this serum was mixed at 40° C with 1·5 ml of 2% agarose in CFD, 0·6 ml of EA suspension (5%) and 1 ml of CFD, and poured on to pre-warmed primed slides. After setting, 1 mm wells were cut and the test sera added.

The zones of haemolysis were read at 6–8 h with a graduated magnifier.

Assay of C6 (Lachmann, *et al.*, 1973). Human serum with a low C3 content (R3) was prepared by incubation with zymosan for 45 minutes. Bathing of EA in R3 for 75 min produced EA coated with C 1423 (EAC 1423). C1 and C2 were removed from these cells by storage, yielding EAC 43; 1·5 ml of a 2% suspension of EAC 43 was mixed with 1·5 ml of 2% agarose, and 0·3 ml of serum from a rabbit genetically deficient in C6 (Lachmann, 1970). The gel was poured, test sera applied, and haemolysis measured as in the C4 assay.

Assay of C7. The method based on 'reactive lysis' of non-sensitized cells (E) was used (Lachmann & Thompson, 1970). C56 was separated in the euglobulin fraction from 'acute phase' (post-operative or post-partum) sera previously activated by zymosan. An aliquot (0·2 ml) of the C56 preparation was mixed with 0·3 ml of E (10% suspension), and 1·5 ml of 2% agarose. The gel was poured, test sera applied and haemolysis measured as in the C4 assay.

Conversion products of C4 and C3. These were demonstrated by two-dimensional immuno-electrophoresis (Laurell, 1965). In the first dimension, electrophoresis of test sera was carried out in 2% agarose containing 0·01 M disodium ethylene-diamine tetraacetic acid (EDTA) for 2–3 h at 150 volts. The strip containing the serum proteins was placed on a larger slide the rest of which was then covered with 2% agarose plus antiserum against human C4 or C3, respectively. The second dimension was run at 90° to the first, so that the separated proteins moved into the gel with the antiserum. Precipitin lines formed where optimum antigen-antibody proportions were established.

Esterolytic activities. These were measured in a Radiometer titrator at 37° C and at pH 7·2 or 7·4. Activating agents, suramin solutions, 50 μl of test serum,

and substrates, were added to 0·9% saline so that the total volume was 2 ml. For C1 esterase activation or activity 2–25 mM ATEe was used as substrate (added as 0·5 M ATEe in 2-ethoxy-ethanol). The activation or activities of thrombin, plasmin, or kallikreins were measured using 0·1–20 mM BAEe as substrate.

Plasma clotting. The recalcification clotting time and the kaolin clotting time were measured as described by Margolis (1958).

Fibrinolytic effects. Standard clots consisting of 33% serum, 0·4% bovine fibrinogen, 0·05 M phosphate buffered saline (pH 7·4), activators and inhibitors, were clotted by thrombin, 1 u/ml and the time of lysis recorded.

Kinin formation. The isolated rat uterus was used as described by Eisen (1963). Human plasma heated at 60° C for 60 min was used as kinin-yielding substrate for purified enzymes. The kinin-forming enzyme pre-kallikrein was activated in serum to kallikrein by dilution and by exposure to silicates (glass or kaolin); 10 mM ε-amino caproic acid and heparin 10 u/ml did not reduce the increase in kinin formation or BAEe-splitting, showing that these activities were not due to plasmin or clotting factors.

N-acetyl-L-tyrosine ethyl ester monohydrate (ATEe) and *N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEe) were obtained from British Drug Houses; Alton-Bell extract from Diagnostic Reagents Ltd.; Complement fixation diluent (CFD; isotonic barbitone buffer, pH 7·2) tablets from Oxoid Ltd.; Agar and agarose from Industrie Biologique Française, S.A.; sheep red cells in Alsever's solution and horse anti-sheep haemolytic serum from Wellcome Reagents Ltd.; antisera against human C3 and C4 from Hyland Ltd.

Suramin (Germanin) and highly purified hog pancreatic kallikrein (1080 Frey units/mg) were kindly given by Bayer Ltd., Germany. Human plasmin (40·4 Remmert-Cohen u/ml) was a gift from the Michigan Department of Health.

Human plasma kallikrein was purified by the method of Webster & Pierce (1960).

Results

Haemolytic effects of complement

Fong & Good (1972) reported that suramin 14–15 μg/ml inhibited total human and rat haemolytic complement by 87%, provided the concentration of the drug was maintained until the complement reaction was terminated. Higher concentrations inhibited complement by more than 90%. In the present experiments, 5 μM suramin (7 μg/ml) reduced CH 50 titres by 23–25%, 10 μM (14 μg/ml) by 43–50%, 50 μM (70 μg/ml) by 69–76%, and 100 μM (143 μg/ml) by more than 80%. These reductions of CH 50 were found only if suramin concentrations were kept constant when sera were diluted.

Assays using haemolytic plates suggested that suramin inhibited both the early and late phases of the complement reaction (Figure 1). In the method adapted from an assay for C4 (Lachmann *et al.*, 1973), the agarose contained sensitized cells (EA), C4 deficient serum (R4) and suramin. The serum in the wells supplied C4. In the conditions of the test, suramin could inhibit all steps of the haemolytic reaction.

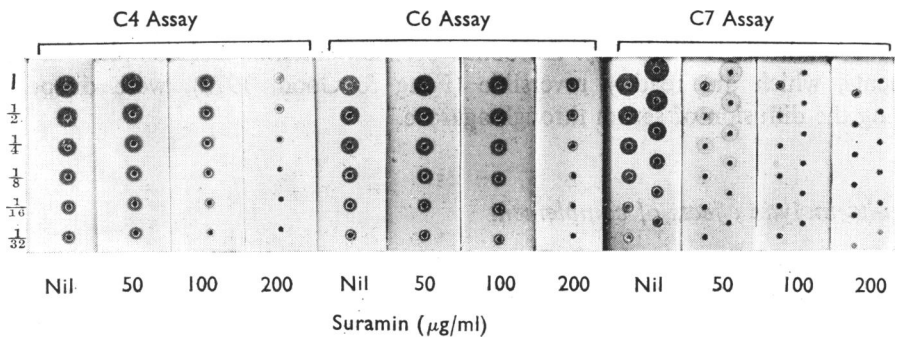

FIG. 1. Assays on haemolytic plates. The action of suramin (concentrations in μg/ml gel at bottom of plates) in modified C4, C6, and C7 assays is shown. Serum dilutions at left margin. Note: in C4 and C6 assays, reductions in zones of lysis are related to suramin concentrations; in C7 assays, suramin 50 μg/ml renders lysis incomplete in undiminished zones, and 100 μg/ml abolishes lysis.

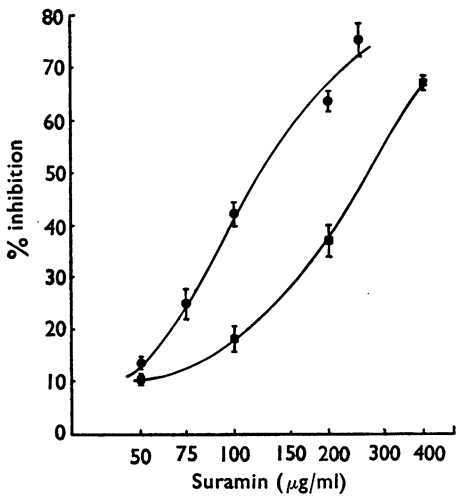

FIG. 2. Suramin dose-response curves in modified haemolytic C4 (●) and C6 (■) assays. Suramin was more potent in C4 assays where it could act on all stages of complement, than in C6 assays where only C3–9 could be inhibited.

In the modified C6 method, agarose was prepared with suramin, EAC 43, and C6 deficient rabbit serum which contributed C5, 7, 8, 9. The serum in the well supplied C6. Suramin could inhibit the C3–9 steps, but not the C1–4–2 steps and proved less potent than in the C4 method (Figure 2).

The reactive lysis (Lachmann & Thompson, 1970) of non-sensitized red cells (E) was also inhibited by suramin. In this method, E, concentrated C56 fragment, and suramin were added to the agarose. C7, 8, 9 diffused from the serum in the well. Possible sites of inhibition were at the C6, 7, 8, 9 steps. In plates containing suramin 50 μg/ml, partial haemolysis was seen in zones of the same diameter as in control plates; 100 μg/ml completely inhibited haemolysis (Figure 1).

In all three haemolytic plate assays, suramin was effective only when present in the agarose; when incubated with the serum which was subsequently placed

into the wells, suramin inhibited only in concentrations of 2·5–10 mg/ml (1·75–7 mM). It is likely that the bonds between suramin and complement components, which are readily reversible (Fong & Good, 1972), were dissociated during the diffusion of serum through agarose.

Non-haemolytic effects of complement

Inhibition of C1 activation in the fluid phase was studied in sera from patients with hereditary angio-oedema (HAO) who are deficient in C1 inhibitor. In a proportion of these sera, spontaneous activation of the C1s subcomponent to C1 esterase is observed (Lundh, Laurell, Wetterqvist, White & Granerus, 1968; Donaldson, 1968). At 37° C, maximum activity (hydrolysis of 0·8–1·5 μmol ATEe per min by 1 ml of serum) is attained in 40–120 min (Eisen & Loveday, 1972). The development of this activity was inhibited by 0·1–2 mM suramin (0·14–2·86 mg/ml); in some experiments, 10–50 μM (14–70 μg/ml) suramin slightly enhanced the development of C1 esterase activity (Figure 3). The inhibition appeared to be directed primarily against the activation of the precursor (C1s). Active C1 esterase isolated from human serum was less effectively inhibited; the

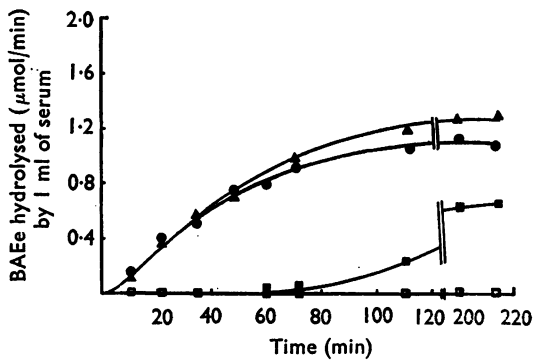

FIG. 3. Spontaneous generation of C1 esterase in HAO serum at 37° C and pH 7·2 (●). Effect of 10 μM (▲), 100 μM (■), and 1 mM (□) suramin.

hydrolysis of 25 mM ATEe was reduced by 0·5 mM suramin to about 70% of the control rate, by 0·2 mM to 85%; inhibition by 0·1 mM suramin was not consistent and ranged from 0–5%. Lineweaver-Burke plots of the hydrolysis of 4–25 mM ATEe (Fig. 4, I) showed that suramin reduced the maximal velocity and increased the apparent K_m, which suggested a mixed competitive and non-competitive mode of inhibition.

C1 activity was also assessed by the conversion of C4 to post-active C4. C1 was activated in normal serum by precipitated cryoglobulins (4·2 mg/ml), and the resulting C4 conversion was demonstrated by two-dimensional immuno-electrophoresis; 70–700 μM suramin (0·1–1·0 mg/ml) had no inhibitory effect.

The conversion of C3 to post-active C3 (β1C to β1A) which also occurs in this system, was equally unaffected by suramin (Figure 5).

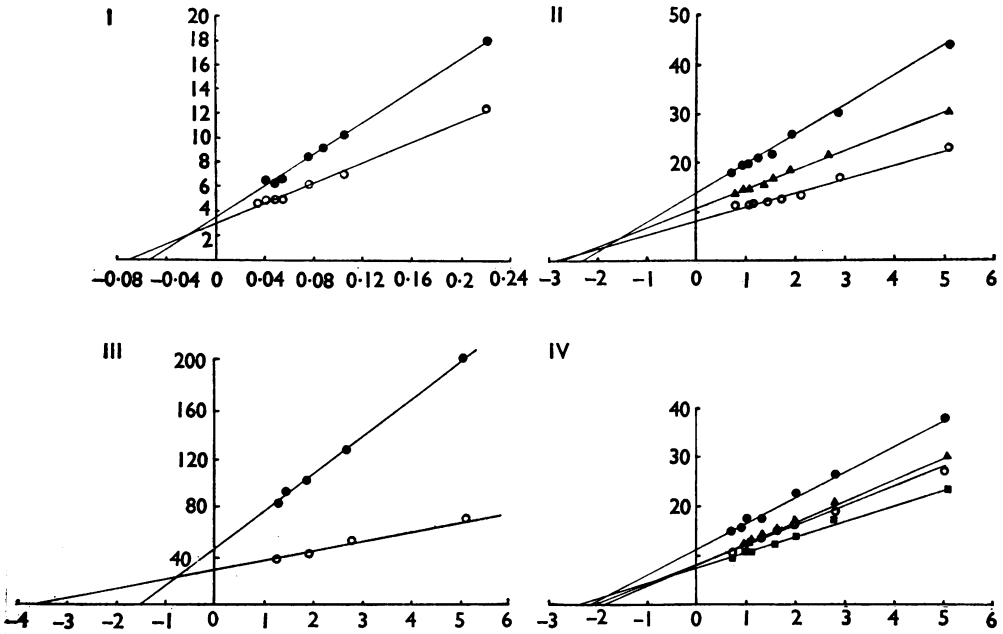

FIG. 4. Effect of suramin on esterolytic activities. Lineweaver-Burke plots: reciprocal of substrate concentration 1/mM on the abscissae and reciprocal of reaction velocity 1/(μmol/min) on the ordinates. Apparant Michaelis-Menten constants at 37° C of the enzyme alone (K_m) and in presence of inhibitor (K_p), and corresponding maximal velocities of reactions (V_{max} and V_p, respectively) were calculated from the least squares regression lines. I. Hydrolysis of ATEe at pH 7·2 by C1 esterase alone (○; K_m=15·55, V_{max}=0·345) and with 0·5 mM suramin (●; K_p=20·0, V_p=0·301). II. Hydrolysis of BAEe at pH 7·4 by plasmin alone (○; K_m=0·352, V_{max}=0·124) and with 0·2 mM suramin (▲; K_p=0·365, V_p=0·093) or 0·5 mM suramin (●; K_p=0·429, V_p=0·071). III Hydrolysis of BAEe at pH 7·4 by human plasma kallikrein alone (○; K_m=0·276, V_{max}=0·059) and with 0·1 mM suramin (●; K_p=0·695, V_p=0·023). IV. Hydrolysis of BAEe at pH 7·4 by hog pancreatic kallikrein alone (○; K_m=0·488, V_{max}=0·121), and with 0·05 mM suramin (■; K_p=0·438, V_p=0·134), 0·2 mM suramin (▲; K_p=0·559, V_p=0·126), or 0·5 mM suramin (●; K_p=0·477, V_p=0·087). Note: The potent inhibition of plasma kallikrein and the weak inhibition of C1 esterase, plasmin, and pancreatic kallikrein were not of the competitive type.

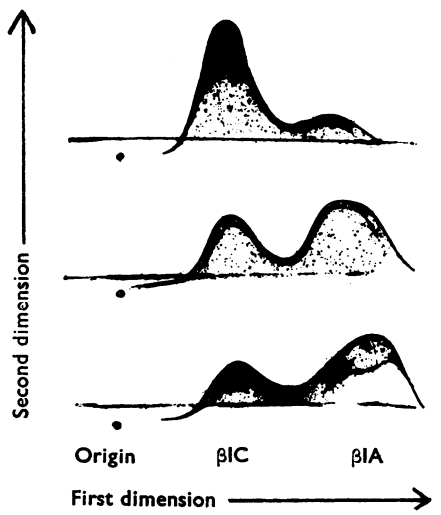

FIG. 5. Activation of C3 by cryoglobulin. Two-dimensional immuno-electrophoresis of pre-active (βIC) and post-active (βIA) component C3. The small proportion of βIA in control serum (I) was increased after incubation with cryoglobulin 4·2 mg/ml serum (II); this was not prevented by suramin 1 mg/ml (III).

Blood coagulation

Suramin prolonged the plasma recalcification clotting time (RCT) more than the kaolin time (KCT), which suggests that the drug acts mainly against the later stages of clotting, and not against the early contact phase involving clotting factors XII and XI. Indeed, a major part of the anti-coagulant effect of suramin must be attributed to inhibition of the terminal clotting stage, the interaction of thrombin and fibrinogen (Table 1).

In contrast to the action on fibrinogen, the hydrolysis by thrombin of 0·1–20 mM BAEe was not inhibited by suramin.

TABLE 1. *Inhibition of blood clotting by suramin.*

A. Effect on recalcification clotting time (RCT) and kaolin clotting time (KCT) of human plasma.

Suramin		*RCT*	*KCT*
mg/ml	(μM)	(s)	(s)
0	(0)	255	90
0·25	(175)	365	90
0·375	(262)	380	115
0·5	(350)	510	130
0·75	(525)	860	140
1·0	(700)	1700	310
1·5	(1·050)	3000	480

B. Effect on clotting of bovine fibrinogen (4 mg/ml) by bovine thrombin (i u/ml).

Suramin		
mg/ml	(μM)	(s)
0	(0)	120
0·087	(62·5)	360
0·175	(125)	1200
0·35	(200)	1800
0·7	(500)	1800

Fibrinolysis

The lysis induced in standard clots (see **Methods**) by streptokinase in final concentrations of 15–70 units/ml was not inhibited by 0·05–0·5 mM suramin (71–715 μg/ml). Since suramin was incubated with plasma before the addition of streptokinase, fibrinogen and thrombin, the results showed that neither the activation of plasminogen nor the activity of plasmin were inhibited by the drug. However, inhibition of both these processes was evident when BAEe was used as substrate. In normal serum, 0·1–0·5 mM suramin depressed the induction of esterolysis by streptokinase (Fig. 6, I). The activity of highly purified human plasmin was less potently inhibited. The inhibition appeared to be largely non-competitive, since suramin altered both V_{max} and K_m (Fig. 4, II).

Plasma kinin formation

The activation of the intrinsic plasma kinin forming system by dilution and/or contact with silicates was inhibited by suramin. The kinin formation developing in human plasma diluted ten times with de Jalon's solution and kept in glass containers was reduced by 0·1 mM suramin to approximately 50% of the rate in the control sample, and by 0·3 mM to less than 20%. The intense activation of the prekallikrein in human serum by kaolin was monitored by the hydrolysis of BAEe which is a substrate for plasma kallikrein ; it was inhibited by 0·1–0·5 mM suramin (Fig. 6, II).

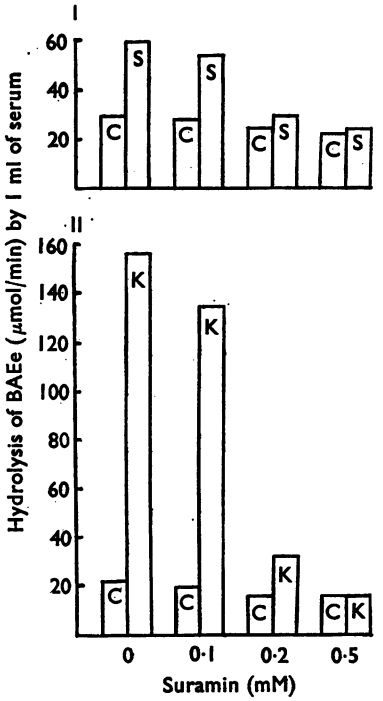

FIG. 6. Inhibition by suramin of plasminogen activation by streptokinase (I), and of plasma prekallikrein by kaolin (II). C=hydrolysis of 5 mM BAEe by 0·1 ml of human serum diluted to 2 ml, at pH 7·4 and 37° C. S=hydrolysis on addition of streptokinase 500 u/ml. K=hydrolysis on addition of kaolin 2 mg/ml.

The same concentration range of suramin was effective against purified active human serum kallikrein. When heated plasma was used as substrate, 0·1 mM suramin had no inhibitory effect; 0·3 mM reduced kinin formation to 70–80% of the control rate, and 0·5 mM to 50%. The hydrolysis of BAEe by purified human serum kallikrein was potently inhibited by 0·1–0·2 mM suramin, and almost abolished by 0·5 mM. The inhibition was not competitive (Fig. 4, III).

The release of kinin from heated human plasma by highly purified hog pancreatic kallikrein was not consistently inhibited by 0·1–0·5 mM suramin. The weak inhibition of BAEe hydrolysis was not of the competitive type (Fig. 4, IV); low concentrations of suramin (25–50 μM) slightly enhanced the splitting of BAEe by pancreatic kallikrein. Human urinary kallikrein was not inhibited by suramin.

Discussion

The present findings suggest that the potency with which suramin inhibits complement and its individual components varies a great deal with the conditions of the assay. The cause of these differences may lie in the reversibility of the suramin-enzyme bonds (Fong & Good, 1972). These bonds are likely to dissociate at different rates in different experimental conditions.

In agreement with Fong & Good (1972), considerable inhibition of the total haemolytic sequence was found when assays were carried out with suspensions of sensitized cells. However, inhibition of this sequence was much weaker when measured by a plate method derived from a C4 assay.

When induced by cryoglobulin, the activation of C1s to C1 esterase and the subsequent conversion of C4, were not inhibited by suramin. In their haemolytic assays Fong & Good (1972) described potent inhibition by suramin and they reported that the activation of C1 esterase was less readily inhibited than its action on C4. The reverse was found in the present esterolytic tests: suramin was more effective against the activation of C1 esterase in HAO serum, than against active C1 esterase.

Similarly, the haemolytic tests by Fong & Good (1972) suggested that the activation of C3 by the C142 complex was inhibited when the C142 complex was adsorbed on to sensitized cells (EAC 142). This is supported by the present finding that suramin is more effective when acting on all stages of the complement reaction, than it is when acting on late stages only. However in immuno-electrophoresis experiments where cryoglobulin was used as the activating agent, suramin did not inhibit the conversion of C3 (β1C to β1A) by C142.

The modified plate assays of C6 and of C7 show that inhibition of the stages C6–C9 undoubtedly contributes to the effect of suramin on the total haemolytic complement reaction. Potent inhibition of C8 by suramin has been reported by Stolfi (1970).

The action of suramin on clotting and fibrinolytic factors also depended on the conditions of the experiments. The fibrinolytic system was not inhibited when acting on its principal natural substrate fibrin. Hydrolysis of the synthetic substrate BAEe was inhibited, the activation of plasminogen being more susceptible than the activity of plasmin.

Suramin inhibited the fibrinogen-fibrin conversion by thrombin, but not its action on BAEe. It is likely that the inhibition of thrombin accounts for only a part of the anticoagulant effect of suramin and that several of the proteolytic steps of blood clotting are inhibited.

The effect on the intrinsic kinin-forming enzymes in plasma differed in that suramin inhibited the actions on both the natural and the synthetic substrates. Moreover, activation as well as activities of plasma kallikrein were inhibited. The fact that pancreatic and urinary kallikrein were far less affected, shows that suramin, like many other inhibitors, acts unequally on different kallikreins.

Sub-inhibitory concentrations of suramin slightly potentiated the esterolytic, but not other actions of the examined proteases. It remains to be seen whether potentiating effects will be evident *in vivo*.

The *in vitro* inhibition of complement activites and plasma kinin formation implies that suramin may be useful in investigating the role of these systems in inflammation and related processes. Such *in vivo* work would then establish whether these inhibitory properties could be used clinically.

We are grateful to Professor C. A. Keele for valuable suggestions and comments. Suramin (Germanin) and hog pancreatic kallikrein were kindly given by Bayer, Germany, and human plasmin by the Michigan Department of Health.

REFERENCES

DONALDSON, V. H. (1968). C1 activation in hereditary angioneurotic oedema plasma: role of urokinase and inhibitors. *J. appl. Physiol.*, **25**, 416–424.

EISEN, V. (1963). Observations on intrinsic kinin-forming factors in human plasma: the effect of acid, acetone, chloroform, heat and euglobulin separation. *J. Physiol. (Lond.)*, **166**, 496–513.

EISEN, V. & LOVEDAY, C. (1972). Activation of arginine and tyrosine esterase in serum from patients with hereditary angio-oedema. *Br. J. Pharmac.*, **46**, 157–166.

EISEN, V. & LOVEDAY, C. (1973). Intrinsic activation of plasma kinin formation and complement. *Int. Arch. Allergy appl. Immunol.*, **45**, 91.

FONG, J. S. C. & GOOD, R. A. (1972). Suramin—a potent reversible and competitive inhibitor of complement systems. *Clin. exp. Immunol.*, **10**, 127–138.

LACHMANN, P. J. (1970). C6-deficiency in rabbits. In: *Protides of the biological fluids.* ed. H. Peeters. pp. 301–310. Oxford, Pergamon Press.

LACHMANN, P. J., HOBART, M. J. & ASTON, W. P. (1973). Complement Technology. In: *Experimental Immunology*, ed. Weir, D. M. pp. 5.1–5.17 Oxford, Blackwell Scientific Publications.

LACHMANN, P. J. & NICOL, P. (1973). Reaction mechanism of the alternative pathway of complement fixation. *Lancet*, **i**, 465–467.

LACHMANN, P. J. & THOMPSON, R. A. (1970). Reactive Lysis: the complement-mediated lysis of unsensitized cells. 1. The characterization of the indicator & its identification as C7. *J. exp. Med.*, **131**, 629–641.

LUNDH, B., LAURELL, A., WETTERQVIST, H., WHITE, T. & GRANERUS, G. (1968). A case of hereditary angioneurotic oedema successfully treated with ε-aminocaproic acid. *Clin. exp. Immunol.*, **3**, 733–745.

LAURELL, C. B. (1965). Antigen-Antibody crossed electrophoresis. *Analyt. Biochem.*, **10**, 358–361.

MARGOLIS, J. (1958). The kaolin clotting time. *J. clin. Path.*, **II**, 406–409.

OSLER, A. G., STRAUSS, J. H. & MAYER, M. M. (1952). Diagnostic complement fixation. 1. A method. *Am. J. Syph. Gonorrhea vener. Dis.*, **36**, 140–154.

STOLFI, R. L. (1970). An analogue of guinea pig C8: In vitro generation and inhibitory activity. *J. Immunol.*, **104**, 1212–1219.

WEBSTER, M. E. & PIERCE, J. V. (1960). Studies on plasma kallikrein and its relationship to plasmin. *J. Pharmac. exp. Ther.*, **130**, 484–491.

WILLS, E. D. & WORMALL, A. (1950). Studies on Suramin. 9. The action of the drug on some enzymes. *Biochem. J.*, **47**, 158–170.

(*Received June* 11, 1973)