[8] Hackney, J. F., Gross, S. R., Aronow, L., and Pratt, W. B., *Molec. Pharmac.*, **6**, 500 (1970).
[9] Thompson, E. B., Tomkins, G. M., and Curran, J. C., *Proc. natn. Acad. Sci. U.S.A.*, **56**, 296 (1966).
[10] Yang, S. S., Lippman, M. E., and Thompson, E. B., *Endocrinology* (in the press).
[11] Pratt, W. B., and Aronow, L., *J. biol. Chem.*, **241**, 5244 (1966).
[12] Lippman, M. E., Halterman, R. H., Leventhal, B. G., Perry, S., and Thompson, E. B., *J. clin. Invest.*, **52**, 1715 (1973).
[13] Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., and Tomkins, G. M., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1892 (1972).
[14] Santi, D. V., Sibley, C., Perriard, E., Tomkins, G. M., and Baxter, J. D., *Biochemistry, N.Y.*, **12**, 2412 (1972).
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randell, R. J., *J. biol. Chem.*, **193**, 256 (1951).

Anticoagulant Action of Heparin

ANTITHROMBIN III (heparin cofactor) is known to inhibit thrombin[1] and Factor X_a[1–3] (activated Factor X). We have purified antithrombin[4] from human plasma by a series of chromatographic and electrophoretic separation techniques. The homogeneity of the final product is demonstrated by disc gel electrophoresis, SDS gel electrophoresis and immunoelectrophoresis[4]. Using this preparation, we have shown that antithrombin forms a 1 : 1 stoichiometric complex with thrombin which cannot be dissociated with denaturing and reducing agents. Addition of heparin dramatically accelerates the rate of formation of this complex. Complex formation is completely dependent on an interaction between the serine active centre of thrombin and an arginine reactive site of antithrombin. Furthermore, ε-amino lysyl groups of antithrombin serve as binding sites for the highly negatively charged heparin. Based on these data and other evidence, we have proposed that heparin acts to accelerate inhibitor function by binding to antithrombin and inducing an allosteric modification in it, which renders the arginine in its reactive site more accessible to the serine in the active centre of thrombin[4].

This mechanism suggested that all serine proteases within the coagulation cascade may be neutralised in a similar manner. To test this hypothesis we have examined the interaction of antithrombin and heparin with Factor XI_a (activated Factor XI), another serine protease[5] generated during coagulation, whose physical properties and substrate specificity differ considerably from those of Factor X_a and thrombin.

Factor XI_a was purified from human plasma by DEAE-cellulose chromatography, celite adsorption-elution, hydroxyapatite chromatography, and polyacrylamide P-150 gel filtration according to the method of Amir *et al.*[6] with minor modifications. The final product is free of coagulation Factors I, II, V, VII, VIII, IX and X as judged by fibrinogen determinations[7] and one-stage assays using congenitally deficient plasmas as substrates[8].

To ascertain whether antithrombin inactivates Factor XI_a, antithrombin was incubated with Factor XI_a at final concentrations of 64 units ml^{-1} and 100 units ml^{-1} respectively, and residual Factor XI_a activity was sequentially measured as a function of time. As shown in Fig. 1, there is a progressive decline in Factor XI_a activity. No significant reduction in this activity is, however, observed when buffer is substituted for antithrombin. When the neutralisation of Factor XI_a activity is plotted in semi-log fashion (not shown), the kinetics closely approximate those of a first order reaction.

To determine whether antithrombin is responsible for a significant proportion of the plasma's ability to inhibit Factor XI_a, we removed antithrombin from plasma by specific immunoprecipitation and measured the residual capacity of these samples to neutralise this activated intermediate. Plasmas deficient in C$\bar{1}$ esterase inhibitor (C$\bar{1}$INH) were employed in these experiments, since it is known to be responsible for 20% of the plasma's Factor XI_a inhibitory capacity[19] and activation of the complement system during immunoprecipitation would result in its reduction. As shown in Table 1, specific immunoprecipitation of 71–80% of plasma antithrombin by a γ-globulin concentrate directed against this protein produces, in all cases, corresponding declines in the ability of these samples to inhibit Factor XI_a. In each instance, non-specific effects of γ-globulin addition have been accounted for by comparing these results with duplicate samples to which we have added a "bland" γ-globulin concentrate obtained from non-immunised rabbits. From these data we estimate that antithrombin is responsible for about 50% of these plasmas' capacity to neutralise Factor XI_a. Using the data of Ratnoff *et al.*[10], on the relative importance of plasma C$\bar{1}$INH as an antagonist of Factor XI_a, we suggest that antithrombin is responsible for about 40% of the Factor XI_a inhibitory capacity of normal plasma, with the residual 40% inhibitory capacity due perhaps to the inhibitor recently isolated by Amir *et al.*[11].

These three plasma protease inhibitors only slowly neutralise Factor XI_a activity. If the antithrombin-Factor XI_a interaction

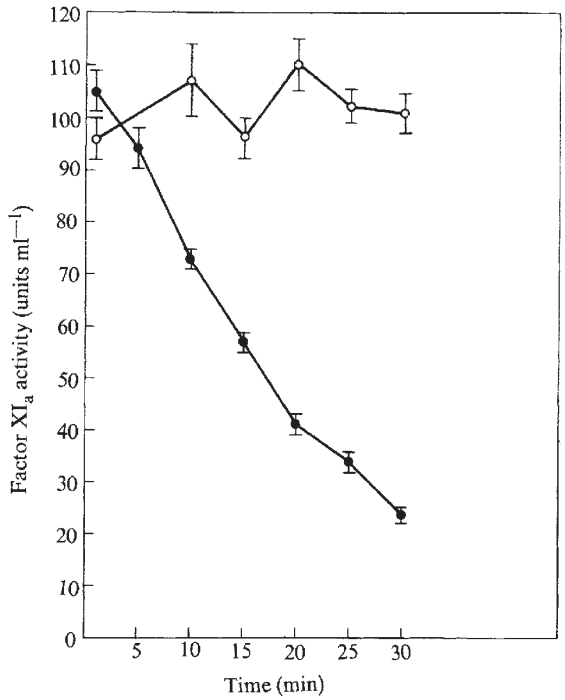

Fig. 1 Inhibition of Factor XI_a by antithrombin. Factor XI_a was incubated with buffer or antithrombin in albuminised plastic tubes at 37° C. 0.02 ml of Factor XI_a was added to either 0.38 ml of 0.15 M NaCl in 0.01 M Tris (*p*H 7.5) or 0.18 ml of this buffer and 0.2 ml antithrombin. The final concentrations of Factor XI_a and antithrombin used were 100 units ml^{-1} (0.002 AU ml^{-1}) and 64 units ml^{-1} (0.060 AU ml^{-1}), respectively. Mixtures were separately incubated for varying periods of time and assayed in triplicate for residual Factor XI_a. Each point represents the mean obtained for two separate incubation mixtures ±s.e.m. ○—○, Factor XI_a+buffer; ●—●, factor XI_a+antithrombin. Factor XI_a activity was measured by a modification of the method of Nossel[21] utilising cephalin[22] and plasmas congenitally deficient in this coagulation protein. The initial concentrations of Factor XI_a preparations were estimated by comparison with the Factor XI content of pooled plasma obtained from twenty-five normal subjects. Assays of Factor XI levels in plasma were performed by the method of Rapaport *et al.*[23] with the mean value of this plasma component arbitrarily set at 100 units ml^{-1}. Antithrombin (heparin cofactor) activity was determined by a modification of the method of Gerandas[24] in the absence of heparin or by the method of Abildgaard[9] in the presence of heparin. Purified human thrombin[25] (3,200 NIH units mg^{-1}) was employed in both types of activity measurements. The concentration of antithrombin was also obtained by immunological assay using the technique of Laurell[26] with monospecific antisera prepared by immunizing rabbits with the purified protein. Activity and immunological measurements were in excellent agreement and were compared to a pool of twenty-five normal plasmas. Unless otherwise indicated, the concentrations of antithrombin cited represent an average of all types of determinations. The mean concentration of antithrombin was arbitrarily set at 100 units ml^{-1}.

were to be dramatically accelerated by heparin, then the addition of this anticoagulant to plasma would transform antithrombin from a plasma inhibitor of some importance to the primary antagonist of Factor XI_a action. To test this possibility we examined the time-dependent decay of Factor XI_a in the presence of antithrombin and heparin.

Methodological problems make such measurements difficult. Heparin is known to inhibit (through antithrombin) the action of Factor X_a on prothrombin[1-2] as well as that of thrombin on fibrinogen[1], and a valid determination of the proteolytic activity of Factor XI_a depends on the degree to which this species triggers these terminal reactions of the coagulation cascade. Therefore heparin as well as antithrombin–heparin complexes must be removed from incubation mixtures before assaying them for Factor XI_a. Well-recognised approaches to inhibiting heparin's action such as protamine and heparinase were initially examined. Protamine was found not only to inhibit heparin but also to reduce directly the activity of Factor XI_a. Heparinase at concentrations of 0.13 mg of crude enzyme ml^{-1} (ref. 12) was shown to rapidly destroy free heparin but only slowly affect heparin bound to antithrombin. The addition of 9 mg of DEAE-cellulose to test incubations, centrifugation of these mixtures and use of the supernatants, proved, however, to be a rapid means of selectively eliminating free or bound heparin without altering the level of Factor XIa.

Several experiments demonstrated the validity of this approach: First, when heparin and Factor XI_a were incubated together and processed in the above fashion, no significant decrease in Factor XI_a activity was detected (Fig. 2). Second, when antithrombin and heparin were incubated together for 5 min, the mixtures treated with DEAE-cellulose, and the resulting supernatants incubated with Factor XI_a, only a minimal reduction in Factor XI_a activity was seen (Fig. 2). Third, when Factor XI_a was incubated with buffer and treated with DEAE-cellulose, the supernatants obtained showed no significant reduction in the initial Factor XI_a activity (Fig. 2).

To test whether heparin accelerates the interaction between Factor XI_a and antithrombin, mixtures of antithrombin, heparin, and Factor XI_a at final concentrations of 36 units ml^{-1}, 0.7 units ml^{-1}, and 100 units ml^{-1}, respectively, were incubated together for 1, 3, or 5 min before the addition of DEAE-cellulose. After centrifugation, the respective supernatants were analysed and Factor XI_a activity was found to be virtually absent in all incubation mixtures (Fig. 2). In other experiments (not shown), shorter periods of incubation (0.1 to 0.5 min) revealed the same degree of Factor XI_a inhibition. These results are to be compared with those obtained when the inactivation was examined in identical conditions, but with the omission of heparin. In these mixtures only a minimal time-dependent reduction in Factor XI_a activity was observed (Fig. 2). Therefore heparin dramatically accelerates the formation of a Factor XI_a-antithrombin complex. On the basis of the neutralisation of Factor XI_a by antithrombin in the absence of heparin, as well as our knowledge of the mechanism of inhibitor action, we may confidently presume that inactivation of Factor XI_a follows this interaction.

Thus Factor XI_a, like Factor X_a and thrombin, is a serine protease which is slowly but progressively inhibited by antithrombin in the absence of heparin and virtually instantaneously neutralised by antithrombin in the presence of this anticoagulant. On the basis of these data and the known mechanism of action of antithrombin[4], we predict that antithrombin will be shown to inactivate the other remaining serine proteases of the coagulation cascade, Factor VII_a (ref. 13), Factor IX_a (ref. 14) and Factor XII_a (ref. 15), and that heparin will accelerate each of these interactions. Indeed, Aronson, using antithrombin prepared by us, has already confirmed this hypothesis for Factor IX_a (personal communication).

Furthermore this linkage of the anticoagulant action of heparin to the early "contact phase" of the coagulation cascade suggests that antithrombin may play a significant role in maintaining nonthrombogenic properties of artificial as well as natural surfaces. When heparin is covalently bonded to a variety of materials, they prove to be highly thromboresistant[16,17]. It is probable that they owe this property to "activated" antithrombin, bound at the blood-surface interface, which can rapidly neutralise serine proteases generated during the initial contact phase of clotting and thus abort

Table 1 The Factor XI_a Inhibitory Activity in C1INH-deficient Plasmas Depleted of Antithrombin

Sample	Initial antithrombin concentration (units ml^{-1})	Initial C1INH concentration (units ml^{-1})	% Antithrombin-heparin cofactor activity removed by immunoprecipitation	Factor XI_a removed after 15 min of incubation at 37° C (units ml^{-1})
Buffer	—	—	—	7
C1INH-deficient plasma 1	100	8.7		
a +γ-globulin concentrate directed against antithrombin			80	56
b +"bland" γ-globulin concentrate			0	75
C1INH-deficient plasma 2	100	12.0		
a +γ-globulin concentrate directed against antithrombin			71	39
b +"bland" γ-globulin concentrate			0	73
C1INH-deficient plasma 3	110	19.0		
a t+γ-globulin concentrate directed against antithrombin			76	55
b+"bland" γ-globulin concentrate			0	70

Plasmas deficient in C1INH were obtained from three patients with hereditary angioneurotic oedema using two-syringe technique, plastic vessels and sodium citrate as anticoagulant (0.38% final concentration). They were individually assayed for antithrombin (see legend to Fig. 1) and C1INH by the technique of Laurell with monospecific sera (normal range: 44–177 units ml^{-1}). Samples were then stored at −70° C. Sera were obtained from rabbits immunised with purified antithrombin as well as rabbits which had not been immunised. The γ-globulin fractions were prepared by the method of Stanworth[27], concentrated to 100 AU ml^{-1}, dialysed against 0.15 M NaCl in 0.01 M Tris (*p*H 7.5) and heated to 60° C for 1 h. These γ-globulin concentrates did not inhibit Factor XI_a nor did they exhibit antithrombin or heparin cofactor activity. Monospecificity of the γ-globulin concentrate directed against antithrombin was demonstrated by immunoelectrophoresis and Ouchterlony double diffusion. C1INH deficient plasmas (0.3 ml) were added to both types of γ-globulin concentrates (0.9 ml) and the mixtures were incubated for 30 min at 37° C and 90 min at 4° C. The samples were centrifuged at 15,000*g* and 4° C for 20 min. The resulting supernatants were assayed for residual heparin cofactor activity (measurement of antithrombin activity in the presence of heparin, see legend to Fig. 1). In addition, residual Factor XI_a inhibitory activity was determined by adding 0.2 ml of supernatant to 0.18 ml of 0.15 M NaCl in 0.01 M Tris (*p*H 7.5) and 0.02 ml of Factor XI_a (final concentration of 100 units ml^{-1}). After 15 min of incubation at 37° C, the mixtures were assayed in triplicate for Factor XI_a activity (see legend to Fig. 1). Controls were obtained for the same period of incubation by replacing the supernatants with 0.15 M NaCl in 0.01 M Tris (*p*H 7.5). Figures given represent the means obtained for duplicate incubation mixtures prepared on two separate days. The relative Factor XI_a inhibitory capacity of plasmas (not given) was estimated by a plot of plasma dilution against the log of the residual Factor XI_a activity at 15 min.

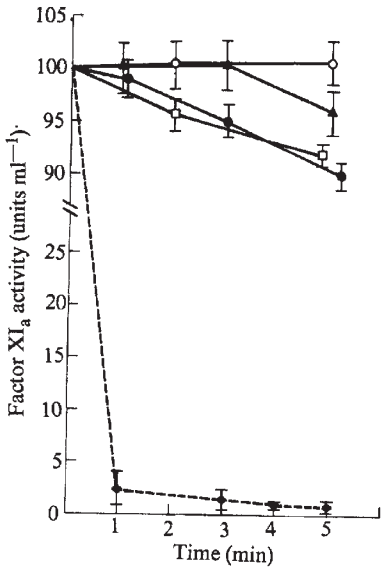

Fig. 2 Inhibition of Factor XI_a by antithrombin and heparin. Two distinct sequences of addition of Factor XI_a to other components were employed to obtain the above data. The first incubation sequence used duplicate samples of 1.6 ml composed of Factor XI_a added to either buffer, heparin, antithrombin or antithrombin and heparin. In this instance, Factor XI_a was employed at a final concentration of 100 units ml^{-1} while heparin and antithrombin, if present, were used at final concentrations of 0.7 units ml^{-1} and 36 units ml^{-1}, respectively. All samples were incubated for 1–5 min at 37° C, dried DEAE-cellulose (9 mg) was added with gentle agitation, the mixtures were immediately centrifuged at 15,000*g* for 30 s and the resulting supernatants were assayed in triplicate for residual Factor XI_a activity. The second incubation sequence used duplicate samples of 1.2 ml consisting of antithrombin and heparin at final concentrations of 48 units ml^{-1} and 0.93 units ml^{-1}, respectively. Both samples were incubated at 37° C for 5 min, dried DEAE-cellulose (9 mg) was added with gentle agitation, the mixtures were centrifuged at 15,000*g* for 30 s and the resulting supernatants were obtained. Factor XI_a was added to these supernatants in a 1 : 3 (v/v) ratio such that its final concentration was 100 units ml^{-1}; the samples were incubated at 37° C for 2–5 min, and the residual Factor XI_a activities were determined in triplicate. Values given represent the mean Factor XI_a activity ±s.e.m. In all cases, components have been either dialysed against or extensively diluted in buffer (0.15 M NaCl in 0.01 M Tris, *p*H 7.5). ○—○, Factor XI_a + buffer; ▲—▲, factor XI_a + heparin; □—□, factor XI_a + heparin-antithrombin-treated supernatant; ●—●, factor XI_a + antithrombin; ◆—◆, factor XI_a + heparin + antithrombin.

the production of the terminal serine proteases responsible for thrombin formation. By analogy, since heparin has been isolated from vessel walls[18,19], as well as other organs[20], it may prove fruitful to reinvestigate vascular endothelial thromboresistance in terms of heparin or heparin-like material bound to surfaces. The presence of such substances coupled with the unique properties of antithrombin suggests that this inhibitor is the critical barrier against activated coagulation factors at the vessel wall interface and thus provides a crucial link between surface properties and the coagulability of blood.

We thank Drs C. Alper and F. Rosen, Children's Hospital, Boston, Massachusetts, for providing the CĪNH deficient plasmas and measuring their residual CĪNH concentrations. We also thank Dr H. S. Kingdon, University of Chicago, for his generous gift of partially purified heparinase and the Platelet Laboratory, Children's Hospital, Boston, Massachusetts, for providing human plasma.

This work was supported by grants from the National Institutes of Health.

PAUL S. DAMUS
MICHAEL HICKS
ROBERT D. ROSENBERG

Departments of Medicine of the Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts

Received July 9; revised September 7, 1973.

1 Dambrose, F. A., Seegers, W. H., and Sedensky, J. A., *Thromb. Diath. haemorrh.*, **26**, 1 (1971).
2 Higgs, R. K., Denson, W. E., and Akman, N., *Br. J. Haemat.*, **19**, 283 (1970).
3 Yin, T. K., and Wessler, S., *J. biol. Chem.*, **246**, 3707 (1971).
4 Rosenberg, R., and Damus, P. S., *J. biol. Chem.*, **248**, 6490 (1973).
5 Kingdon, H. S., Davie, E. W., and Ratnoff, O. D., *Biochemistry*, **3**, 166 (1964).
6 Amir, J., Pensky, J., and Ratnoff, O. D., *J. lab. clin. Med.*, **79**, 106 (1972).
7 Ratnoff, O. D., and Menzie, C., *J. lab. clin. Med.*, **37**, 316 (1951).
8 Hardisty, R. M., and McPherson, J. C., *Thromb. Diath. haemorrh.*, **7**, 215 (1962).
9 Abildgaard, U., *Thromb. Diath. haemorrh.*, **24**, 224 (1970).
10 Ratnoff, O. D., Pensky, J., Donaldson, V. H., and Amir, J., *J. lab. clin. Med.*, **80**, 803 (1972).
11 Amir, J., Ratnoff, O. D., and Pensky, J., *J. lab. clin. Med.*, **80**, 786 (1972).
12 Hutt, E. D., and Kingdon, H. S., *J. lab. clin. Med.*, **79**, 1027 (1972).
13 Nemerson, Y., and Esnouf, M. P., *Proc. nat. Acad. Sci. U.S.A.*, **70**, 310 (1973).
14 Davie, E. W., and Ratnoff, O. D., *The Proteins*, **3**, chap. 16 (Academic Press, N.Y., 1965).
15 Wuepper, K. D., and Cochrane, C. G., *J. exp. Med.*, **135**, 1 (1972).
16 Gott, U. L., Whitten, J. D., and Dutton, R. C., *Science, N.Y.*, **142**, 1297 (1963).
17 Wong, P. S. L., Merrill, E. W., and Salzman, E. W., *Fedn Proc.*, **28**, 441 (1969).
18 Kirk, J. E., *Nature*, **184**, 369 (1959).
19 Gore, I., and Larkey, B. J., *J. lab. clin. Med.*, **56**, 839 (1960).
20 McLean, J., *Am. J. Physiol.*, **41**, 250 (1916).
21 Nossel, H. G., *The Contact Phase of Blood Coagulation* (Blackwell, Oxford, 1964).
22 Bell, W. N., and Alten, H. G., *Nature*, **174**, 880 (1954).
23 Rapaport, S. I., Schiffman, S., and Patch, M. J., *J. lab. clin. Med.*, **59**, 771 (1961).
24 Gerandas, M., *Thromb. Diath. haemorrh.*, **4**, 56 (1960).
25 Rosenberg, R. D., and Waugh, D. F., *J. biol. Chem.*, **245**, 5049 (1970).
26 Laurell, C. B., *Analyt. Biochem.*, **15**, 45 (1966).
27 Stanworth, R., *Nature*, **188**, 156 (1960).

Perception of Polarised Light in Insects by Filter Mechanism

THE response of a single visual cell of an insect, intracellularly recorded[1–3], to polarised light (PL) is relatively small but, as is generally known, insects display a great capability of orientating to polarised light. Here I suggest that this contradiction can be explained with the aid of a filter mechanism.

My theory is based on some well known facts. Many insects have so-called tiered retinulas with one or more basal (or proximal) visual cells and both theoretical[4] and experimental[5,6] results indicate that the microvilli of rhabdomeres exhibit dichroism with a dichroic ratio of 2. Furthermore, the longer the dichroic rhabdomere, the lesser its dichroic ratio, which is 1 in the limit because of 100% absorption for any orientation of the E vector. Thus, a long rhabdomere could hardly act as a PL detector in the eye of an insect.

The principal idea presented here is that a distal rhabdomere serves as a PL filter (analyser) for linearly polarised light from blue sky, whereas the proximal one absorbs or measures the intensity of PL transmitted by the distal rhabdomere (Fig. 1).

Let the intensity of incident PL be I_0. When the E vector of PL is parallel to the long axes of the microvilli, that is, when it is orientated in the x direction, the intensity of light transmitted by the distal rhabdomere and entering the proximal rhabdomere will be $I_{x_d} = I_0 \exp(-x_d) = I_0 \exp(-kl_d)$ where k is the absorption coefficient and l_d is the length of the distal rhabdomere. In turn, $k = 2.3\ \varepsilon c_x$ where ε is the molar extinction coefficient, c_x is the concentration of photopigment molecules orientated in the x direction, and 2.3 is the constant derived from changing from the generally used extinction coefficient $K = \varepsilon c_x$ to the absorption coefficient (k) used here; that is

$$I_{x_d} = I_0 \exp(-2.3\ \varepsilon\ c_x l_d) = I_0 \times 10^{-\varepsilon c_x l_d}$$