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KINETICS OF REACTION OF HUMAN C1-INHIBITOR WITH THE HUMAN COMPLEMENT SYSTEM PROTEASES C1r and C1s

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Summary

The interactions of the two classical serine proteases of the complement system with their natural inhibitor, C1-inhibitor, have been studied. C1r and C1s react with C1-inhibitor to form complexes which contain 1 mol of C1-inhibitor per mol of protease monomer. The complexes are not degraded in the presence of excess protease, and are not dissociated by strong denaturing agents. Rate constants and dissociation constants for these reactions fall within the normal range for protease-protease inhibitor interactions. The affinity of both proteases for C1-inhibitor is similar, but C1s reacts more rapidly than does C1r. The presence of Ca^{2+} decreases the rate at which C1r complexes with C1-inhibitor, but does not affect the reactivity of C1s. The C1r-(C1-inhibitor) reaction is also inhibited by extremes of ionic strength, and has a more marked temperature-dependence than the C1s-(C1-inhibitor) interaction.

Heparin stimulates the rate of the reaction of C1s with C1-inhibitor by a factor of 14—15. The C1r-(C1-inhibitor) interaction is also accelerated by heparin, but the effect is much smaller than for C1s. Neither protease is inhibited by heparin alone. In contrast to the effect of heparin, flufenamic acid was found to inhibit the action of C1-inhibitor.

The incorporation of C1r into C1 bound to immune complexes was found to increase the reactivity of C1r towards C1-inhibitor. C1s reactivity, however,

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Abbreviations: DIP-C1s, DIP-C1r, respectively, C1s and C1r which have been totally inactivated with diisopropyl phosphorofluoridate; EDTA, ethylenediaminetetraacetic acid.

The nomenclature of the components of complement is that recommended by the World Health Organization [Bull. W.H.O. (1968) 39, 935—936]. An enzymically active component is indicated by a superscript bar, e.g. C1r.

was not stimulated by this treatment. The enhancement of $C\bar{1}r$ activity by strong binding to antibody-antigen-C1q complexes and to $C\bar{1}s$, parallels earlier work (Dodds, A.W., Sim, R.B., Porter, R.R. and Kerr, M.A. (1978) Biochem. J. 175, 383—390) on the activation of the C1r proenzyme, and demonstrates that the other components of the antibody-antigen-C1 complex act as modifiers of the activities of both activated and proenzymic C1r.

Introduction

The humoral immune defence system, complement, is activated by a number of stimuli associated with entry of foreign material into the circulation. One mode of activation of complement, the interaction of the first component of complement, C1, with antibody-antigen complexes, is now relatively well understood [1]. $C\bar{1}$ is a glycoprotein complex consisting of three distinct types of subcomponent, C1q, C1r and C1s, bound together in the presence of Ca^{2+}. Binding of the C1q subunit in C1 to antibody in immune complexes leads to sequential proteolytic activation of the C1r and C1s subunits [2]. The activated forms of these subunits, $C\bar{1}r$ and $C\bar{1}s$, are both serine proteases [3], and $C\bar{1}s$ initiates the proteolytic activation of subsequent components in the complement reaction sequence [1].

Once activated, these two proteases, like other circulating plasma proteases, become subject to control by endogenous protease inhibitors. $C\bar{1}r$ and $C\bar{1}s$ are both inhibited by $C\bar{1}$-inhibitor [4,5], a well-characterised plasma protease inhibitor first isolated by Schultze et al. [6]. It has been shown that inhibition of $C\bar{1}r$ and $C\bar{1}s$ by $C\bar{1}$-inhibitor is accompanied by formation of a complex, containing 1 molecule of $C\bar{1}$-inhibitor per molecule of protease. The complexes formed do not dissociate in sodium dodecyl sulphate and urea [7—9]. Recent studies indicate that $C\bar{1}$-inhibitor is the only plasma protease inhibitor which reacts with $C\bar{1}r$ and $C\bar{1}s$ [10].

Qualitative studies of the formation of complexes between isolated $C\bar{1}s$ and $C\bar{1}$-inhibitor have been reported [8], but little further information is available on the reaction of isolated $C\bar{1}r$ with $C\bar{1}$-inhibitor. $C\bar{1}$-inhibitor may be regarded as a pseudo-substrate of $C\bar{1}r$ and $C\bar{1}s$, and although these two proteases are very similar in size, structure and mode of proteolytic activation [3,11,12] they are distinct in their proteolytic substrate specificities [11,12]. It is therefore likely that differences in their interaction with $C\bar{1}$-inhibitor will exist. The present study was undertaken to compare directly and quantitatively the characteristics of the reactions between each protease and $C\bar{1}$-inhibitor.

Materials and Methods

Materials

Human citrated plasma was obtained from the Centre de Transfusion Sanguine, Grenoble. Serum was prepared and stored as in Ref. 9. The sources of commercial products were as follows: $Na^{125}I$, The Radiochemical Centre, Amersham, Bucks, U.K.; lactoperoxidase (Grade B), Calbiochem, San Diego, CA, U.S.A.; materials for polyacrylamide gels, sodium dodecyl sulphate and

iodoacetamide, Merck, Darmstadt, F.R.G.; *N*-α-carbobenzoxy-L-lysine *p*-nitrophenol ester, Interchim, Montluçon, France; heparin (sodium salt), Fluka A.G., Buchs, Switzerland; flufenamic acid (2-{[3-(trifluoromethyl)-phenyl]amino}-benzoic acid), Aldrich-Europe, Beerse, Belgium, hen ovalbumin, Sigma, St. Louis, MO, U.S.A. Other reagents and chemicals were from Merck or from Prolabo, Paris, France.

Proteins

C$\bar{1}$r and C$\bar{1}$s were isolated by either of two methods [3,13] which yield products equivalent in purity and activity. C$\bar{1}$-inhibitor was prepared by the method of Reboul et al. [7]. C1q was isolated as described in Ref. 13. C$\bar{1}$r and C$\bar{1}$s, totally inactivated by diisopropyl phosphorofluoridate (DIP-C$\bar{1}$r, DIP-C$\bar{1}$s), were formed as in Ref. 2.

Immune complexes containing hen ovalbumin and rabbit-antihen ovalbumin IgG antibody were prepared as previously described [9,12].

Isolated proteins in solution were quantified from their specific absorbance at 280 nm [9]. Molecular weight estimates used to calculate molar concentrations were: C1q, 410 000 [1]; C$\bar{1}$r or C$\bar{1}$s monomer, 83 000 [3]; C$\bar{1}$-inhibitor, 100 000 [7] and rabbit IgG, 150 000.

Protein iodination

C$\bar{1}$r and C$\bar{1}$s were labelled with ^{125}I by lactoperoxidase catalysis [10] using an Na^{125}I solution diluted to a specific activity of 500 μCi/ml. This iodination method has been shown to be without effect on the enzymic activities of C$\bar{1}$r and C$\bar{1}$s, and does not alter the rate at which they react with C$\bar{1}$-inhibitor [10].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Electrophoresis in 5.6% (w/v) polyacrylamide gels in buffers containing sodium dodecyl sulphate was done as described by Fairbanks et al. [14]. Preparation of samples for electrophoresis, staining of gels with Coomassie blue, scanning of stained gels, and slicing of gels for determination of radioactivity were done as before [15].

Spectrophotometric assay of C$\bar{1}$s and estimation of C$\bar{1}$-inhibitor activity

The *N*-α-carbobenzoxy-L-lysine *p*-nitrophenol esterase activity of C$\bar{1}$s was determined at pH 6.0 [2,11] using a Beckman Acta III or Leres spectrophotometer, maintained at 37°C by means of a Huber Ministat water circulator. Linear increase in absorbance at 340 nm was measured continuously for 3 min.

To determine the inhibitory activity of C$\bar{1}$-inhibitor towards C$\bar{1}$s, samples of C$\bar{1}$s were incubated with C$\bar{1}$-inhibitor in 5 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, containing 5 mM $CaCl_2$ or 5 mM EDTA. At various times a portion (50—100 μl) of the incubation mixture was withdrawn and diluted to 3.0 ml with 100 mM sodium phosphate, 100 mM NaCl, 15 mM EDTA, pH 6.0. The residual C$\bar{1}$s esterase activity in the 3 ml sample was then measured as noted above. Dilution of the C$\bar{1}$s + C$\bar{1}$-inhibitor mixture and lowering of the pH to 6.0 reduces the rate of the C$\bar{1}$-inhibitor-C$\bar{1}$s interaction to negligible values, permitting an accurate measurement of the remaining C$\bar{1}$s activity.

Measurement of the extent of complex formation between C$\bar{1}$r or C$\bar{1}$s and C$\bar{1}$-inhibitor

The extent of inhibition of C$\bar{1}$s by C$\bar{1}$-inhibitor can be measured conveniently and rapidly by estimating C$\bar{1}$s esterase activity, as described above. C$\bar{1}$r, however, has only very weak esterase activity [11,16,17] and no rapid, direct assay for C$\bar{1}$r esterase activity is available. Therefore, in order to compare directly the rates of interaction of C$\bar{1}$r and C$\bar{1}$s with C$\bar{1}$-inhibitor, the rates of formation of the sodium dodecyl sulphate and urea-stable complexes between the two proteases and C$\bar{1}$-inhibitor were measured. The formation of such complexes has been shown to correlate with inhibition of the proteases [9] and so measurement of complex formation is essentially equivalent to measurement of inhibition.

Samples of ^{125}I-labelled C$\bar{1}$r or of ^{125}I-labelled C$\bar{1}$s were incubated with C$\bar{1}$-inhibitor in 5 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, containing 5 mM $CaCl_2$ or 5 mM EDTA. At various times, portions of 50 μl were withdrawn from the incubation mixture, and diluted immediately with 50 μl of 200 mM Tris-HCl, 8 M urea, 2% (w/v) sodium dodecyl sulphate, 40 mM iodoacetamide, pH 8.0, and incubated for 4 min at 100°C. The samples were then loaded onto sodium dodecyl sulphate polyacrylamide gels, and the complexes of radiolabelled C1r or C$\bar{1}$s with C$\bar{1}$-inhibitor were separated from free, unreacted C$\bar{1}$r, C$\bar{1}$s and C$\bar{1}$-inhibitor by electrophoresis. Gels were stained with Coomassie blue, destained and sliced into 2 mm segments. Radioactivity in the slices was measured on an Intertechnique CG2000 gamma-counter. The progress of reaction between C$\bar{1}$r or C$\bar{1}$s and C$\bar{1}$-inhibitor was calculated from the proportion of the ^{125}I radioactivity associated with the C$\bar{1}$r-(C$\bar{1}$-inhibitor) or C$\bar{1}$s-(C$\bar{1}$-inhibitor) complexes at various incubation times, as described previously [10,15]. The specific radioactivity of the ^{125}I-labelled C$\bar{1}$r or C$\bar{1}$s was adjusted so that each polyacrylamide gel was loaded with 2500—3000 cpm.

Calculation of kinetic constants for the reaction of C$\bar{1}$r or C$\bar{1}$s with C$\bar{1}$-inhibitor

The overall rates of the reactions shown in Eqn. 1

$$\begin{aligned} &C\bar{1}r + C\bar{1}\text{-inhibitor} \xrightarrow{k_1} C\bar{1}r\text{-}(C\bar{1}\text{-inhibitor}) \\ &C\bar{1}s + C\bar{1}\text{-inhibitor} \xrightarrow{k_1} C\bar{1}s\text{-}(C\bar{1}\text{-inhibitor}) \end{aligned} \quad (1)$$

were determined experimentally as described above, using ^{125}I-labelled C$\bar{1}$r or C$\bar{1}$s, and examining complex formation by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Rates of protease-protease-inhibitor complex formation were studied at pH 7.4 and 37°C in the presence of Ca^{2+} or of EDTA, by incubating mixtures of different concentrations of ^{125}I-labelled-C$\bar{1}$r or C$\bar{1}$s and C$\bar{1}$-inhibitor. The concentration ranges examined were: C$\bar{1}$r, 0.2—4.9 μM (16.6—407 μg/ml); C$\bar{1}$s, 0.2—4.4 μM (16.6—366 μg/ml) and C$\bar{1}$-inhhibitor, 1.2—10.2 μM (120—1022 μg/ml). The progress of the reaction was found to conform to the second-order rate equation (Eqn. 2)

$$v = k_1 [P] [I] \quad (2)$$

where v is the reaction velocity and k_1 is the rate constant of association. [P]

and [I] represent the concentrations of the protease and of C$\bar{1}$-inhibitor, respectively. The kinetic constant, k_1, was calculated by fitting data to the integrated rate equation as described in [18]. This equation is of the form

$$\log_{10}\frac{P_0(I_0 - C)}{I_0(P_0 - C)} = k_1 t\frac{I_0 - P_0}{2.303} \quad (3)$$

where P_0, I_0, represent the concentrations of protease or of C$\bar{1}$-inhibitor, repectively, at time zero, and C is the concentration of protease-(protease-inhibitor) complex at time t.

Calculation of the dissociation constant for the reaction of C$\bar{1}$r or C$\bar{1}$s with C$\bar{1}$-inhibitor

The dissociation constant, K_D, for the reaction between C$\bar{1}$r or C$\bar{1}$s and C$\bar{1}$-inhibitor was determined in a manner similar to that described by Wiman and Collen [19]. ^{125}I-labelled C$\bar{1}$r or C$\bar{1}$s (1.2 μM, 100 μg/ml) was incubated with an equivalent molar quantity of C$\bar{1}$-inhibitor, and also with a 2-, 4-, 8-, and 16-fold molar excess of C$\bar{1}$-inhibitor, for 1 h at 37°C. Incubations were done in 5 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, containing 5 mM $CaCl_2$ or 5 mM EDTA. The extent of formation of complexes was determined by electrophoresis, as described above. K_D was calculated from Eqn. 4,

$$K_D = \frac{P_e I_e}{C_e} \quad (4)$$

where P_e, I_e and C_e represent, respectively, the concentrations of protease, of C$\bar{1}$-inhibitor and of the protease-(C$\bar{1}$-inhibitor) complex at equilibrium.

Results

Formation of complexes between C$\bar{1}$r or C$\bar{1}$s and C$\bar{1}$-inhibitor

On incubation of C$\bar{1}$r and C$\bar{1}$s with C$\bar{1}$-inhibitor, complexes are formed which remain stable even under the strong denaturing conditions used in preparation of samples for sodium dodecyl sulphate polyacrylamide gel electrophoresis [7—9]. Scans of Coomassie blue-stained gels illustrating C$\bar{1}$r-(C$\bar{1}$-inhibitor) and C$\bar{1}$s-(C$\bar{1}$-inhibitor) complexes, formed in the presence either of excess protease or of excess C$\bar{1}$-inhibitor, are shown in Fig. 1.

Formation of complexes of similar stability between various protease-(protease-inhibitor) pairs has been reported, e.g., plasmin-(C$\bar{1}$-inhibitor) [8]; trypsin-α_2 antiplasmin; plasmin-α_2 antiplasmin [20]; thrombin-antithrombin III [21] and trypsin-α_1 antitrypsin [22]. In the majority of such cases, it has been shown that in the presence of an excess of the protease involved, the inhibitor, or the protease-(protease-inhibitor) complex itself undergoes proteolytic degradation [8,20—22]. As shown in Fig. 1, however, when C$\bar{1}$-inhibitor is incubated with a large excess of C$\bar{1}$r or C$\bar{1}$s, only one type of complex is formed, and no degradation of the complex by the excess of free protease is detected. Similarly, no degradation of free C$\bar{1}$-inhibitor is observed when C$\bar{1}$-inhibitor is in excess. Harpel and Cooper [8], examining C$\bar{1}$s-(C$\bar{1}$-inhibitor) interaction, also concluded that C$\bar{1}$s does not degrade C$\bar{1}$-inhibitor. Thus, the C$\bar{1}$r-(C$\bar{1}$-inhibitor) and C$\bar{1}$s-(C$\bar{1}$-inhibitor) complexes are stable both to dissocia-

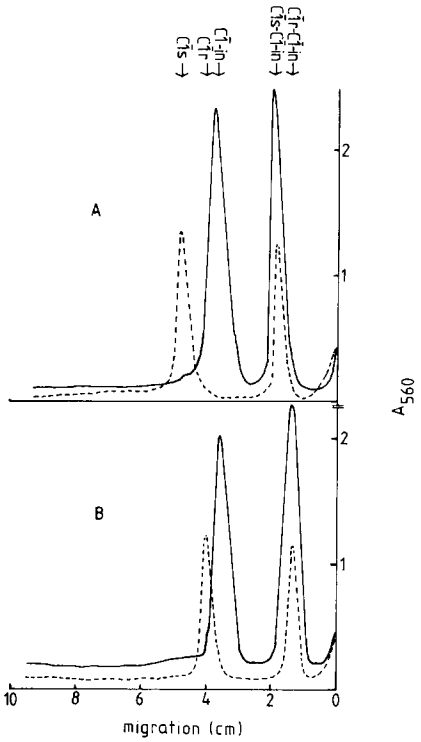

Fig. 1. Scans of sodium dodecyl sulphate polyacrylamide gels of mixtures of $C\bar{1}r$ or $C\bar{1}s$ and $C\bar{1}$-inhibitor. $C\bar{1}s$ + $C\bar{1}$-inhibitor (A) or $C\bar{1}r$ + $C\bar{1}$-inhibitor (B) were incubated for 1 h at 37°C, then run on sodium dodecyl sulphate polyacrylamide gels without reduction of disulphide bonds. ———, mixtures of 15 μg of protease with a 3-fold molar excess of $C\bar{1}$-inhibitor; - - - - - -, mixtures of 5 μg of $C\bar{1}$-inhibitor with a 3-fold molar excess of protease.

tion by denaturing agents and to proteolysis, and are therefore always readily separable from excess free $C\bar{1}r$ or $C\bar{1}s$ or free $C\bar{1}$-inhibitor by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

Kinetic and dissociation constants

Typical curves showing the rates of formation of complexes between $C\bar{1}r$ or $C\bar{1}s$ and $C\bar{1}$-inhibitor are shown in Fig. 2. The progress of the reaction was found to conform to the second-order rate equation (Eqn. 2), and the simplified integrated form of the equation (Eqn. 3) described the reaction up to about 70% completion. The fit of the reaction rate data to Eqn. 3 is shown in Fig. 2C. The reactions illustrated in Fig. 2 all reach completion in less than 1 h, justifying the use of a 1 h incubation to determine the dissociation constant, as described in Materials and Methods.

The calculated values for the kinetic constants of association, and the dissociation constants are shown in Table I. These results demonstrate that $C\bar{1}r$ and $C\bar{1}s$ have similar affinities for $C\bar{1}$-inhibitor, but that $C\bar{1}r$ reacts with the inhibitor more slowly than does $C\bar{1}s$. The presence of Ca^{2+} does not affect the rate at which $C\bar{1}s$ combines with $C\bar{1}$-inhibitor, but the rate of the $C\bar{1}r$-($C\bar{1}$-inhibitor) reaction is slowed by a factor of two to three in the presence of Ca^{2+},

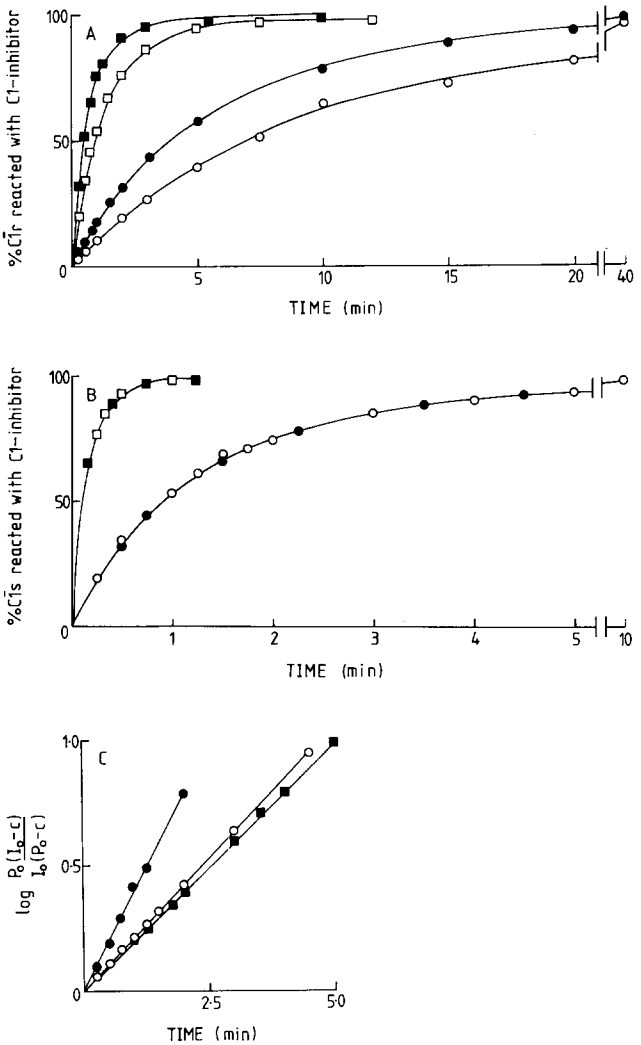

Fig. 2. Rates of reaction of $C\bar{1}r$ and $C\bar{1}s$ with $C\bar{1}$-inhibitor. $C\bar{1}r$ or $C\bar{1}s$ and $C\bar{1}$-inhibitor were incubated at 37°C, and the extent of formation of $C\bar{1}r$-(C1-inhibitor) or $C\bar{1}s$-($C\bar{1}$-inhibitor) complexes was estimated by the sodium dodecyl sulphate polyacrylamide gel electrophoresis method described in Materials and Methods. The curves shown are: (A) $C\bar{1}r$ (0.6 μM) + $C\bar{1}$-inhibitor (1.2 μM) in the presence of EDTA (●) or of Ca^{2+} (○); $C\bar{1}r$ (4.9 μM) + $C\bar{1}$-inhibitor (10.2 μM) in the presence of EDTA (■) or Ca^{2+} (□). (B) $C\bar{1}s$ (0.6 μM) + $C\bar{1}$-inhibitor (1.2 μM) in the presence of EDTA (●) or of Ca^{2+} (○); $C\bar{1}s$ (4.4 μM) + $C\bar{1}$-inhibitor (10.2 μM) in the presence of EDTA (■) or of Ca^{2+} (□). Results shown are means of two experiments at each concentration. (C), data from parts A and B have been fitted to the integrated second-order rate equation (see Eqn. 3 in text). For clarity, some time points are omitted. $C\bar{1}r$ (4.9 μM) + $C\bar{1}$-inhibitor (10.2 μM) in the presence of EDTA (●) or of Ca^{2+} (○); $C\bar{1}s$ (0.6 μM) + $C\bar{1}$-inhibitor (1.2 μM) in the presence of either EDTA or of Ca^{2+} (■).

compared with the rate seen in EDTA. The dissociation constants for both reactions are, however, unaffected by the presence or absence of Ca^{2+}, and the values shown in Table I are the means of values which include determinations in the presence of Ca^{2+} or of EDTA.

The lower velocity of the $C\bar{1}r$-($C\bar{1}$-inhibitor) interaction supports previous

TABLE I

VALUES OF REACTION CONSTANTS FOR THE INTERACTION OF $C\bar{1}r$ OR $C\bar{1}s$ WITH $C\bar{1}$-INHIBITOR

k_1 and K_D (see text for definition) were calculated from results obtained at 37°C in the presence of 5 mM $CaCl_2$ or 5 mM EDTA. Values of k_1 and K_D are the means ± S.D. of 5—8 determinations. Values for the activation energy were calculated from the best straight line and standard deviation of two or three rate determinations at each temperature.

	$C\bar{1}r$ + $C\bar{1}$-inhibitor	$C\bar{1}s$ + $C\bar{1}$-inhibitor
k_1 (in Ca^{2+}) ($M^{-1} \cdot s^{-1}$)	$(1.53 \pm 0.3) \cdot 10^3$	$(1.25 \pm 0.2) \cdot 10^4$
k_1 (in EDTA) ($M^{-1} \cdot s^{-1}$)	$(2.84 \pm 0.2) \cdot 10^3$	$(1.20 \pm 0.15) \cdot 10^4$
K_D (Ca^{2+} or EDTA) (M)	$(1.21 \pm 0.2) \cdot 10^{-7}$	$(9.6 \pm 0.5) \cdot 10^{-8}$
Activation energy (kcal · mol^{-1})	44.3 ± 4.0	11.7 ± 0.5

observations [7] that $C\bar{1}r$ in solution does not compete effectively with $C\bar{1}s$ for $C\bar{1}$-inhibitor. The inhibitory effect of Ca^{2+} on the $C\bar{1}r$-($C\bar{1}$-inhibitor) reaction confirms a previous suggestion by Laurell et al. [23]. Ca^{2+} also has an inhibitory effect on the proteolytic activity of $C\bar{1}r$ [12,17]. The reactivity of $C\bar{1}s$ with $C\bar{1}$-inhibitor, like the proteolytic and esterolytic activities of $C\bar{1}s$ [12,24], is unaffected by Ca^{2+}.

Effect of temperature on reaction rates

The effect of temperature on the interaction of $C\bar{1}r$ or $C\bar{1}s$ with $C\bar{1}$-inhibitor was tested by determining the initial velocities of these reactions over the temperature range 15°—37°C. The rates determined were fitted to the Arrhenius equation. The Arrhenius plot, shown in Fig. 3, was found to be linear for both reactions over the temperature range tested. The slopes of the plots for the $C\bar{1}r$-($C\bar{1}$-inhibitor) or $C\bar{1}s$-($C\bar{1}$-inhibitor) reactions were not significantly altered when the reactants were incubated in the presence of Ca^{2+}. Values for the activation energies, calculated from the slope of the graph, are shown in Table I. The value obtained for the $C\bar{1}s$-($C\bar{1}$-inhibitor) interaction, 11.7 kcal/mol, is in close agreement with that reported by Loos et al. [25] for the reaction between guinea-pig $C\bar{1}s$ and $C\bar{1}$-inhibitor from the same species. Similar values have been calculated for various hydrolytic reactions of $C\bar{1}s$, e.g., hydrolysis of amino acid esters (10.4—12.0 kcal/mol) [24,26].

The decrease in reaction rate with falling temperature is much more marked for $C\bar{1}r$ than for $C\bar{1}s$. The reaction of $C\bar{1}r$ with $C\bar{1}$-inhibitor is very slow at 15°C and at 20°C. At these temperatures, incubation for several hours is required to obtain 50% complex formation in the presence of physiological concentrations of $C\bar{1}r$ and $C\bar{1}$-inhibitor. This is reflected in the high value for the activation energy (Table I). The rate of proteolysis by $C\bar{1}r$ of its physiological substrate, C1s, is also strongly temperature-dependent, with an activation energy of 30—32 kcal/mol [27,28].

Effect of ionic strength on reaction rates

The effect of varying the ionic strength of the incubation medium in the range of 40—775 mM on the reactions between $C\bar{1}$-inhibitor and the two proteases is shown in Fig. 4. The $C\bar{1}r$-($C\bar{1}$-inhibitor) reaction occurs optimally

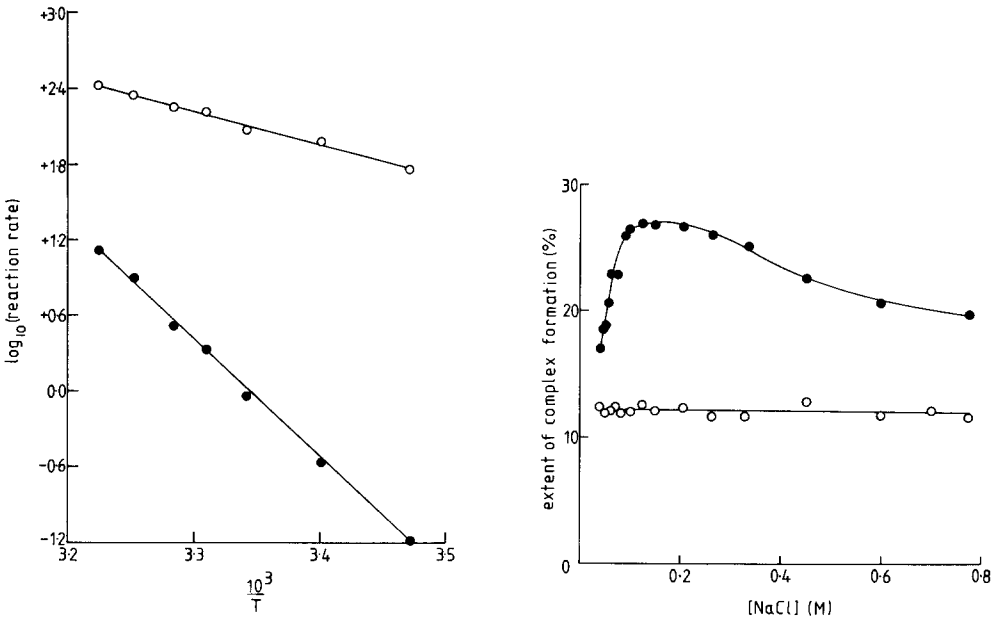

Fig. 3. Effect of temperature on the rate of reaction of $C\bar{1}r$ or $C\bar{1}s$ with $C\bar{1}$-inhibitor. $C\bar{1}r$ (0.6 μM) or $C\bar{1}s$ (1.2 μM) were incubated with a 2-fold molar excess of $C\bar{1}$-inhibitor at various temperatures between 15 and 37°C in the presence of 5 mM EDTA. The rate of uptake of $C\bar{1}r$ or $C\bar{1}s$ into complexes with $C\bar{1}$-inhibitor was calculated at each temperature from graphs of the type shown in Fig. 2A, B. The logarithm of the initial reaction rate for $C\bar{1}r$ (•) and $C\bar{1}s$ (○) is shown as a function of the absolute temperature, T. Two or three determinations of initial reaction rate were performed at each temperature.

Fig. 4. The effect of ionic strength on the reaction between $C\bar{1}r$ or $C\bar{1}s$ and $C\bar{1}$-inhibitor. $C\bar{1}r$ or $C\bar{1}s$ (final concentration 0.6 μM) was incubated at 37°C with a 2-fold molar excess of $C\bar{1}$-inhibitor in 5 mM triethanolamine-HCl, 5 mM EDTA, pH 7.4, containing 45—775 mM NaCl. The percentage of $C\bar{1}r$ taken up into $C\bar{1}r$-($C\bar{1}$-inhibitor) complexes was determined after 2 min (•) by sodium dodecyl sulphate polyacrylamide gel electrophoresis, as described in Materials and Methods. The percentage of $C\bar{1}s$ taken up in complexes was determined after 10 s (○).

between 110 and 300 mM. A sharp decrease in reaction rate is seen below 100 mM, and a more gradual decrease occurs above 300 mM. In contrast, variation in salt strength over this range has no significant effect on the $C\bar{1}s$-($C\bar{1}$-inhibitor) interaction. The same pattern of results is obtained in the presence of Ca^{2+} or of EDTA. Preincubation (5 min, 37°C) either of the protease alone, or of $C\bar{1}$-inhibitor alone, at the ionic strength used in each test did not alter the results obtained.

The inhibition of the $C\bar{1}r$-($C\bar{1}$-inhibitor) interaction at high ionic strength is likely to be a direct effect on $C\bar{1}r$ itself, since similar effects are seen in the hydrolysis of amino acid esters by $C\bar{1}r$ [17] and in the cleavage of proenzymic C1s by $C\bar{1}r$ [28,29]. High ionic strength does not appear to affect the reactivity of $C\bar{1}$-inhibitor, since its reaction with $C\bar{1}s$ (Fig. 4) is not inhibited at high ionic strength. The effect on the $C\bar{1}r$-($C\bar{1}$-inhibitor) interaction at low ionic strength may reflect the low solubility of $C\bar{1}r$ [28], and possible inhibition of the reaction by formation of soluble $C\bar{1}r$ aggregates. No visible precipitation occurred during the time course of the reaction.

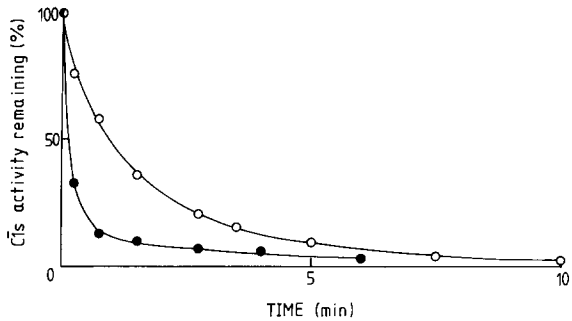

Fig. 5. The effect of heparin on inhibition of C̄1s esterase activity by C̄1-inhibitor. C̄1s (1.6 μM) was incubated in 5 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, at 15°C with a 4-fold molar excess of C̄1-inhibitor, in the absence of heparin (○) or with 5 μg/ml heparin (●). At various times, 50-μl samples of the mixture were withdrawn, and the remaining *N*-α-carbobenzoxy-L-lysine *p*-nitrophenol esterase activity of C̄1s was measured as described in Materials and Methods.

Effect of heparin on reaction rates

Heparin has been shown to potentiate the inhibition of C̄1 by C̄1-inhibitor [30], and so tests were carried out to determine whether this action affected the inhibition of both C̄1r and C̄1s. Results obtained with C̄1s using the spectrophotometric assay system described in Materials and Methods, demonstrated that heparin does greatly enhance the rate at which C̄1s is inhibited by C̄1-inhibitor (Fig. 5). Heparin alone, in agreement with previous findings [30,31] has no effect on the esterolytic activity of C̄1s up to the highest concentration of heparin tested, 150 μg/ml.

Further studies on the effect of heparin on both C̄1r-(C̄1-inhibitor) and C̄1s-(C̄1-inhibitor) reactions were carried out using the sodium dodecyl sulphate polyacrylamide gel electrophoresis assay system. Results are shown in Fig. 6. The rate of reaction of C̄1s with C̄1-inhibitor increases linearly with heparin concentration up to about 15 μg/ml heparin, and then increases more slowly. The C̄1r-(C̄1-inhibitor) interaction is also accelerated in the presence of heparin, but the effect is much smaller than for C̄1s. The maximum enhancement of reaction rate observed for C̄1r is 2—2.5-fold, while for C̄1s, the reaction rate can be increased by a factor of 14—15. As is the case for C̄1s, the esterolytic activity of C̄1r is not affected by heparin alone [17]. The enhancement of reaction rates observed was independent of the presence of Ca^{2+} or of EDTA.

Effect of flufenamic acid on C̄1-inhibitor

Flufenamic acid, an in vitro fibrinolytic agent, has been suggested to promote plasmin action by decreasing the reactivity of various protease inhibitors which act on plasmin [32]. The effect of flufenamate on C̄1-inhibitor was investigated in the C̄1s-(C̄1-inhibitor) system, and results are shown in Fig. 7. Concentrations of flufenamate between 1 mM and 3 mM were found to arrest almost completely the inhibition of C̄1s by a 2-fold molar excess of C̄1-inhibitor. Pre-incubation of C̄1-inhibitor with flufenamate was necessary to obtain this effect. Flufenamic acid has an aromatic structure, and is known to inhibit chymotrypsin [33]. As shown in Fig. 7, flufenamate also inhibits C̄1s activity,

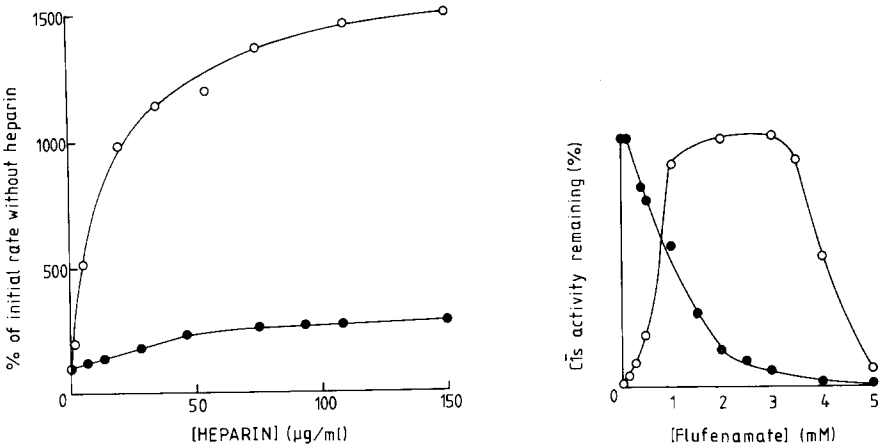

Fig. 6. Relationship of heparin concentration to the rate of reaction of $C\bar{1}r$ or $C\bar{1}s$ with $C\bar{1}$-inhibitor. $C\bar{1}r$ (•) or $C\bar{1}s$ (○) at a final concentration of 0.6 μM was incubated at 37° C with a 2-fold molar excess of $C\bar{1}$-inhibitor, in the presence of 5 mM EDTA and various concentrations of heparin. The time-course of formation of protease-($C\bar{1}$-inhibitor) complexes was followed by the sodium dodecyl sulphate polyacrylamide gel method as shown, e.g. in Fig. 2A, B. Initial rates of raction at each heparin concentration were calculated, and are shown relative to the initial rates in the absence of heparin which are taken as 100%.

Fig. 7. The effect of flufenamic acid on $C\bar{1}$-inhibitor and on $C\bar{1}s$. Curve 1 (•) shows the effect of flufenamic acid on $C\bar{1}s$ alone. $C\bar{1}s$ (0.6 μM) was incubated for 1 h at 37° C in 5 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, with various concentrations of flufenamic acid. The remaining *N*-α-carbobenzoxy-L-lysine *p*-nitrophenol esterase activity of $C\bar{1}s$ was then measured. Curve 2 (○) shows the effect of flufenamic acid on $C\bar{1}$-inhibitor. $C\bar{1}$-inhibitor (1.2 μM) was incubated for 1 h at 37° C with various concentrations of flufenamic acid in the same buffer. The remaining capacity of $C\bar{1}$-inhibitor to inhibit $C\bar{1}s$ was then tested by adding $C\bar{1}s$ to the $C\bar{1}$-inhibitor such that the final molar ratio $C\bar{1}s$: $C\bar{1}$-inhibitor was 1 : 2. Incubation was continued for 15 min at 37° C and the residual esterase activity of $C\bar{1}s$ was measured as above. Flufenamic acid was used as a stock 100 mM solution, adjusted to pH 7.4 with NaOH.

but inhibition of $C\bar{1}s$ occurs at a higher concentration of flufenamate than does the observed inactivation of $C\bar{1}$-inhibitor. At concentrations greater than 3 mM, however, in this system, the direct effect of flufenamate on $C\bar{1}s$ become predominant. A previous report [34] documented an effect of flufenamate on the whole complement system which is consistent with the inhibition of $C\bar{1}s$ seen here.

These results demonstrate that flufenamate is a potent inhibitor of isolated $C\bar{1}$-inhibitor, and confirm earlier indirect evidence of this phenomenon presented by Kluft [33].

Effect of uptake of $C\bar{1}r$ and $C\bar{1}s$ into $C\bar{1}$ bound to immune complexes

In the blood, C1r and C1s exist predominantly as macromolecular C1, i.e., as a firm Ca^{2+}-dependent complex of two C1r plus two C1s monomers, or four C1r plus four C1s monomers bound to one molecule of C1q [1]. On activation of macromolecular C1 by immune complexes, both C1r and C1s become cleaved and activated, but they remain together in the $C\bar{1}$ complex, bound in the immune aggregates [1,2,15]. Thus in plasma, $C\bar{1}r$ and $C\bar{1}s$ are likely to become available for interaction with $C\bar{1}$-inhibitor while they are incorporated into a large complex, rather than reacting as independent free proteases. The effect of the presence of an excess of various combinations of other $C\bar{1}$ subcomponents,

and of insoluble antibody-antigen complexes on the reactivities of $C\bar{1}r$ and $C\bar{1}s$ towards $C\bar{1}$-inhibitor was therefore studied. In these experiments, in mixtures in which both $C\bar{1}r$ and $C\bar{1}s$ were present, one of the two proteases was used in the form completely inactivated by diisopropyl phosphorofluoridate (DIP-$C\bar{1}r$, DIP-$C\bar{1}s$). These inactivated molecules do not react with $C\bar{1}$-inhibitor [9] but their ability to interact with other C1 subcomponents to form the $C\bar{1}$ complex is unimpaired [2,15]. Use of these forms simplifies interpretation, by preventing competition of $C\bar{1}r$ and $C\bar{1}s$ for the $C\bar{1}$-inhibitor which is available.

Results are shown in Table II. In the presence of EDTA, which greatly weakens or prevents the binding of $C\bar{1}r$ or $C\bar{1}s$ to each other or to C1q, the rates of reaction of $C\bar{1}r$ or $C\bar{1}s$ with $C\bar{1}$-inhibitor are not significantly altered by the presence of other $C\bar{1}$ subcomponents or of immune complexes.

In the presence of Ca^{2+}, however, the following binding reactions occur [1,2,28,36]. (a) In the absence of immune aggregates, strong complexes between $C\bar{1}r$ and $C\bar{1}s$ are formed, which are likely to bind to C1q. $C\bar{1}r$ and $C\bar{1}s$ individually do not bind to C1q. (b) In the presence of immune aggregates, C1q binds to the aggregates, and $C\bar{1}r$ alone, or $C\bar{1}r$-$C\bar{1}s$ complexes bind strongly to the bound C1q. $C\bar{1}s$ alone does not bind to antibody-antigen-C1q complexes. $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}r$-$C\bar{1}s$ complexes do not interact with immune aggregates in the absence of C1q.

As shown in Table II, under conditions where $C\bar{1}r$ does not bind to the other

TABLE II

THE EFFECT OF IMMUNE COMPLEXES AND OTHER $C\bar{1}$ SUBCOMPONENTS ON THE REACTION OF $C\bar{1}r$ AND $C\bar{1}s$ WITH $C\bar{1}$-INHIBITOR

$C\bar{1}r$ or $C\bar{1}s$ was mixed with large excesses of other $C\bar{1}$ subcomponents or of immune complexes (IC), and incubated at 37°C with 2-fold molar excess of $C\bar{1}$-inhibitor. Incubation was done in a total volume of 0.1 ml of 5 mM triethanolamine-HCl, 145 mM NaCl, pH 7.4, containing 5 mM Ca^{2+} or 5 mM EDTA. Initial rates of formation or protease-($C\bar{1}$-inhibitor) complexes were calculated from graphs of the type shown in Fig. 2A, B. The rate observed with $C\bar{1}r$ in 5 mM Ca^{2+}, in the absence of other components, is expressed as 1.0, and other rates are shown relative to this value.

Sample No.	Components present (μg)						Initial rate of reaction of $C\bar{1}r$ or $C\bar{1}s$ with $C\bar{1}$-inhibitor	
	$C\bar{1}r$	$C\bar{1}s$	DIP-$C\bar{1}r$	DIP-$C\bar{1}s$	C1q	IC	Ca^{2+}	EDTA
1	5	—	—	—	—	—	1.0	1.9
2	5	—	—	20	—	—	0.8	2.0
3	5	—	—	—	20	—	1.0	2.0
4	5	—	—	—	—	200	1.0	2.0
5	5	—	—	20	20	—	0.7	1.8
6	5	—	—	20	—	200	0.8	2.0
7	5	—	—	—	20	200	0.9	1.8
8	5	—	—	20	20	200	3.7	1.9
9	—	5	—	—	—	—	8.1	7.9
10	—	5	20	—	—	—	7.9	8.1
11	—	5	—	—	20	—	8.3	8.0
12	—	5	—	—	—	200	8.0	8.1
13	—	5	20	—	20	—	7.7	7.9
14	—	5	20	—	—	200	8.0	8.0
15	—	5	—	—	20	200	7.8	8.3
16	—	5	20	—	20	200	7.3	8.1

components present (lines 3 and 4), its reactivity towards $C\bar{1}$-inhibitor is identical to that observed with $C\bar{1}r$ alone in Ca^{2+}. When binding of $C\bar{1}r$ to $C\bar{1}s$ or to C1q plus $C\bar{1}s$ is expected (lines 2, 5 and 6), a slight decrease in the rate of reaction with $C\bar{1}$-inhibitor is observed. This may be due to some steric interference of the $C\bar{1}r$ active site by $C\bar{1}s$ binding as discussed in [15]. When $C\bar{1}r$ is bound to insoluble C1q-antibody-antigen complexes (Table II, line 7), a slight decrease in reactivity is again observed. Such a decrease may be due to insolubilization of $C\bar{1}r$. However, when $C\bar{1}r$ is bound firmly both to $C\bar{1}s$ and to antibody-antigen-C1q complexes (Table II, line 8) a 4-fold increase in reactivity is seen.

To summarise, the reactivity of $C\bar{1}r$ towards $C\bar{1}$-inhibitor in the various mixtures is decreased slightly by either of two influences: - (a) binding to $C\bar{1}s$ or (b) when it become insolubilized. When the whole $C\bar{1}$ complex is assembled on immune aggregates, however, (line 8), a net increase in reactivity of $C\bar{1}r$ is observed, even though $C\bar{1}r$ is in this case both insolubilized and bound to $C\bar{1}s$.

For $C\bar{1}s$, binding to $C\bar{1}r$ (Table II. lines 10 and 14) or to $C\bar{1}r$ plus C1q (line 13) has no significant effect on the reactivity of $C\bar{1}s$ towards $C\bar{1}$-inhibitor. Insolubilization of $C\bar{1}s$ occurs only when $C\bar{1}r$, C1q and immune complexes are present (line 16) and this reduces slightly the reactivity of $C\bar{1}s$. No enhancement of $C\bar{1}s$ reactivity is seen in any of the mixtures.

Previous work [7,9] has shown that while free isolated $C\bar{1}r$ does not compete effectively with $C\bar{1}s$ for $C\bar{1}$-inhibitor, $C\bar{1}r$ bound to immune complexes does compete. The results presented here are consistent with this finding, and show that the alteration in relative reactivities is mainly an enhancement effect on $C\bar{1}r$. To confirm the enhancing effect seen for $C\bar{1}r$, titration experiments

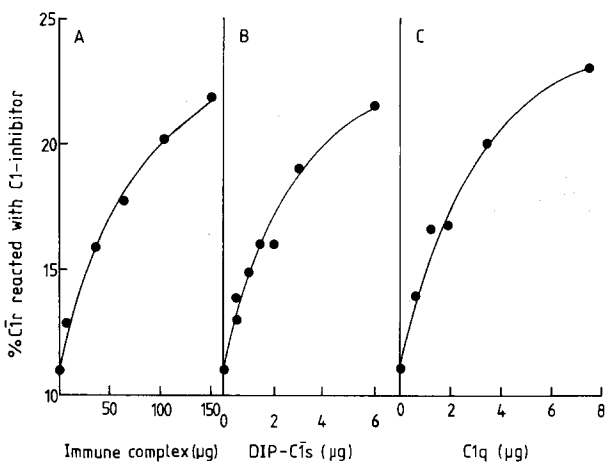

Fig. 8. The effect of increasing quantities of DIP-$C\bar{1}s$, C1q and immune complexes on the rate of reaction of $C\bar{1}r$ with $C\bar{1}$-inhibitor. $C\bar{1}r$ (5 µg) was incubated in 5 mM triethanolamine-HCl, 145 mM NaCl, 5 mM $CaCl_2$, pH 7.4, with a 2-fold molar excess of $C\bar{1}$-inhibitor in the presence of: (A) DIP-$C\bar{1}s$ (5 µg) + C1q (5 µg) + increasing quantities of immune complexes. (B) C1q (5 µg) + immune complexes (100 µg) + increasing quantities of DIP-$C\bar{1}s$. (C) DIP-$C\bar{1}s$ (5 µg) + immune complexes (100 µg) + increasing quantities of C1q. Total incubation volume was 0.1 ml. After 1 min incubation at 37° C, the proportion of $C\bar{1}r$ taken up into complexes with $C\bar{1}$-inhibitor was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

were carried out, varying the quantity of immune complexes, of C1q or of DIP-C$\bar{1}$s added to C$\bar{1}$r. The results which are shown in Fig. 8, demonstrate the absolute requirement for all components of the C$\bar{1}$-immune complex assembly in inducing an increase in the rate of binding of C$\bar{1}$r to C$\bar{1}$-inhibitor.

Discussion

Protease-(protease inhibitor) interactions are generally considered to occur by a two-step mechanism [19,37] consisting of a rapid second-order reaction to form a reversible complex, followed by a slower first-order rearrangement to a more stable or irreversible complex (Eqn. 5).

$$P + I \rightleftharpoons C^* \rightarrow C \qquad (5)$$

Such a mechanism is likely to be applicable to the reactions of C$\bar{1}$r of C$\bar{1}$s with C$\bar{1}$-inhibitor.

In the experiments described here, only the formation of the final product C was monitored. The rate constants determined (Table I) are therefore constants for the overall reaction. The dissociation constants, K_D, shown in Table I are an expression of the reversibility of the first part of the reaction.

Despite the probability of reaction intermediates being present, the overall reaction of C$\bar{1}$r of C$\bar{1}$s with C$\bar{1}$-inhibitor follows second-order kinetics. This is a general finding for reactions of proteases with their inhibitors [37]. The rate constants found for the reactions of C$\bar{1}$r or C$\bar{1}$s with C$\bar{1}$-inhibitor are in the range 10^{-3}—10^{-4} $M^{-1} \cdot s^{-1}$, and are comparable to other values reported for interactions of plasma proteases with plasma protease inhibitors, e.g. the reactions of thrombin with antithrombin III or α_2 macroglobulin [38] and of plasmin with α_1 antitrypsin [39]. Reactions of this type are however, much slower than those between trypsin and inhibitors of plant or tissue origin, e.g., soybean trypsin inhibitor or ovomucoids, for which velocity constants are typically in the range 10^{-5}—10^{-7} $M^{-1} \cdot s^{-1}$ [37].

With some exceptions, dissociation constants for protease-(protease inhibitor) interactions generally lie within the range 10^{-6}—10^{-9} M [19,37] and the constants shown in Table I lie in the higher part of this range.

The effects of temperature, Ca^{2+} and ionic strength on the reactivities of C$\bar{1}$r and C$\bar{1}$s with C$\bar{1}$-inhibitor are similar to the effects produced by these variables on the proteolytic and esterolytic activities of the two enzymes. This justifies the treatment of C$\bar{1}$-inhibitor as a pseudo-substrate. The very large difference in temperature coefficients of C$\bar{1}$r and C$\bar{1}$s (Table I) emphasises the need to study both enzymes at 37°C. Comparisons of the activities of C$\bar{1}$r or C$\bar{1}$s at temperatures lower than 37°C will lead to a considerable underestimate of the relative reactivity of C$\bar{1}$r.

Heparin, as shown in Figs. 5 and 6, enhances the rate at which C$\bar{1}$r and C$\bar{1}$s react with C$\bar{1}$-inhibitor, although the effect is much greater for C$\bar{1}$s.

Heparin is well-known as an accelerator of the inhibition of thrombin by the protease inhibitor, antithrombin III. This plasma protease inhibitor reacts with most of the serine proteases of the blood coagulation and fibrinolysis systems. As discussed by Chan et al. [40], heparin accelerates inhibition of all these proteases by antithrombin III, but the degree of enhancement differs consider-

ably for each protease, as is the case for the reactions of $C\bar{1}$-inhibitor (Fig. 6).

The mechanism by which heparin enhances antithrombin III is not fully established. Rosenberg and Damus [41] have suggested that heparin binds directly to antithrombin III, inducing a change to a more active conformation. Griffith et al. [42], however, have determined by indirect methods that there may also be a requirement for heparin to bind directly to the protease involved in the reaction. The effect of heparin on $C\bar{1}$-inhibitor interactions is at least superficially similar to the effect on antithrombin III in that the enhancement is not the same for each protease, and the two proteases studied here, $C\bar{1}r$ and $C\bar{1}s$, are known to bind heparin [43] although they are not inhibited by heparin alone.

$C\bar{1}$-inhibitor appears to be a major physiological inhibitor of the proteases involved in the initiation of coagulation and fibrinolysis (coagulation factors XIIa, XIa, factor XIIa fragments and kallikrein) [44]. It would be of great interest to determine the effect of heparin on the $C\bar{1}$-inhibitor-mediated inhibition of these proteases. The anticoagulant action of heparin is generally regarded [45] as resulting from stimulation of antithrombin III, but it seems probable, in view of the results presented here, that $C\bar{1}$-inhibitor may also contribute to this anticoagulant action. The effect of heparin in accelerating inhibition of $C\bar{1}r$ and $C\bar{1}s$ is only one of a number of inhibitory effects of heparin on the complement system [46].

An enhancement of reactivity of $C\bar{1}r$ with $C\bar{1}$-inhibitor is seen (Table II and Fig. 8) when $C\bar{1}r$ is incorporated into $C\bar{1}$ bound to antibody-antigen complexes. Ratnoff and Naff [47] have reported that addition of C1q plus C1s to $C\bar{1}r$, in the presence of Ca^{2+}, caused an enhancement of the proteolytic activity of $C\bar{1}r$ towards its substrate, C1s. In the present study, the reactivity of $C\bar{1}r$ was enhanced only if antibody-antigen aggregates, as well as C1q plus $C\bar{1}s$, were present. Ratnoff and Naff, however, used impure preparations of C1q, C1r and C1s and it is probable that IgG aggregates were present in the C1q preparations. Such IgG aggregates would act in the same way as the antibody-antigen aggregates used in the present study. The increase in activity seen by Ratnoff and Naff was variable, further suggesting that it may have been caused by variable quantities of aggregated IgG.

The activation of proenzymic C1r has also been shown to occur only when C1r is incorporated into C1 bound to immune aggregates [2,28]. This effect may be interpreted as the enhancement of the inherent proteolytic activity of C1r proenzyme caused by binding to antibody-antigen-C1q complexes and to C1s. From the results shown in Table II and Fig. 8, and from the data of Ratnoff and Naff [47] it is evident that the activity of $C\bar{1}r$ is also increased by the same binding reactions.

The demonstration that the activities of both $C\bar{1}r$ and of proenzymic C1r are modified by strong binding of immune-complex-bound C1q and of C1s (or $C\bar{1}s$) further strenghthens the analogy between C1 activation and the activation of plasminogen by streptokinase. In the latter system, the activities of both plasmin and of its proenzyme, plasminogen, are modified by strong binding of streptokinase [48]. This phenomenon of induction of zymogen activation and modulation of protease activity by the same modifier proteins may prove to be a more general protease control mechanism.

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