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Fluid-phase interaction of C1 inhibitor (C1 Inh) and the subcomponents C1r and C1s of the first component of complement, C1

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Interactions between proenzymic or activated complement subcomponents of C1 and C1 Inh (C1 inhibitor) were analysed by sucrose-density-gradient ultracentrifugation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The interaction of C1 Inh with dimeric C1r in the presence of EDTA resulted into two bimolecular complexes accounting for a disruption of C1r. The interaction of C1 Inh with the Ca^{2+}-dependent $C1r_2$–$C1s_2$ complex (8.8 S) led to an 8.5 S inhibited C1r–C1s–C1 Inh complex (1:1:2), indicating a disruption of $C1r_2$ and of $C1s_2$ on C1 Inh binding. The 8.5 S inhibited complex was stable in the presence of EDTA; it was also formed from a mixture of C1r, C1s and C1 Inh in the presence of EDTA or from bimolecular complexes of C1r–C1 Inh and C1s–C1 Inh. C1r II, a modified C1r molecule, deprived of a Ca^{2+}-binding site after autoproteolysis, did not lead to an inhibited tetrameric complex on incubation with C1s and C1 Inh. These findings suggest that, when C1 Inh binds to $C1r_2$–$C1s_2$ complex, the intermonomer links inside $C1r_2$ or $C1s_2$ are weakened, whereas the non-covalent Ca^{2+}-independent interaction between $C1r_2$ and $C1s_2$ is strengthened. The nature of the proteinase–C1 Inh link was investigated. Hydroxylamine (1 M) was able to dissociate the complexes partially (pH 7.5) or totally (pH 9.0) when the incubation was performed in denaturing conditions. An ester link between a serine residue at the active site of C1r or C1s and C1 Inh is postulated.

Complement subcomponent C1 Inh (C1 inhibitor) exists in human blood at concentrations high enough to block a number of proteinases participating in different systems such as blood clotting, fibrinolysis, kallikreins and complement (Donaldson, 1979). This glycoprotein has been shown to be the exclusive inhibitor of C1 proteinases C1r and C1s (Sim *et al.*, 1979*b*; Ziccardi, 1981). Beyond this double capacity towards both subcomponents of component C1 (Arlaud *et al.*, 1978), which may reflect a control on the activation of C1 at the level of C1r and on the activity of C1 at the level of C1s, a point of interest was raised from the fact that the binding of C1 Inh to C1r and C1s leads to the disruption of C1 (Laurell *et al.*, 1978; Arlaud *et al.*, 1979*a*; Sim *et al.*, 1979*a*; Ziccardi & Cooper, 1979). This dissociation leaves C1q bound to C1 activator and liberates two moieties each composed of C1r, C1s and C1 Inh, which are thus able to undergo catabolic degradation independently of C1q. More recently this last observation was extended when C1q receptors were described on phagocytes: these receptors can bind C1q and hence indirectly its activators when the stem part of C1q is revealed on disruption of C1, thus leading to a phagocytic action immediately after C1 activation (Tenner & Cooper, 1980, 1981). Apart from its physiopathological interest, underlined by disturbances characterizing hereditary angioneurotic oedema due to a congenital defect in C1 Inh, this subcomponent appears to be an excellent probe for C1 subcomponents. Previous reports have given details on the reaction of C1 Inh with C1r and C1s present in the C1 complex (Laurell *et al.*, 1978; Arlaud *et al.*, 1979*a*; Sim *et al.*, 1979*a*; Ziccardi & Cooper, 1979). In the present work the interaction of C1 Inh with C1r, C1s and $C1r_2$–$C1s_2$ complex has been studied. Most of the previous results were obtained by sodium

Abbreviations used: the nomenclature of complement components is that recommended by the World Health Organisation (1968).

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dodecyl sulphate / polyacrylamide - gel electrophoresis, which preserves the proteinase–inhibitor link but precludes the study of inter-proteinase bonds; we decided to analyse these interactions by sucrose-density-gradient ultracentrifugation. The link between $C\bar{1}$ Inh and each proteinase of $C\bar{1}$ was also studied. The preliminary results indicate that the binding of $C\bar{1}$ Inh leads to a weakening of the inter-monomer non-covalent bond in $C\bar{1}r_2$, responsible for the previously described disruption of $C\bar{1}$ into two inhibited complexes, and to a reinforcement of the non-covalent interaction between the inhibited proteinases in each $C\bar{1}$ Inh–$C\bar{1}$r–$C\bar{1}$s–$C\bar{1}$ Inh complex. These findings reflect a net modification of the A-chains or $C\bar{1}$r and $C\bar{1}$s, involved in the intermonomer of inter-dimer links in $C\bar{1}r_2$–$C\bar{1}s_2$ complexes, on binding of $C\bar{1}$ Inh to the active sites located in the B-chains. These observations also support the involvement of non-covalent interactions between $C\bar{1}$r and $C\bar{1}$s in component $C\bar{1}$ and are discussed in connection with other Ca^{2+}-dependent links also contributing to the stability of $C\bar{1}$.

Materials and methods

Materials

Outdated human citrated plasma was obtained from the Centre de Transfusion Sanguine, Grenoble, France. Serum was prepared from plasma and stored as described previously (Arlaud *et al.*, 1979*a*).

The sources of commercially available materials were as follows: yeast alcohol dehydrogenase, ox liver catalase, horse spleen apoferritin and lactoperoxidase (purified grade), Calbiochem, Laboratoires Eurobio, Paris, France; di-isopropyl phosphorofluoridate and iodoacetamide, Sigma, Coger, Paris, France; sodium dodecyl sulphate and methylamine hydrochloride, Merck, Laboratoire Merck–Clevenot S.A., Division Chimie, Paris, France; hydroxylamine hydrochloride, BDH, Laboratoire Merck–Clevenot S.A., Division Chimie, Paris, France; materials for polyacrylamide-gel electrophoresis, Eastman, Touzart et Matignon, Paris, France; other reagents and chemicals were from Merck and Prolabo, Paris, France. Radioactive materials, namely $Na^{125}I$ (specific radioactivity 16.0mCi/μg of I) was from The Radiochemical Centre, Amersham, Bucks., U.K.; [^{14}C]-methylamine hydrochloride (specific radioactivity 41.2mCi/mmol) and di-[^{3}H]isopropyl phosphorofluoridate (specific radioactivity 6.5Ci/mmol) were from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. Materials for liquid-scintillation counting of radioactivity were purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Components of complement and other proteins

$C\bar{1}$r and $C\bar{1}$s were purified by a method described previously (Arlaud *et al.*, 1979*b*). During a 5h incubation period at 37°C, $C\bar{1}$r underwent two proteolytic cleavages, which led to the successive removal of two fragments, α (M_r 35000) and β (M_r 7000–11000), from each subunit, leaving a dimeric molecule of smaller size (M_r 110000) named $C\bar{1}$r II (Arlaud *et al.*, 1980*b*). A modified technique was used for the purification of proenzymic C1s (Arlaud *et al.*, 1980*b*). Purified proenzymic C1r was obtained with a two-step technique in which outdated human citrated plasma is directly chromatographed on anti-$C\bar{1}$s antibody immobilized on Sepharose/polyacrylamide (Villiers *et al.*, 1981). C4 was prepared by the method of Reboul *et al.* (1979). $C\bar{1}$ Inh was purified as previously described (Reboul *et al.*, 1977).

Anti-$C\bar{1}$s, anti-($C\bar{1}$ Inh) and other antisera were raised in rabbits by the method of Porter (1955). Purification of anti-$C\bar{1}$s, anti-($C\bar{1}$ Inh) and other antibodies and coupling to Sepharose-4B was as described by Arlaud *et al.* (1977*b*) for anti$C\bar{1}$r immunoglobulin G antibody.

Concentrations of purified proteins were determined from their specific absorbance at 280nm by using respectively, for $C\bar{1}$r (C1r), $C\bar{1}$s (C1s) and C1 Inh, $A^{1\%}_{1cm} = 11.5$ (Sim *et al.*, 1977), $A^{1\%}_{1cm} = 9.5$ (Sim *et al.*, 1977) and $A^{1\%}_{1cm} = 4.5$ (Harpel, 1976). A value of $A^{1\%}_{1cm} = 14.0$ was used for purified rabbit immunoglobulins. Molecular weights were taken as 85000 for $C\bar{1}$r (C1r), 85000 for $C\bar{1}$s (C1s), 100000 for $C\bar{1}$ Inh and 150000 for rabbit immunoglobulin G.

Sucrose-density-gradient ultracentrifugation

Samples were sedimented as described by Martin & Ames (1961) in linear 5–20% (w/v) sucrose gradients at 4°C for 15h (110000*g*; $r_{av.}$ 9cm) in a TST 54 rotor in a Kontron TGA 50 ultracentrifuge. Yeast alcohol dehydrogenase (7.6S), ox liver catalase (11.4S) and horse spleen apoferritin (17.6S) were used as standards for measurement of $s_{20,w}$. Standards were detected in the eluates by their absorbance at 280nm; complement proteins were detected, after counting of radioactivity, by using a Coomassie Blue staining method (Bradford, 1976). The fractions of eluates submitted to sodium dodecyl sulphate / polyacrylamide - gel electrophoresis were pooled, extensively dialysed against distilled water and then freeze-dried.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Non-reduced samples were incubated in 4M-urea/1% (w/v) sodium dodecyl sulphate/10mM-iodoacetamide/0.1M-Tris/HCl buffer, pH8.0, for 1h at 37°C. Reduced samples were incubated for 1h at

37°C in the same buffer but with iodoacetamide replaced by 140mM-2-mercaptoethanol, and were then alkylated with 140mM-iodoacetamide for 20min at 37°C. Gels containing 5% acrylamide were prepared as described by Fairbanks *et al.* (1971) and run at 5mA/gel. Staining of gels with Coomassie Blue was as described by Weber & Osborn (1969). Gels loaded wth ^{125}I-labelled samples were cut into 1mm slices for direct counting of radioactivity in an MR 480 Kontron γ-radiation counter. Gels loaded with [^{14}C]methylamine-treated samples were cut into 0.5mm slices, and the slices were grouped into fours and submitted to a digestion in 1ml of 30% (w/v) H_2O_2 in the counting vials for 15h at 55°C. After cooling and addition of 10ml of scintillation fluid (Patterson & Greene, 1965), [^{14}C]methylamine radioactivity was measured in an SL 3000 Counter (Intertechnique).

Labelling of proteinases by di-[1,3-^{3}H]isopropyl phosphorofluoridate

Labelling of C$\bar{1}$r and C$\bar{1}$s by di-[1,3-^{3}H]isopropyl phosphorofluoridate was performed as described by Arlaud *et al.* (1980*a*).

Interaction of hydroxylamine and methylamine with complexes formed between C$\bar{1}$r or C$\bar{1}$s and C$\bar{1}$ Inh or with isolated proteins

C$\bar{1}$r (0.4mg/ml), C$\bar{1}$s (1mg/ml), C$\bar{1}$ Inh (2mg/ml) or their complexes (C$\bar{1}$ Inh/proteinase ratio 1.5:1.0, w/w) were treated with nucleophiles as described by Owen (1975). In non-denaturing conditions, protein solutions (1 vol.) were incubated with an equal volume of hydroxylamine (2M) or methylamine (100mM) at pH7.5 or 9.0 for 3h at 25°C, unless specified otherwise. In denaturing conditions, proteins or their complexes were first treated with $\frac{1}{4}$vol. of 10% (w/v) sodium dodecyl sulphate, and the mixtures were heated at 60°C for 10min. The samples were dialysed twice against 2 litres of 0.1% (w/v) sodium dodecyl sulphate/50mM-NaCl. Then the sample was made 50% (v/v) with respect to the nucleophile solution at pH7.5 or 9.0. After 1h at 25°C, the mixture was dialysed for 3h against 1 litre of 0.1% (w/v) sodium dodecyl sulphate/50mM-NaCl, except where specified otherwise. This material, as well as a control sample from which the nucleophile had been omitted, were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

C$\bar{1}$s esterase activity

C$\bar{1}$s esterase activity was measured as described by Arlaud *et al.* (1980*a*), with *p*-tosyl-L-arginine methyl ester as substrate. C$\bar{1}$s activity on C4 was measured as described by Reboul *et al.* (1979).

Estimation of C$\bar{1}$ Inh activity

The inhibitory activity of C$\bar{1}$ Inh was estimated from the residual *p*-tosyl-L-arginine methylesterase activity after the incubation of C$\bar{1}$ Inh with C$\bar{1}$s for 30min at 37°C (Arlaud *et al.*, 1977*b*).

Results

Iodination of C$\bar{1}$r (C1r), C$\bar{1}$s (C1s) and C$\bar{1}$ Inh

The influence of iodination on individual subcomponents was investigated; after being labelled in the conditions described in the Materials and methods section C$\bar{1}$ Inh has an apparent sedimentation coefficient of 4.3S in the presence of Ca^{2+} or EDTA. Purified unlabelled C$\bar{1}$ Inh showed a tendency to form aggregates on storage, and this appeared to be increased with the iodinated molecule. However, the radiolabelled C$\bar{1}$ Inh retained all the inhibitory activity of the unlabelled C$\bar{1}$ Inh.

^{125}I-labelling of proenzymic or activated C1r in the presence of EDTA did not alter the sedimentation coefficient measured in the presence of EDTA: a value of 7.1S was found, as reported by other authors (Valet & Cooper, 1974*b*; Ziccardi & Cooper, 1976; Arlaud *et al.*, 1980*a*). In the presence of Ca^{2+} both forms of C1r had a strong tendency to form aggregates, which precluded the study of iodination in these conditions. The sedimentation coefficient of C$\bar{1}$r II, a fragment of C$\bar{1}$r generated by autoproteolysis of activated C$\bar{1}$r (Arlaud *et al.*, 1980*b*), was not influenced by iodination. C$\bar{1}$s normally behaved as a 4.5S protein in the presence of EDTA and 6.1S in the presence of Ca^{2+}, values that indicate respectively a monomeric and a dimeric state for this molecule; the same observations were also valid for C1s. Iodination of proenzymic or activated C1s in the presence of Ca^{2+} revealed no gross modification of the behaviour of the protein on centrifugation in the presence of Ca^{2+} or EDTA. However, when C1s was labelled in the presence of EDTA either in the proenzymic or activated form it always behaved as a 4.5S monomer on centrifugation in the presence of either EDTA or Ca^{2+}, indicating that the iodination of monomeric C1s or C$\bar{1}$s prevented its subsequent dimerization in the presence of Ca^{2+}.

Fluid-phase interactions between C$\bar{1}$ Inh and proteinases isolated from C$\bar{1}$

C$\bar{1}$r–C$\bar{1}$ Inh interaction. C$\bar{1}$r normally appeared as a 7.1S protein on sucrose-density-gradient ultracentrifugation in the presence of EDTA, this value corresponding to a dimeric organization of the molecule (Fig. 1*a*). After incubation of ^{125}I-labelled C$\bar{1}$r with an excess of C$\bar{1}$ Inh and subsequent sucrose-density-gradient ultracentrifugation, a single radioactive peak was detected sedimenting at 6.5S

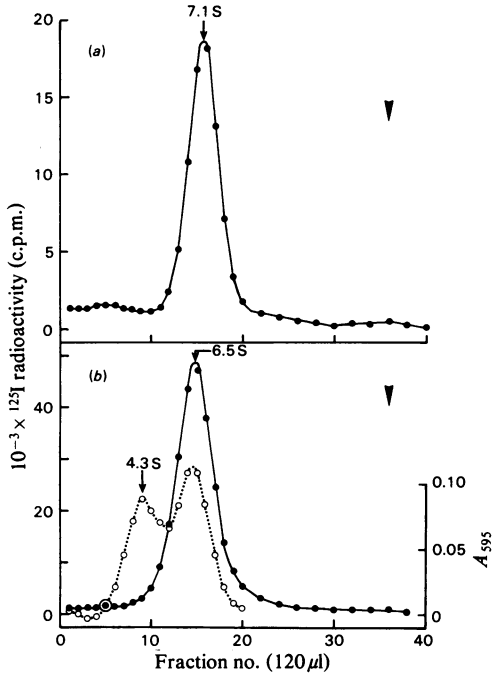

Fig. 1. *Formation of complexes containing monomeric C1r and C1 Inh*

(*a*) Purified ^{125}I-labelled C$\bar{1}$r. The sample (150 μl) applied to the gradient contained 100 μg of C$\bar{1}$r/ml in 5 mM-triethanolamine/HCl/145 mM-NaCl buffer, pH 7.4, containing apoferritin (1.7 mg/ml); 2 mM-EDTA was present in the gradient. (*b*) C$\bar{1}$r–C$\bar{1}$ Inh complex. A mixture containing ^{125}I-labelled C$\bar{1}$r (200 μg/ml) and an excess of C$\bar{1}$ Inh (333 μg/ml) was incubated for 30 min at 37°C in 5 mM-triethanolamine/HCl/145 mM-NaCl/2 mM-EDTA buffer, pH 7.4, and cooled. Apoferritin (1.7 mg/ml) was added before run. Samples (150 μl) were analysed by sucrose-density-gradient ultracentrifugation in the presence of 2 mM-EDTA. Fractions were collected from the top of the gradient and counted for ^{125}I radioactivity (●). Protein (○) was determined as described in the Materials and methods section. The position of apoferritin is shown by the arrow.

(Fig. 1*b*) coincident with a major protein peak, the second protein peak (4.3 S) corresponding to the excess of C$\bar{1}$ Inh. The total absence of any 7.1 S protein thus strongly suggests the involvement of one monomer of C$\bar{1}$r in association with C$\bar{1}$ Inh in the 6.5 S peak (Chesne *et al.*, 1980).

When C$\bar{1}$r and C$\bar{1}$ Inh were incubated in the presence of Ca^{2+} or when the EDTA incubation described above was followed by an incubation and centrifugation in the presence of Ca^{2+}, complexes sedimenting at 9.1 S were observed, corresponding probably to aggregates.

Similar observations were obtained on incubation of C$\bar{1}$r II, a proteolysed form of C$\bar{1}$r described by Arlaud *et al.* (1980*b*), with C$\bar{1}$ Inh. Isolated C$\bar{1}$r II behaved, on sucrose-density-gradient centrifugation, as a 6.0 S protein; after incubation with an excess of C$\bar{1}$ Inh, a 5.8 S complex was formed, corresponding to the association of one molecule of C$\bar{1}$ Inh with one monomer of C$\bar{1}$r II. This 5.8 S value was not altered in the presence of Ca^{2+}, which is in keeping with previous observations indicating that a Ca^{2+}-binding site is lost on proteolysis of C$\bar{1}$r leading to C$\bar{1}$r II (Villiers *et al.*, 1980). By the same method, no interaction was detected between unlabelled or iodinated proenzymic C1r and C$\bar{1}$ Inh.

C$\bar{1}$s–C$\bar{1}$ Inh interaction. In the presence of EDTA C$\bar{1}$s behaved as a monomer, confirming previous observations (Valet & Cooper, 1974*a*), and was found able to form a 6.1 S complex on incubation with C$\bar{1}$ Inh, as shown previously.

In the presence of Ca^{2+}, C$\bar{1}$s formed a dimeric 6.1 S complex, and the incubation of this dimeric form with C$\bar{1}$ Inh led to a 7.7 S tetrameric complex. When the proteinase was iodinated in the presence of EDTA, the incubation of ^{125}I-labelled C$\bar{1}$s with C$\bar{1}$ Inh led to the formation of a bimolecular 6.1 S complex in the presence of either EDTA or Ca^{2+}.

C$\bar{1}$s labelled in the presence of Ca^{2+} behaved like unlabelled C$\bar{1}$s on incubation with C$\bar{1}$ Inh. As noted for proenzymic C1r, no interaction was detected between proenzymic C1s and C$\bar{1}$ Inh by the above-described methods.

Interactions between C$\bar{1}$r–C$\bar{1}$s association and C$\bar{1}$ Inh

As shown previously (Arlaud *et al.*, 1980*a*), purified C$\bar{1}$r and C$\bar{1}$s were associated into an 8.8 S complex in the presence of Ca^{2+}, corresponding to a C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ structure. A similar value of 8.8 S was found when ^{125}I-labelled C$\bar{1}$s, labelled in the presence of EDTA and then incubated with C$\bar{1}$r in the presence of Ca^{2+}, was analysed by sucrose-density-gradient ultracentrifugation in the presence of Ca^{2+}. Both 8.8 S complexes dissociated into 7.1 S (C$\bar{1}$r) and 4.5 S (C$\bar{1}$s) peaks on density-gradient ultracentrifugation in the presence of EDTA; monomeric C$\bar{1}$r and C$\bar{1}$s were identified when the complexes were submitted to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This indicates that two iodinated monomeric C$\bar{1}$s molecules can participate in the C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ complex without forming the individual C$\bar{1}$s$_2$ Ca^{2+}-dependent structure observed with the unlabelled C$\bar{1}$s.

In the case of the iodination of C$\bar{1}$r no gross effect of the label could be detected on the formation of the 8.8 S C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ complex.

The ability to reconstruct a C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ complex from the purified individual proteinases was used to analyse the interactions between purified C$\bar{1}$ Inh and the two subcomponents of the complex.

As reported previously for $C\bar{1}$ (Laurell *et al.*, 1978; Arlaud *et al.*, 1979*a*; Ziccardi & Cooper, 1979; Sim *et al.*, 1979*a*, 1980; Chesne *et al.*, 1980), $C\bar{1}$ Inh is able to bind to $C\bar{1}r$ and $C\bar{1}s$ in their association complex. Incubation of $C\bar{1}r_2$–$C\bar{1}s_2$ complex with $C\bar{1}$ Inh resulted in the formation of an inactive 8.5 S complex consisting of two molecules of $C\bar{1}$ Inh and of one monomer of each of proteinase $C\bar{1}r$ and $C\bar{1}s$ (Ziccardi & Cooper, 1979; Sim *et al.*, 1980); the proteinases are individually strongly linked to the $C\bar{1}$ Inh, as revealed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis of the complex. The qualitative composition of the

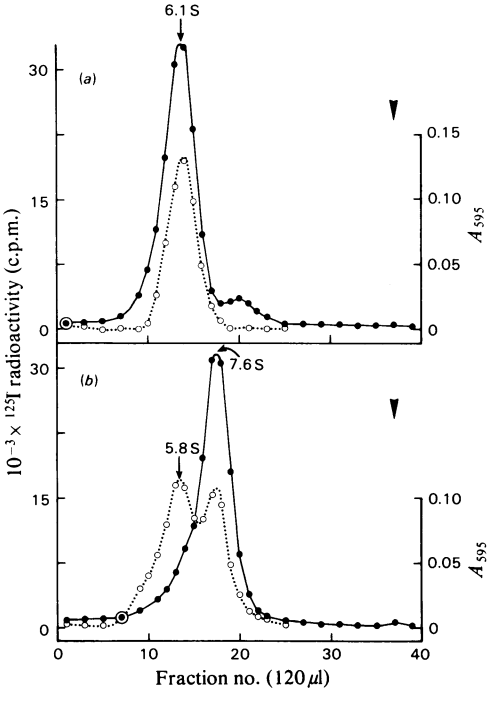

Fig. 2. *Effect of proteolysed $C\bar{1}r$ ($C\bar{1}r$ II) on the formation of complexes with $C\bar{1}s$ or (and) $C\bar{1}$ Inh*

(*a*) A mixture containing $C\bar{1}r$ II (280 μg/ml) and ^{125}I-labelled dimeric $C\bar{1}s$ (280 μg/ml) was incubated at 0°C in 5 mM-triethanolamine/HCl/145 mM-NaCl/2 mM-$CaCl_2$ buffer, pH 7.4, containing apoferritin (1.7 mg/ml). (*b*) $C\bar{1}r$ II (280 μg/ml) and ^{125}I-labelled dimeric $C\bar{1}s$ (280 μg/ml) were incubated for 30 min at 37°C with $C\bar{1}$ Inh (350 μg/ml) in 5 mM-triethanolamine/HCl/145 mM-NaCl/2 mM-$CaCl_2$ buffer, pH 7.4. Apoferritin (1.7 mg/ml) was added as standard. Samples (150 μl) were analysed by sucrose-density-gradient ultracentrifugation in the presence of 1 mM-$CaCl_2$. Fractions were collected from the top of the gradient and counted for ^{125}I radioactivity (●). Protein (○) was determined as described in the Materials and methods section. The position of apoferritin is shown by the arrow.

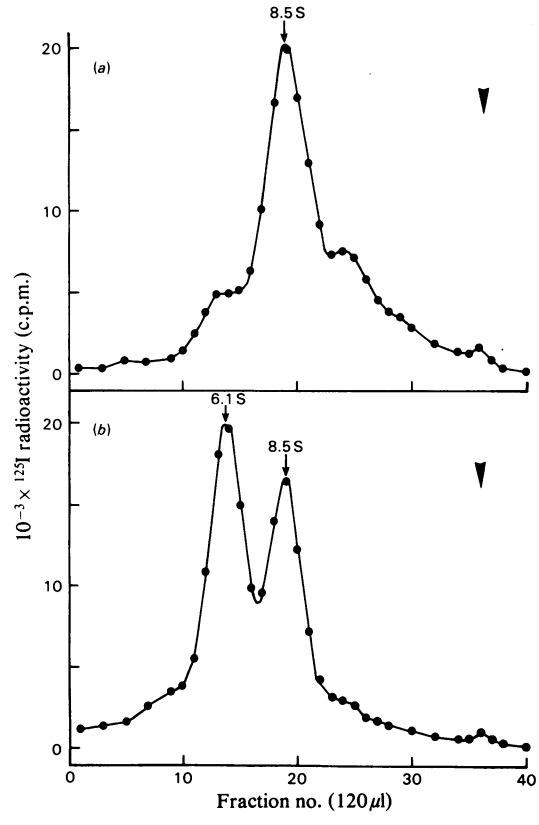

Fig. 3. *Formation of a $C\bar{1}$ Inh–$C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ Inh complex in EDTA from isolated $C\bar{1}r$, $C\bar{1}s$ and $C\bar{1}$ Inh*

(*a*) ^{125}I-labelled $C\bar{1}r$ (167 μg/ml) and $C\bar{1}s$ (167 μg/ml) in 5 mM-triethanolamine/HCl/145 mM-NaCl/2 mM-EDTA buffer, pH 7.4, were added to $C\bar{1}$ Inh (444 μg/ml) in the same buffer. After 30 min incubation at 37°C, the mixture was cooled. Apoferritin (1.7 mg/ml) was added before the run. (*b*) as in (*a*), but with ^{125}I-labelled $C\bar{1}s$ (167 μg/ml). Samples (150 μl) were analysed by sucrose-density-gradient ultracentrifugation in the presence of 2 mM-EDTA. Fractions were collected from the top of the gradient and counted for ^{125}I radioactivity. The position of apoferritin is shown by the arrow.

inhibited 8.5 S complex was verified in separate density-gradient ultracentrifugation experiments with ^{125}I-labelled $C\bar{1}r$ or ^{125}I-labelled $C\bar{1}s$ and specific antibodies against $C\bar{1}s$ or $C\bar{1}$ Inh: the binding of antibodies to the inhibited complexes shifted the radioactivity to the bottom of the gradient. Similar results were obtained with $C\bar{1}r_2$–$C\bar{1}s_2$ complexes including dimeric or monomeric labelled $C\bar{1}s$ or labelled $C\bar{1}r$.

In contrast with $C\bar{1}r_2$–$C\bar{1}s_2$ complexes, this $C\bar{1}$ Inh–$C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ Inh complex was not influenced by the addition of EDTA, a finding that confirms

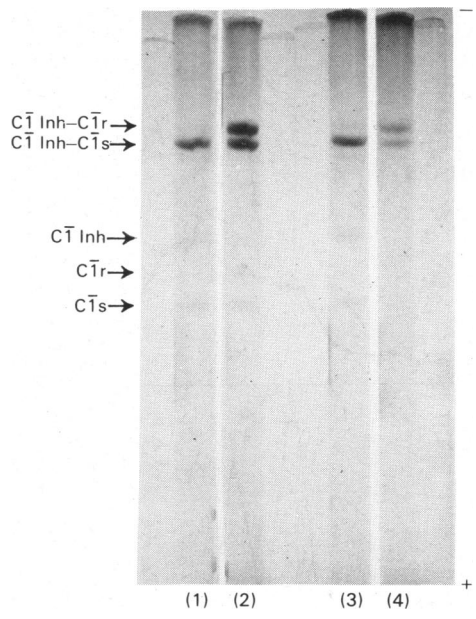

Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis of 8.5 S complexes formed from isolated C1r, C1s and C1 Inh*

After ^{125}I-radioactivity counting, fractions were pooled, dialysed against distilled water and freeze-dried as described in the Materials and methods section. Dry material was dissolved in distilled water (150 μl), and a 75 μl sample was submitted to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions. Pooled fractions from Fig. 3(*a*): gel 1, fractions 5–16; gel 2, fractions 17–22. Pooled fractions from Fig. 3(*b*): gel 3, fractions 5–16; gel 4, fractions 17–22. Pooled fractions 23–30 (not shown) were identified as C1 Inh–C1r aggregates.

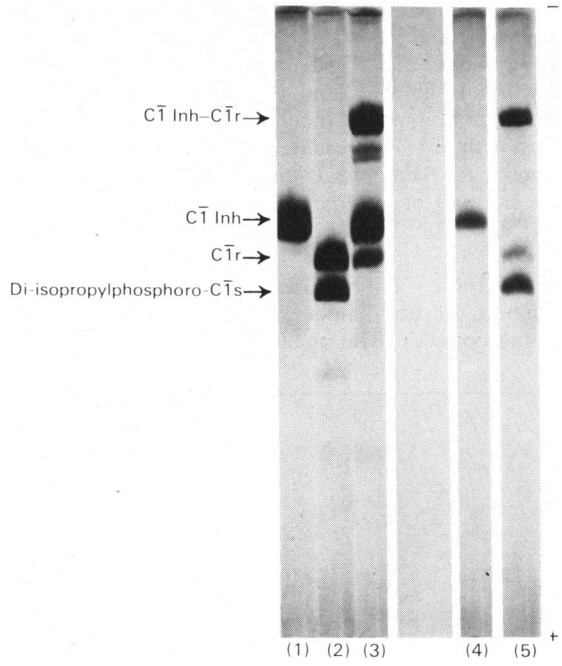

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis of complexes formed by C1r, di-isopropylphosphoro-C1s and C1 Inh*

A mixture containing ^{125}I-labelled C1r (180 μg/ml) and C1s (180 μg/ml) blocked by di-isopropyl phosphorofluoridate in 5 mM-triethanolamine/HCl/145 mM-NaCl/2 mM-$CaCl_2$ buffer, pH 7.4, was added to a C1 Inh solution (333 μg/ml) in the same buffer. After incubation for 30 min at 37°C the mixture was cooled in ice. Apoferritin (1.7 mg/ml) was added to the sample before analysis by sucrose-density-gradient ultracentrifugation in the presence of 1 mM-$CaCl_2$. Fractions were collected from the top of the gradient and counted for ^{125}I radioactivity. The fractions corresponding to a 7.8 S peak were pooled, dialysed against distilled water and freeze-dried as described in the Materials and methods section. Dry material was dissolved in distilled water (150 μl), and a 75 μl sample was submitted to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis analysis under non-reducing conditions. Gel 1, reference C1 Inh; gel 2, reference C1r plus di-isopropylphosphoro-C1s; gel 3, reference C1 Inh–C1r complex and free C1 Inh and C1r; the two light bands under the C1 Inh–C1r complex were identified as complexes formed between C1 Inh and partially proteolysed C1r; gel 4, pool of fractions 5–14 of the gradient; gel 5, pool of fractions 15–20 of the gradient, corresponding to a 7.8 S peak.

that there is a reinforcement of the non-covalent interaction between C1r and C1s when these proteinases are bound to C1 Inh. This finding was supported by results obtained with mixtures of C1r II and C1s incubated, in the presence of Ca^{2+}, with C1 Inh; no tetrameric complex could be detected between C1r II and C1s incubated and analysed in the presence of Ca^{2+} (Fig. 2*a*). In the presence of C1 Inh, C1r II and C1s formed 5.8 S C1 Inh–C1r II and 7.6 S $C1s_2$–C1 Inh_2 association complexes (Fig. 2*b*). It thus can be concluded that the cohesion of the C1–Inh–C1r–C1s–C1–Inh complex does not appear to be due to a direct C1-Inh–C1-Inh interaction.

In order to understand how the tetrameric proteinase–inhibitor complex is formed either from the maximum octameric $C1r_2$–$C1s_2$–C1 Inh_4 association or from individual proteinase–C1 Inh dimeric complexes, the association of these individual complexes was investigated. When C1r–C1 Inh complexes were incubated with ^{125}I-labelled dimeric C1s–C1 Inh complexes (7.7 S) in the presence of Ca^{2+} and subsequently analysed by sucrose-density-

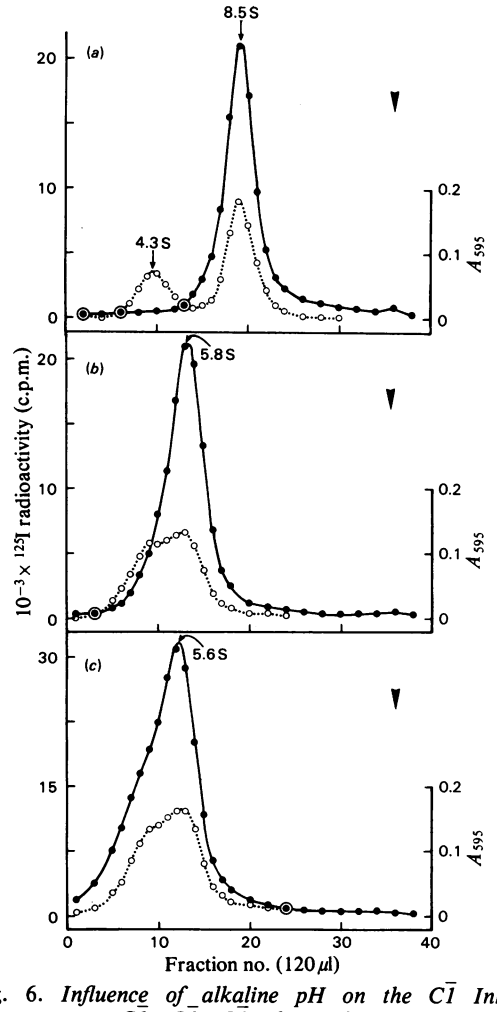

Fig. 6. *Influence of alkaline pH on the C$\bar{1}$ Inh–C$\bar{1}$r–C$\bar{1}$s–C$\bar{1}$ Inh complex*

Three mixtures containing C$\bar{1}$r (176.5 μg/ml) and C$\bar{1}$s (176.5 μg/ml) in 5 mM-triethanolamine/HCl/145 mM-NaCl/2 mM-$CaCl_2$ buffer, pH 7.4, were added to C$\bar{1}$ Inh solutions (588 μg/ml) in the same buffer. After 30 min incubation at 37°C, 170 μl samples were dialysed for 6 h against (*a*) 20 mM-Tris/HCl/145 mM-NaCl buffer pH 7.5, or (*b*) and (*c*) 30 mM-Na_2CO_3/NaOH/20 mM-glycine/150 mM-NaCl buffer, pH 11.0. Apoferritin (1.7 mg/ml) was added to each sample before the run. Samples (150 μl) were analysed by sucrose-density-gradient ultracentrifugation in the absence of EDTA or Ca^{2+}. Fractions were collected from the top of the gradient and counted for ^{125}I radioactivity (●). Protein (○) was determined as described in the Materials and methods section. The position of apoferritin is shown by the arrow. The initial inhibited complexes were prepared from ^{125}I-labelled C$\bar{1}$s (*a* and *c*) or from ^{125}I-labelled C$\bar{1}$r (*b*).

gradient ultracentrifugation in the presence of Ca^{2+} or EDTA, an 8.5 S complex was obtained. The same result was observed when the label was in C$\bar{1}$r or in monomeric C$\bar{1}$s. When purified C$\bar{1}$r and C$\bar{1}$s were incubated with C$\bar{1}$ Inh in the presence of EDTA the same 8.5 S complex was formed (Figs. 3 and 4).

Influence of di-isopropyl phosphorofluoridate on interactions of C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ complex with C$\bar{1}$ Inh

Di-isopropyl phosphorofluoridate treatment of C$\bar{1}$s was shown previously to prevent its covalent interaction with C$\bar{1}$ Inh (Haines & Lepow, 1964; Bing, 1969; Arlaud *et al.*, 1979*a*). In preliminary experiments we checked also that the binding of di-isopropyl phosphorofluoridate to the active site of C$\bar{1}$s was without effect either on the Ca^{2+}-dependent dimerization of the proteinase or on its Ca^{2+}-dependent association with C$\bar{1}$r.

C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ tetrameric complexes were formed by mixing C$\bar{1}$r and ^{3}H-labelled di-isopropylphosphoro-C$\bar{1}$s as well as ^{125}I-labelled C$\bar{1}$r and unlabelled di-isopropylphosphoro-C$\bar{1}$s; both complexes behaved as 8.8 S components on sucrose-density-gradient ultracentrifugation. When these complexes were incubated with C$\bar{1}$ Inh, a new value of 7.8 S was found for the resulting reaction products. This result, and the analysis of the 7.8 S peak by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 5), indicate that the complex is formed by the association of C$\bar{1}$ Inh–C$\bar{1}$r complex with di-isopropylphosphoro-C$\bar{1}$s, showing that the C$\bar{1}$r–C$\bar{1}$s interaction within these inhibited complexes is reinforced, as also seen with the C$\bar{1}$r$_2$–C$\bar{1}$s$_2$ tetrameric complexes with C1 Inh.

Analogous results were expected when the effect of di-isopropyl phosphorofluoridate was studied on C$\bar{1}$r, but in this case di-isopropyl phosphorofluoridate binding to C$\bar{1}$r resulted in precipitation of the blocked proteinase, which prevented any studies with di-isopropylphosphoro-C$\bar{1}$r.

Stability of the C$\bar{1}$ Inh–C$\bar{1}$r–C$\bar{1}$s–C$\bar{1}$ Inh complex

Influence of ionic strength and pH. In confirmation of previous observations (Sim *et al.*, 1980), the stability of the 8.5 S C$\bar{1}$ Inh–C$\bar{1}$r–C$\bar{1}$s–C$\bar{1}$ Inh complex was not influenced by increasing the ionic strength up to 1.2 M-NaCl.

The influence of pH was studied over a pH 4.0–11.0 range on complexes sedimenting with an 8.5 S value at pH 7.5 (Fig. 6*a*), composed of ^{125}I-labelled C$\bar{1}$r or ^{125}I-labelled C$\bar{1}$s. Modifications were observed only for the extreme values: a series of peaks ranging from 6 S to 17.6 S was observed after incubation and sucrose-density-gradient centrifugation at pH 4.0, revealing probably a split of the original tetramolecular complex into its two proteinase–C$\bar{1}$ Inh halves, with a subsequent aggregation of these bimolecular complexes. At pH 11.0 (Figs. 6*b* and 6*c*) the original 8.5 S value was shifted

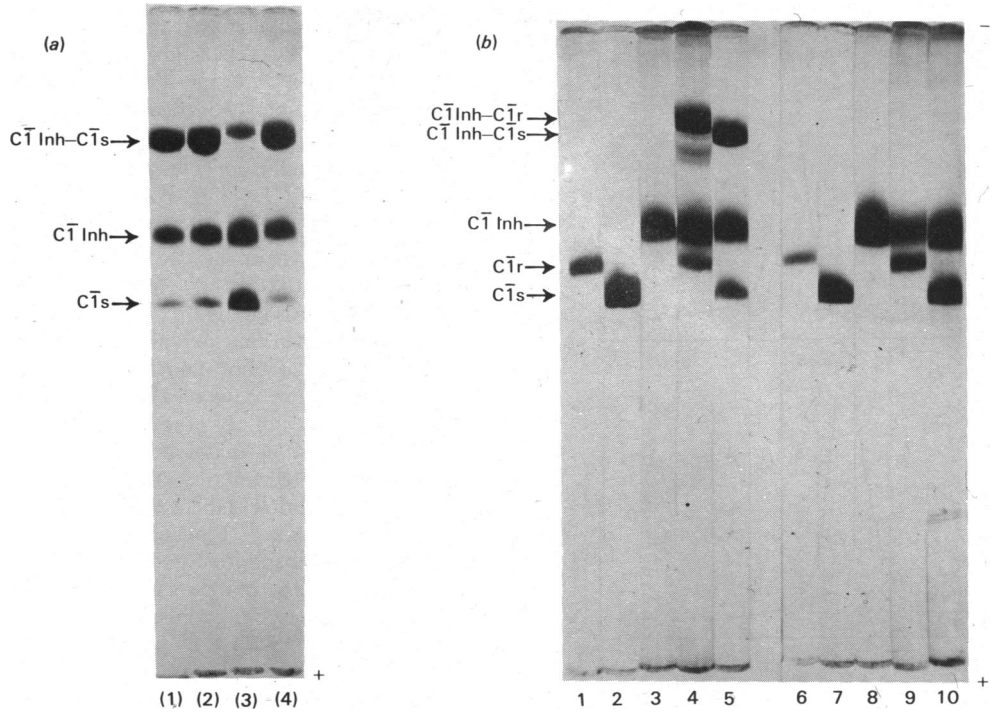

Fig. 7. *Effect of hydroxylamine on the sodium dodecyl sulphate-denaturated C1 Inh–proteinases complexes*
(*a*) C1 Inh–C1s complex, pH 7.5. Purified C1s (160 μg) and C1 Inh (240 μg) in 280 μl of 5 mM-triethanolamine/HCl/145 mM-NaCl buffer, pH 7.4, were incubated for 45 min at 37°C and treated by hydroxylamine as described in the Materials and methods section. Gel 1, control for formation of the initial complex; gel 2, heat- and sodium dodecyl sulphate-denatured complex; gel 3, same as 2, with 1 M-hydroxylamine, pH 7.5; gel 4, same as 2, with 20 mM-Tris/HCl buffer, pH 7.5. (*b*) C1 Inh–C1r and C1 Inh–C1s complexes, pH 9.0. Samples (300 μl) containing C1 Inh (1 mg/ml) and C1s (0.5 mg/ml) or C1 Inh (0.67 mg/ml) and C1r (0.27 mg/ml) were incubated for 30 min at 37°C. C1 Inh–C1r and C1 Inh-C1s complexes and isolated C1r, C1s and C1 Inh were first denatured by sodium dodecyl sulphate and heated as described in the Materials and methods section, and divided each in two portions: one was treated with an equal volume of 2 M-hydroxylamine, pH 9.0; the other, used as a reference, was treated with an equal volume of 20 mM-Tris/HCl buffer, pH 9.0. Incubation and dialysis were as described in the Material and methods section. Samples were dialysed against distilled water and freeze-dried. Dry material was dissolved in distilled water (220 μl), and a 75 μl fraction was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions. Gel 1, reference C1r; gel 2, reference C1s; gel 3, reference C1 Inh; gel 4, reference C1 Inh–C1r complex (with a major band corresponding to C1 Inh in excess and minor bands featuring free C1r and proteolysed C1r–C1 Inh complexes); gel 5, reference C1 Inh–C1s complex (with free C1 Inh and C1s); gel 6, hydroxylamine-treated C1r; gel 7, hydroxylamine-treated C1s; gel 8, hydroxylamine-treated C1 Inh; gel 9, hydroxylamine-treated C1 Inh–C1r complex; gel 10, hydroxylamine-treated C1 Inh–C1s complex.

to a new 5.6–5.8 S value, revealing a lability of the C1r–C1s interaction inside the tetrameric inhibited complex. Sodium dodecyl sulphate/polyacrylamide gel-electrophoresis analysis of the alkaline incubation medium showed mainly C1r–C1 Inh and C1s–C1 Inh complexes, with small amounts of split products from C1s also detected in the upper part of the sucrose density gradient (Fig. 6*c*).

Influence of nucleophiles. The influence of nucleophilic reagents was tested on the C1s–C1 Inh complex prepared from the purified individual proteins, in order to study a putative ester bond involving a serine residue at the active site of C1s and an acidic group in C1 Inh. Treatment with 1 M-hydroxylamine resulted, only under denaturing conditions, in a partial hydrolysis of the C1s–C1 Inh bond at pH 7.5 (Fig. 7*a*) and in its total hydrolysis at pH 9.0 (Fig. 7*b*); a similar result was obtained for the C1r–C1 Inh complex (Fig. 7*b*). Parallel controls at pH 9.0 without nucleophile showed that there was no pH-dependent hydrolysis.

In non-denaturing conditions, attempts to prevent the formation of C1s–C1 Inh complexes from C1s and C1 Inh by hydroxylamine were unsuccessful:

under conditions preserving the total enzymic activity of $C\bar{1}s$, $C\bar{1}s$–C1 Inh complex would be formed in the presence of 1M-hydroxylamine.

Methylamine at concentrations up to 50mM, in the same denaturing condition as for hydroxylamine, had no effect on hydrolysis of the $C\bar{1}s$–$C\bar{1}$ Inh bond.

Discussion

The study of the $C\bar{1}s$–$C\bar{1}$ Inh and $C\bar{1}r$–$C\bar{1}$ Inh interactions required the use of iodinated proteins. In checking the influence of the label on the functions of these proteins it was observed that $C\bar{1}$ Inh and dimeric C1r, $C\bar{1}r$, C1s and $C\bar{1}s$ were not altered in their catalytic or binding activities by iodination. However, in the case of monomeric C1s or $C\bar{1}s$ the binding capacity was modified: the ^{125}I-labelled activated or proenzymic monomer of C1s was no longer able to form dimers in the presence of Ca^{2+}. It was checked that ^{125}I-labelled activated or proenzymic dimeric C1s was able, after monomerization in EDTA, to re-form dimers. This suggests that the binding of iodine to tyrosine residue(s) located on the A-chain of C1s interferes with the monomer–monomer interaction. This behaviour of ^{125}I-labelled $C\bar{1}s$ was used in the study of its interaction with $C\bar{1}r$: monomeric ^{125}I-labelled $C\bar{1}s$ forms a $C\bar{1}r_2$–$C\bar{1}s_2$ tetramer on incubation with $C\bar{1}r$ in the presence of Ca^{2+}, which tends to show that the dimeric form of $C\bar{1}s$ may not be essential for the $C\bar{1}r$–$C\bar{1}s$ interaction.

The interaction between isolated $C\bar{1}s$ and C1 Inh showed that $C\bar{1}s$ labelled in the presence of EDTA was no longer able to form a tetrameric $C\bar{1}s_2$–$C\bar{1}$ Inh_2 complex on incubation with $C\bar{1}$ Inh in the presence of Ca^{2+}, which rules out any Ca^{2+}-dependent interaction within the $C1s_2$–C1 Inh_2 complex between the two molecules of $C\bar{1}$ Inh.

The interaction of $C\bar{1}r$ and $C\bar{1}$ Inh in the presence of EDTA resulted in $C\bar{1}$ Inh–$C\bar{1}r$ dimeric complexes. This observation can be compared with results published by Sim *et al.* (1979*a*), showing that in $C\bar{1}$ the reaction between $C\bar{1}$ Inh and $C\bar{1}r$ is the rate-limiting step for the dissociation of the first component of complement: the effect of $C\bar{1}$ Inh on dimeric $C\bar{1}r$ may be attributed to a conformational change imposed on $C\bar{1}r$ by the binding of $C\bar{1}$ Inh on the B-chain of the proteinase and transmitted to the A-chain, which participate in the inter-monomer link (Arlaud *et al.*, 1980*a*). This would result in a weakening of the inter-monomer bond in $C\bar{1}r_2$ in component $C\bar{1}$.

When $C\bar{1}$ Inh was incubated with $C\bar{1}r$ and $C\bar{1}s$ in the presence of Ca^{2+}, an 8.5S complex was formed that was probably similar to the 9.0S complex described by Ziccardi & Cooper (1979) from disassembly of aggregated immunoglobulin G–$C\bar{1}$ by $C\bar{1}$ Inh. This complex was stable in the presence of EDTA, and could be formed from isolated components in the presence of EDTA, a finding that adds to the preceding observation: the binding of $C\bar{1}$ Inh to the B-chain of $C\bar{1}r$ or $C\bar{1}s$ results in a strengthening of the interaction between $C\bar{1}r$ and $C\bar{1}s$ within the $C\bar{1}$ Inh–$C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ Inh complex with a decrease in the $C\bar{1}r$–$C\bar{1}r$ interaction. In both cases the area of interaction may be located in the A-chain of the proteinases (Arlaud *et al.*, 1980*a*), which is in agreement with a conformational change taking place in the A-chain on the binding of $C\bar{1}$ Inh to the B-chain.

A similar interaction appeared also in the complexes formed from the interaction of $C\bar{1}$ Inh with di-isopropylphosphoro-$C\bar{1}r_2$–$C\bar{1}s_2$ or $C\bar{1}r_2$–di-isopropylphosphoro-$C1s_2$ complexes. One may then question whether (1) the binding of one $C\bar{1}$ Inh molecule with the subsequent modification of one A-chain in the proteinase linked to $C\bar{1}$ Inh is able to reinforce the $C\bar{1}r$–$C\bar{1}s$ interaction within the inhibited complex or (2) the binding of di-isopropyl phosphorofluoridate to one of the two proteinases of the complex mimics the binding of C1 Inh. This latter hypothesis appears unlikely, as di-isopropyl phosphorofluoridate treatment of $C\bar{1}r_2$ does not lead to monomerization of the proteinase.

In any case this strengthening of the $C\bar{1}r$–$C\bar{1}s$ interaction confirms the existence of a non-Ca^{2+}-dependent affinity between the two proteinases, as shown previously (Arlaud *et al.*, 1980*a*). The role of Ca^{2+} is difficult to discuss in the absence of any data on the possible presence of clustered Ca^{2+} in the $C\bar{1}$ Inh–$C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ Inh complexes. The results obtained in the case of the incubation of $C\bar{1}$ Inh with mixtures of $C\bar{1}s$ and $C\bar{1}r$ II, a fragment of $C\bar{1}r$ lacking a Ca^{2+}-binding site involved in the $C\bar{1}r$–$C\bar{1}s$ interaction, tend to prove that the peptide missing in $C\bar{1}r$ II plays a direct role in addition to its binding capacity for Ca^{2+}, as no tetrameric inhibited complex was detected. As it was found possible, from the individual $C\bar{1}r$–$C\bar{1}$ Inh and $C\bar{1}s$–$C\bar{1}$ Inh complexes, to re-form a tetrameric $C\bar{1}$ Inh–$C\bar{1}r$–$C\bar{1}s$–$C\bar{1}$ Inh 8.5S complex on mixing [an observation that is at difference with the previous findings by Ziccardi & Cooper (1979)], a direct role of Ca^{2+} within this tetrameric complex is unlikely.

From the results obtained by hydroxylaminolysis, both the $C\bar{1}r$–$C\bar{1}$ Inh and $C\bar{1}s$–$C\bar{1}$ Inh interactions appear to be due to ester bonds, with the probable implication of the serine of the B-chain of the proteinases. Minta & Aziz (1981) have produced chemical evidence for the implication of a lysine residue of $C\bar{1}$ Inh in the $C\bar{1}s$–$C\bar{1}$ Inh interaction; it cannot be excluded that modification of the ε-amino group of a lysine residue induces perturbations on a neighbouring putative carboxylic group. Obviously

more work is needed on this precise point, as we were unable to get more direct evidence for the involvement of a carboxylic group from a labelling with [^{14}C]methylamine. The overall structure of C$\bar{1}$ Inh, with a large carbohydrate moiety, does not facilitate a direct chemical approach on the native molecule. In our attempt to denature C$\bar{1}$ Inh mildly for such studies we noticed that concentrations of mercaptoethanol as high as 25 mM were required to abolish C$\bar{1}$ Inh activity. This observation reflects probably a difficult access to the interior of the C$\bar{1}$ Inh molecule which may account also for the inefficiency of mild nucleophiles on the C$\bar{1}$ Inh–C$\bar{1}$r or C$\bar{1}$ Inh–C$\bar{1}$s bond.

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