Activation of the First Component of Human Complement (C1) by Antibody–Antigen Aggregates

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The activation of subcomponents C1r and C1s in the first component of complement, C1, when bound to antibody–antigen complexes was investigated. Activation was followed both by the splitting of the peptide chains of subcomponents C1r and C1s and by the development of proteolytic activity. For the maximum rate of activation to occur, all components must be present in approximate molar proportions of antibody: C1q: C1r: C1s of 13:1:5:5. For activation of subcomponent C1s, subcomponents C1r or C1r, but not C1r inactivated with iPr_2P -F (di-isopropyl phosphorofluoridate), are effective. For activation of subcomponent C1r, subcomponents C1s, C1s or C1s inactivated with iPr_2P -F are effective. Subcomponent C1s is activated by C1r, and C1r is activated autocatalytically, probably through the formation of an intermediary C1r* in which the peptide chain is unsplit but a conformational change caused by interaction with the other components has led to the formation of a catalytic site able to split subcomponent C1r to C1r.

The first component of complement, C1, is bound by antibody–antigen aggregates and becomes activated to a proteinase which in turn activates components C2 and C4 and hence initiates complement fixation by the classical pathway (reviewed by Reid & Porter, 1975; Porter, 1977). Lepow *et al.* (1963) showed that component C1 contains three proteins, subcomponents C1q, C1r and C1s, which form a complex in the presence of Ca^{2+} . A fourth subcomponent, 'C1t', was also reported to be part of component C1 (Assimé & Painter, 1975), but the component-C1 haemolytic activity of serum can be accounted for in terms of the content of subcomponents C1q, C1r and C1s (Gigli *et al.*, 1976), and it is now apparent that 'subcomponent C1t' is not part of component C1 (Painter, 1977). Component C1 binds to aggregated antibody through subcomponent C1q. In the complex, subcomponent C1r binds directly to C1q, and subcomponent C1s binds directly to C1r (see Porter, 1977). The esterase–proteinase activity developed by component C1 on activation is inhibited

on reaction with iPr_2P -F (Becker, 1956) and is due to activated subcomponent C1s (Lepow *et al.*, 1963). Subcomponent C1r is activated to give C1r, which is a highly specific proteinase capable of splitting one or more bonds in subcomponent C1s, but not in component C2 or C4. Subcomponent C1r can also hydrolyse, at a low rate, a very restricted group of basic amino acid esters (Naff & Ratnoff, 1968; Sim *et al.*, 1977; Volanakis *et al.*, 1977).

Activated subcomponent C1r is believed to be responsible for the activation of subcomponent C1s in the C1 complex (Naff & Ratnoff, 1968; Gigli *et al.*, 1976) and subcomponent C1r has been reported to be autoactivatable both in solution and when component C1 is bound to antibody–antigen aggregates (Ziccardi & Cooper, 1976; Takahashi *et al.*, 1976).

We report that most preparations of subcomponent C1r are relatively stable in solution, and we have investigated the conditions of activation of subcomponent C1r when bound in the component C1 complex to antibody–antigen aggregates. The result shows that subcomponent C1r is autoactivatable only when bound in the component C1 complex to antibody–antigen aggregates and only if the whole C1 complex is present.

Materials and Methods

iPr_2P -F was purchased from Aldrich, Gillingham, Dorset, U.K., α -N-Boc-L-lysine *p*-nitrophenyl ester from Calbiochem, Hereford, U.K., *p*-nitrophenyl

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968). Activated components are indicated by a bar, e.g. C1̄. iPr_2P -F, diisopropyl phosphorofluoridate; iPr_2P -C1r, iPr_2P -C1s, subcomponents C1r and C1s respectively, inactivated with iPr_2P -F; Boc-, benzyloxycarbonyl-; -ONp; *p*-nitrophenyl ester.

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p'-guanidinobenzoate HCl (Gertler *et al.*, 1974) from Vega Fox Biochemicals, Tucson, AZ, U.S.A., and *p*-nitrophenyl *N*²-acetyl-*N*¹-benzylcarbazate (Elmore & Smyth, 1968) was a gift from Professor D. T. Elmore, Department of Biochemistry, Queens University, Belfast.

The subcomponents C1q, C1r, C1s, C1̄r and C1̄s were prepared as described by Gigli *et al.* (1976). They were mixed in equal weight ratios (molar proportions approx. C1q:C1r:C1s=1:5:5) to give maximal haemolytic activity of the whole component C1 (Gigli *et al.*, 1976). Antibody-antigen aggregates were prepared from rabbit anti-ovalbumin and ovalbumin, treated with 10mM-*i*Pr₂P-F and kept in suspension (10mg/ml) in 0.15M-NaCl at 2°C (Gigli *et al.*, 1976). In activation experiments antibody-antigen aggregates were added in 12 molar excess of antibody over subcomponent C1q in buffers containing 50mM-Tris/HCl, 100mM-NaCl and 5mM-CaCl₂, pH7.4. Activation experiments were done at 37°C.

Assay of components

Haemolytic assays of components and subcomponents were carried out in veronal/NaCl buffers as described previously (Gigli *et al.*, 1976).

The extent of activation of subcomponent C1s in component C1-antibody-antigen complexes was measured by incubating subcomponents C1q + C1r + C1s with antibody-antigen in the proportions described above. After incubation, samples were diluted with 100mM-sodium phosphate/100mM-NaCl/10mM-EDTA, pH6.0 at 0°C, and centrifuged (1500g, 10min). Subcomponent C1s activity in the supernatant was measured by hydrolysis of α -*N*-Boc-L-Lys-ONp at pH6.0 (Sim *et al.*, 1977). This extraction procedure dissolves all subcomponent C1s and also all subcomponent C1r from the bound component C1 complex. Dilution, lowering the pH to 6.0, the low temperature and the high ionic strength of the pH6.0 buffer, however, decrease further activation of subcomponent C1s by C1̄r in the supernatant to a negligible rate. The assay thus measures only activation occurring before resolubilization.

Activation of subcomponent C1r in the C1 complex was measured by incubating mixtures of subcomponents C1q, C1r, *i*Pr₂P-C1̄s and antibody-antigen. Subcomponent C1r was then resolubilized as described for subcomponent C1s above, and activity of subcomponent C1̄r in the supernatant was measured by activation of added subcomponent C1s (Sim *et al.*, 1977).

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate-containing buffers

Activation of subcomponents C1̄r and C1̄s was also monitored by electrophoresis in polyacrylamide gels

in buffers containing sodium dodecyl sulphate (Fairbanks *et al.*, 1971). Reduction of samples was by heating to 100°C for 4min in 50mM-dithiothreitol/4M-urea/1% (w/v) sodium dodecyl sulphate/0.1M-Tris/HCl, pH8.0, and for a further 2min after addition of 120mM-iodoacetamide. Unreduced samples were heated for 6min at 100°C in 20mM-iodoacetamide/4M-urea/1% (w/v) sodium dodecyl sulphate/0.1M-Tris/HCl, pH8.0. Subcomponents C1̄r and C1̄s move in this gel system as proteins of apparent mol.wt. 83000 after reduction, whereas subcomponents C1̄r and C1̄s after reduction gave two chains, one (C1̄r and C1̄s 'a' chain) of apparent mol.wt. 58000, and the other C1̄r 'b' chain, of apparent mol.wt. 36000, and C1̄s 'b' chain of apparent mol.wt. 29000 (Sim *et al.*, 1977).

Preparation of *i*Pr₂P-F-inactivated subcomponents C1̄r and C1̄s

The subcomponents were inactivated by addition of 2.5M-*i*Pr₂P-F in propan-2-ol to solutions of C1̄r or C1̄s (0.1–1mg/ml in 0.1M-sodium phosphate, pH7.4) to give a final concentration of 5mM-*i*Pr₂P-F. The mixture was left for 1h at 37°C, *i*Pr₂P-F was again added to 5mM and the mixture incubated at 37°C for 1h more; the solutions were then dialysed against 5mM-sodium acetate (pH5.5)/0.15M-NaCl at 2°C overnight. The haemolytic and enzymic activity of subcomponents C1̄r and C1̄s were decreased to less than 1% of the original values.

Treatment of subcomponents C1̄r and C1̄s with *p*-nitrophenyl-*p*'-guanidinobenzoate

In order to investigate the effect of *p*-nitrophenyl-*p*'-guanidinobenzoate on subcomponents C1̄r and C1̄s in conditions similar to those occurring during activation, the activation complex was prepared as described above and the subcomponent-C1̄s activity measured. *p*-Nitrophenyl-*p*'-guanidinobenzoate was added to the suspension at a final concentration of 1mM and incubated at 37°C for 15min. The precipitate was centrifuged (1500g 10min) and washed four times in buffer containing 25mM-Tris/HCl, 50mM-NaCl, 5mM-CaCl₂ and 5% (w/v) glucose, pH7.4, to remove all unchanged *p*-nitrophenyl-*p*'-guanidinobenzoate, and the subcomponent C1̄s activity measured again.

The effect of *p*-nitrophenyl-*p*'-guanidinobenzoate on subcomponent C1̄r activity was tested in a similar manner, except that the complex was formed by using inactivated subcomponent *i*Pr₂P-C1̄s instead of subcomponent C1̄s, and the subcomponent C1̄r measured as described by Sim *et al.* (1977).

Results

Stability of subcomponents C1r and C1s

Though as reported previously (Gigli *et al.*, 1976) the unactivated subcomponents were stable over weeks at 2°C and pH 5.5 in the presence or absence of Ca²⁺, some preparations of both subcomponents were converted partially into the active enzymes on incubation at 37°C, pH 7.4. This occurred more frequently with subcomponent C1r than with C1s. Substantial stabilization of subcomponent C1r and complete stabilization of subcomponent C1s was achieved by incubating with 5mM-iPr₂P-F in 0.1M-Tris/HCl buffer, pH 7.4, containing 5mM-Ca²⁺ at 37°C for 30min. In Fig. 1 the development of the ability to convert subcomponent C1s into C1s in EDTA-containing buffers is shown for three preparations of subcomponent C1r relative to activated C1r. None of the subcomponent C1r preparations showed initial activity, but this developed to 30% after 100min in one case and to 25% in 240min in another.

In studies of a large number of subcomponent C1r preparations, all of constant haemolytic specific activity, rates of activation have been found to be variable, but generally much lower than those reported by Ziccardi & Cooper (1976). The rate of activation, as well as the time-lag before activation was detectable, showed no correlation with subcomponent C1r concentration, indicating that the activation observed does not correspond to an inter-molecular autocatalytic process.

It is likely that this spontaneous activation in solution of subcomponent C1r was due to traces of contaminating proteinases not readily inactivated by iPr₂P-F and possibly themselves present as zymogens. However, attempts to remove completely the contaminating proteinase(s) have not been successful.

Further support for this view was obtained by incubating subcomponent C1r or C1s with an equal weight of subcomponent C1r at 37°C in EDTA-containing buffers. Subcomponents C1s was activated rapidly by C1r, but subcomponent C1r showed little or no activation as judged by enzyme assay (Fig. 2). It is concluded that subcomponent C1r, like C1s, is not auto-activatable in solution, contrary to the report of Ziccardi & Cooper (1976). Subcomponent C1r formed from 'autoactivation' of C1r in solution has been reported to be inactive in reconstituting C1 haemolytic activity (Ziccardi & Cooper, 1976; Assimeh *et al.*, 1978). However, the subcomponent C1r prepared as described by Gigli *et al.* (1976) is active in reconstruction of component C1 (Table 1), suggesting that apparent autocatalysis in solution may result in a degraded form of C1r.

Activation of subcomponents C1r and C1s in component C1 bound to antibody-antigen aggregates

Activation of subcomponent C1s. When component C1 is reconstituted from the subcomponents C1q, C1r and C1s in the molar proportions 1:5:5, i.e. optimum conditions for haemolytic activity (Gigli *et al.*, 1976), and is mixed with 12-fold molar excess of antibody-antigen aggregates over C1q in buffer containing 50mM-Tris/HCl, 100mM-NaCl and 5mM-Ca²⁺, pH 7.4, the rates of activation of subcomponents C1r and C1s are similar, when followed by electrophoresis of the mixtures in polyacrylamide gels in

Fig. 1. Stability of three different subcomponent C1r preparations

Samples containing 5µg of subcomponent C1r in 100µl of 57mM-sodium phosphate/5mM-EDTA, pH 7.4, were incubated at 37°C. Preparations: □, 1; △, 2; ●, 3. The ordinate shows the percentage activity relative to 5µg of subcomponent C1r as measured by activation of subcomponent C1s.

Fig. 2. Incubation of subcomponent C1r with C1r. For this, 6µg of subcomponent C1r (○), 6µg of C1r (□), or 6µg of both (△) in 70µl of 50mM-Tris/HCl/100mM-NaCl/10mM-EDTA, pH 7.4, were incubated at 37°C, and C1r activity was measured by activation of subcomponent C1s.

Table 1. Component C1 haemolytic activity of mixtures of subcomponents

The concentration of each subcomponent was 1 mg/ml, i.e. approximate molar proportions C1q:C1r:C1s of 1:5:5. iPr_2P -C1r and iPr_2P -C1s are C1r and C1s respectively which have been inactivated by treatment with iPr_2P -F. For details see the text.

Subcomponents added	$10^3 \times$ C1 haemolytic activity (units/ml)
C1q, C1r, C1s	1500
C1q, C1r, C1s	1250
C1q, C1r, C1s	3500
C1q, iPr_2P -C1r, C1s	100
C1q, C1r, iPr_2P -C1s	<50
C1q, C1s	75
C1q, C1r	<50
C1r, C1s	<50

Fig. 3. Activation of subcomponent C1s in mixtures of antibody-antigen aggregates with subcomponents C1q, C1r and C1s, at 37°C

Mixtures containing 90 μ g of antibody antigen, 12 μ g of subcomponent C1q, 12 μ g of subcomponent C1s, and 12 μ g of subcomponent C1r (●), C1r (○), iPr_2P -C1r (□) or no C1r (△), suspended in 100 μ l of 50 mM-Tris/100 mM-NaCl/5 mM-CaCl₂, pH 7.4, were incubated at 37°C. The reaction was stopped at various times and the C1s formed measured by its rate of hydrolysis of α -N-Boc-Lys-ONp.

buffers containing sodium dodecyl sulphate. If subcomponent C1r is replaced by C1r, activation of subcomponent C1s is somewhat more rapid as followed by assay of esterase activity, and if the subcomponent C1r is first inactivated with iPr_2P -F little activation of subcomponent C1s can be detected (Fig. 3). Subcomponent C1r has been shown to activate subcomponent C1s in solution containing Ca²⁺, and more rapidly in the presence of EDTA (Gigli *et al.*, 1976; Ziccardi & Cooper, 1976). Our results demonstrate that activation of subcomponent C1s in the component C1 complex is also by sub-

Fig. 4. Initial stage of the activation of subcomponent C1s, in mixtures containing antibody-antigen aggregates with subcomponents C1q, C1r and C1s at 37°C

Activation mixtures containing 500 μ g of antibody-antigen, 100 μ g of subcomponent C1q, 100 μ g of subcomponent C1s and 100 μ g of subcomponent C1r (●) or no subcomponent C1r (○) in 700 μ l of 50 mM-Tris/HCl/100 mM-NaCl/5 mM-CaCl₂, pH 7.4, preincubated before mixing, were incubated at 37°C. The reaction was stopped at various times, and the C1s formed measured by its rate of hydrolysis of α -N-Boc-Lys-ONp.

component C1r, as suggested by earlier workers (Naff & Ratnoff, 1968; Sakai & Stroud, 1974; Valet & Cooper, 1974), since little activation of subcomponent C1s occurs if C1r inactivated with iPr_2P -F is used to form the complex. There is no evidence of autoactivation of subcomponent C1s by C1s either in solution (Gigli *et al.*, 1976) or when bound in the component C1 complex to antibody-antigen aggregates. The slight activation of subcomponent C1s observed when C1r is omitted or when iPr_2P -C1r is used to form the component C1 complex is the same (Fig. 3) and is considered to be the result of trace contaminant proteinases. When the rate of subcomponent C1s activation is followed at short time intervals a lag phase of about 2 min is apparent (Fig. 4), suggesting a lag in the activation of subcomponent C1r (see below).

Haemolytic assays (Table 1) are in agreement with the conclusion that subcomponent C1r activates C1s, because in the component C1 complex containing iPr_2P -C1r or in the absence of C1r little haemolytic activity develops. The higher activity of mixtures of subcomponents C1q + C1r + C1s than of subcomponents C1q + C1r + C1s is probably due to the activation of components C2 and C4 by the bound subcomponent C1s continuing throughout the whole incubation period rather than only the part of the time after activation of subcomponent C1s.

Activation of subcomponent C1r

The rate of activation of subcomponent C1r was followed both by conversion from one peptide chain of apparent mol.wt. 83000 into two peptide chains of apparent mol.wts. 58000 and 36000 and by development of the ability to convert subcomponent C1s into a form capable of hydrolysing the substrate α -N-Boc-L-Lys-ONp at 25°C and pH 6.0 (Sim *et al.*, 1977).

When the splitting of subcomponent C1r was followed by polyacrylamide-gel electrophoresis, the rate of activation of subcomponent C1r in the C1q-C1r-C1s-antibody-antigen complex was the same whether subcomponent C1s, C1s or iPr_2P -C1s was used. However, if subcomponent C1s was omitted, no activation of subcomponent C1r occurred (Fig. 5, Fig. 7). Subcomponent C1s was therefore essential in the complex for subcomponent C1r activation to occur, but it had no proteolytic role, since iPr_2P -C1s was equally effective. If 10mM- iPr_2P -F was added to the C1q-C1r-C1s-antibody-antigen activation mixture, subcomponent C1r was activated, but little activation of subcomponent C1s occurred, presumably because the C1r was inactivated by iPr_2P -F as it was formed.

The absolute requirement for antibody aggregates, C1q and iPr_2P -C1s for subcomponent C1r activation is shown in Fig. 6. Two of the three components were kept constant while the third was varied and equivalent and near-maximal activity was obtained with Ab-Aq/C1q/C1r/C1s in the proportions 5:1:1:1 by wt. and 13:1:5:5 by molar proportions. This approximates to the activation mixture that has been used in other experiments (12:1:5:5). Activation of subcomponent C1r was negligible in the absence of any one of the three other components of the mixture.

When iPr_2P -C1s was used in activation mixtures the rate of activation of subcomponent C1r could be followed by its capacity to activate subcomponent C1s (added subsequently) (Sim *et al.*, 1977) as the iPr_2P -C1s had no interfering enzyme activity. The rate of activation of subcomponent C1r under the conditions used is shown in Fig. 7. At short time intervals there is a lag phase comparable with that seen when the activation of subcomponent C1s is followed, and it is concluded that in both experiments this is a measure of the autocatalytic activation of subcomponent C1r.

However, in the antibody-antigen-component C1 complex, activation of subcomponent C1s, but not of

Fig. 5. Electrophoresis in polyacrylamide gel in buffers containing sodium dodecyl sulphate of antibody-antigen aggregates and subcomponents C1q, C1r with or without C1s inactivated with iPr_2P -F in the presence and absence of p -nitrophenyl- p' -guanidinobenzoate/HCl

Key to gels: (1) antibody-antigen aggregate, C1q, C1r and iPr_2P -C1s, 0min; (2) antibody-antigen aggregate, C1q, C1r and iPr_2P -C1s, 15 min, 37°C; (3) antibody-antigen aggregate, C1q and C1r, 15 min, 37°C; (4) antibody-antigen aggregate, C1q, C1r and iPr_2P -C1s + 1 mM- p -nitrophenyl- p' -guanidinobenzoate, 15 min, 37°C. Each sample was electrophoresed before (U) and after reduction (R). In the unreduced samples the subcomponent C1r moves slightly more slowly than the subcomponent C1s. In the reduced samples, iPr_2P -C1s and subcomponent C1r split into a and b chains and move as indicated. Subcomponent C1r is not split on reduction and moves more slowly than before reduction.

Fig. 6. Absolute requirement for antibody-antigen aggregates, subcomponent C1q and subcomponent C1s, for activation of subcomponent C1r

(a) Activation mixtures containing 25 μg of antibody-antigen aggregates, 5 μg of subcomponent C1q and 5 μg of subcomponent C1r, with increasing amounts of iPr₂P-C1s in 100 μl of 50 mM-Tris/HCl/100 mM-NaCl/5 mM-CaCl₂, pH 7.4, were incubated for 5 min at 37°C. (b) Activation mixtures containing 25 μg of antibody-antigen aggregates, 5 μg of subcomponent C1r and 5 μg of iPr₂P-C1s, with increasing amounts of subcomponent C1q in 100 μl of 50 mM-Tris/HCl/100 mM-NaCl/5 mM-CaCl₂, pH 7.4, were incubated for 5 min at 37°C. (c) Activation mixtures containing 5 μg of subcomponent C1q, 5 μg of subcomponent C1r and 5 μg of iPr₂P-C1s, with increasing amounts of antibody-antigen aggregates in 100 μl of 50 mM-Tris/HCl/100 mM-NaCl/5 mM-CaCl₂, pH 7.4, were incubated for 5 min at 37°C. ○, Points at which the three activation mixtures are identical.

subcomponent C1r, is inhibited if 10 mM-iPr₂P-F is present in the activation mixture, suggesting either (a) that subcomponent C1r is not activated by C1r or (b) that the rate of reaction of iPr₂P-F with subcomponent C1r is slower than C1r activation by C1r but not slower than subcomponent C1s activation by C1r. Measurement of activation rates of subcomponents C1r and C1s show them to be comparable and when other inhibitors were tested, 1 mM-*p*-nitrophenyl *p*'-guanidinobenzoate/HCl but not 1 mM-*p*-nitrophenyl-*N*²-acetyl-*N*'-benzylcarbazate, would inhibit the activation of both C1r and C1s in the activation mixture (Fig. 5). Further, this inactivation by *p*-nitrophenyl *p*'-guanidinobenzoate/HCl is reversible. If the complex is centrifuged, washed in low-ionic-strength buffer, and re-incubated at 37°C in the isotonic Tris/saline buffer, activation now occurs. This is in contrast with the reaction of subcomponents C1r and C1s with *p*-nitrophenyl-*p*'-guanidinobenzoate/HCl, which is irreversible. If the complex of antibody-antigen, C1q, C1r and C1s, is first allowed to activate, then 1 mM-*p*-nitrophenyl *p*'-guanidinobenzoate/HCl added and the aggregates are spun

down and washed, all C1r and C1s activity is lost. Similarly, subcomponents C1r and C1s are inactivated irreversibly by *p*-nitrophenyl *p*'-guanidinobenzoate/HCl if treated in solution and the reactant is dialysed away. These results suggest that in the activation of subcomponent C1r to C1r an intermediate is formed which reacts rapidly but reversibly with *p*-nitrophenyl *p*'-guanidinobenzoate, and as judged by polyacrylamide-gel electrophoresis (Fig. 5) the reactive form of subcomponent C1r is produced in the complex before the peptide chain is split.

Discussion

Difficulty has been met throughout in obtaining preparations of subcomponent C1r stable at 37°C and at neutral pH. Instability of subcomponent C1s preparations could be eliminated by treatment with iPr₂P-F and it was most probably due to persistent traces of subcomponent C1r or C1r in the subcomponent C1s preparations, as Arlaud *et al.* (1977a,b) have obtained stable subcomponent C1s by treatment with anti-(subcomponent C1r) coupled to Sepharose.

Fig. 7. Activation of subcomponent C1r, as measured by rate of activation of C1s, in mixtures containing antibody-antigen aggregates, C1q, C1r and either iPr_2P -C1s or no C1s

For this $20\mu g$ of antibody-antigen aggregates, $4\mu g$ of subcomponent C1q, $4\mu g$ of subcomponent C1r and $4\mu g$ of iPr_2P -C1s (●) or no subcomponent C1s (○) in $100\mu l$ of $50\text{mM-Tris/HCl}/100\text{mM-NaCl}/5\text{mM-CaCl}_2$, pH 7.4, preincubated before mixing, were incubated at 37°C . The reaction was stopped at various times and the C1r formed measured by its ability to split added subcomponent C1s.

Subcomponent C1r sufficiently stable at 37°C to investigate its activation in antibody-antigen aggregates can be obtained by incubation with iPr_2P -F at 37°C in buffers containing 5mM-Ca^{2+} , but on further incubation in the absence of iPr_2P -F slow activation often occurs. Other proteolytic inhibitors such as soya-bean trypsin inhibitor, Trasylol or ϵ -amino-hexanoic acid did not stabilize subcomponent C1r in solution.

The haemolytic activity of subcomponents C1q and C1s in the absence of subcomponent C1r or in the presence of iPr_2P -C1r, and the slow activation of subcomponent C1s when mixed with subcomponent C1q and antibody-antigen aggregates, are almost certainly due to traces of subcomponent C1r, as found by Arlaud *et al.* (1977a,b). Subcomponent C1s will bind to antibody-antigen aggregates in the presence of Ca^{2+} , subcomponent C1q and subcomponent C1r, C1r or iPr_2P -C1r; but little activation occurs in the last case (Fig. 3), confirming that as in solution (Gigli *et al.*, 1976) subcomponent C1s is activated by C1r when bound in the component C1 complex to antibody-antigen aggregates.

In contrast, subcomponent C1r is activated if C1s inactivated with iPr_2P -F is used in forming the component C1 complex. Though subcomponent C1r in the presence of Ca^{2+} will bind to antibody-antigen-C1q no activation occurs in the absence of subcomponent C1s (Fig. 5, Fig. 7). Subcomponent C1s inactivated with iPr_2P -F has no proteolytic activity,

but it does fulfil the requirements for the activation of the subcomponent C1r in the bound complex, presumably by determining the intermolecular arrangement or conformation of subcomponent C1r. The activation of subcomponent C1r is dependent on the presence in the complex of exact proportions of subcomponent C1r to antibody-antigen aggregates and subcomponents C1q and C1s (Fig. 6).

The kinetics of activation of subcomponent C1r in the complex, whether measured directly (Fig. 7) or indirectly by following activation of subcomponent C1s (Fig. 4), show a lag phase of about 2 min under the conditions used. This suggests that the activation of subcomponent C1r is autocatalytic. Subcomponent C1r will not activate subcomponent C1r in solution (Fig. 2), nor does it appear likely that subcomponent C1r will activate subcomponent C1r when bound in the activation complex, as subcomponent C1r activation is not prevented by the presence of $10\text{mM-}iPr_2P$ -F, though the activation of subcomponent C1s by subcomponent C1r is inhibited. p -Nitrophenyl- p' -guanidinobenzoate/HCl will stop activation of subcomponent C1r in the complex if present, but the inhibition is reversible, as activation occurs if the p -nitrophenyl- p' -guanidinobenzoate/HCl is removed. p -Nitrophenyl- p' -guanidinobenzoate/HCl inactivates subcomponent C1r (and subcomponent C1s) irreversibly, and hence it is probable that p -nitrophenyl- p' -guanidinobenzoate/HCl in the complex is reacting with an activated form of subcomponent C1r, C1r*, able to split subcomponent C1r to give subcomponent C1r. C1r* is an unsplit form of subcomponent C1r which has a catalytic site able to react reversibly with p -nitrophenyl- p' -guanidinobenzoate/HCl and cause the hydrolysis of subcomponent C1r. The formation of C1r* is dependent on the interaction of the four components of the complex, namely antibody-antigen, C1q, C1r and C1s in definite proportions (Fig. 6), which presumably leads to a conformational change in subcomponent C1r to give C1r*. This change is slow and gives rise to the time lag observed under the conditions used here. With the reactants and the products of the reaction in an insoluble complex, a more definite interpretation of the kinetics is not possible. As p -nitrophenyl- p' -guanidinobenzoate/HCl reacts reversibly with C1r*, a direct demonstration that radioactive p -nitrophenyl- p' -guanidinobenzoate/HCl bound to unsplit subcomponent C1r was also impossible. However, the formation of the catalytic intermediary C1r* in the activation process appears to be the most probable explanation of the results obtained.

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