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The Unactivated Form of the First Component of Human Complement, C1

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The first component of complement, C1, was isolated unactivated from human serum by repeated additions of di-isopropyl phosphorofluoridate during isolation. The unactivated subcomponents were also isolated, and evidence is given that the three subcomponents C1q, C1r and C1s account wholly for the activity of component C1 in serum. No evidence could be found for a fourth subcomponent, C1t. The approximate molar proportions of the subcomponents in serum are C1q/C1r/C1s = 1:2:2. Optimum activity by haemolytic assay was found at approximate molar proportions C1q/C1r/C1s of 1:4:4. No activity was found when subcomponents were assayed singly or in pairs, except for subcomponents C1q and C1s, which in molar ratio 1:4 gave 15–20% of the activity of the mixture C1q+C1r+C1s. The proteolytic activity of the isolated subcomponent $\overline{C1s}$ varied according to the method of activation used. Subcomponents C1q+C1r+C1s and C1q+C1s in the presence of antibody–antigen aggregates were activated and inactivated simultaneously, showing a peak of activity and subsequent loss of activity. Both reactions are probably due to proteolysis, and analysis of the peptide bonds split will be necessary to distinguish these two phenomena.

Complement is a complex mixture of blood proteins which are activated sequentially, many by conversion of a proteolytic zymogen into an active proteinase, and which play an important role in the host defence against infection. There are two distinct ways of initiating activation of these proteins (Müller-Eberhard, 1975). The first, or classical pathway, is activated by the interaction with aggregates of IgG† or IgM. A second, the alternative pathway, can be activated by naturally occurring polysaccharides and by antibody–antigen interaction, but in this case activation involves a different mechanism from that used in the initiation of the classical pathway (Reid & Porter, 1975). In both cases activation leads to conversion of the third component (C3) into an active form and subsequent utilization of the late components, C5–C9. In the classical pathway the first component (C1) is converted by antibody–antigen aggregates from C1 into the active form $\overline{C1}$ (Lepow *et al.*, 1956). Component $\overline{C1}$ cleaves components C4 and C2 to give a complex proteolytic enzyme, $\overline{C42}$, which converts component C3 into C3b and C3a (Müller-Eberhard *et al.*, 1967). Component C3b continues in the pathway now common to both the classical and alternative pathways. Knowledge of the molecular mechanisms involved is increasing, but many steps are still unclear. In particular, study of the initiation of the activation of component C1 by antibody–antigen aggregates has been held up by the difficulty of isolating component C1, as it is easily converted into the $\overline{C1}$ form during fractionation, probably owing to non-specific activation by traces of proteolytic enzymes in the serum. This has been overcome by repeated addition of the serine esterase inhibitor Dip-F and by working at pH 5.5. The isolation of component C1 is described in this paper together with studies of the activation of the three subcomponent proteins C1q, C1r and C1s.

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† Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M (macroglobulin) (World Health Organisation, 1964); Dip-F, di-isopropyl phosphorofluoridate. The nomenclature of the components of complement is that recommended by World Health Organisation (1968). Activation of a component is indicated by a bar.

Materials and Methods

Outdated human plasma was obtained from the Churchill Hospital, Oxford, Oxon., U.K. It was made 20mM with $CaCl_2$ and left to clot overnight at 4°C. The clot was removed by centrifugation and the serum stored at −20°C.

Dip-F was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Before use the Dip-F was dissolved in propan-2-ol (dried over Na_2SO_4) at 2.5M and kept for short periods at −20°C. Dip-F and solutions were used in a fume cupboard. Pipettes etc. were immersed in alkali immediately after use. 1,10-Phenanthroline monohydrate (Sigma Chemical Co.) was dissolved in water at 4mM. Iodoacetamide

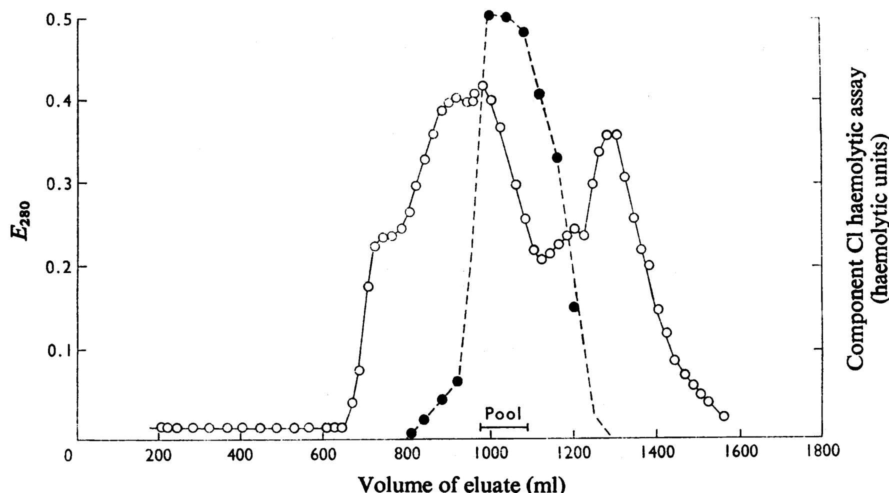


Fig. 1. *Elution of euglobulin from a column of Sepharose 6B*

The column (90cm × 5cm) was eluted with 0.2M-NaCl/0.05M-sodium acetate (pH 5.5)/5mM-$CaCl_2$. Active fractions were pooled as shown. ○, E_{280}; ●, Percentage lysis of sensitized cells in haemolytic assay of component C1.

(Fisons Scientific Reagents, Loughborough, Leics., U.K.) was recrystallized from ethanol.

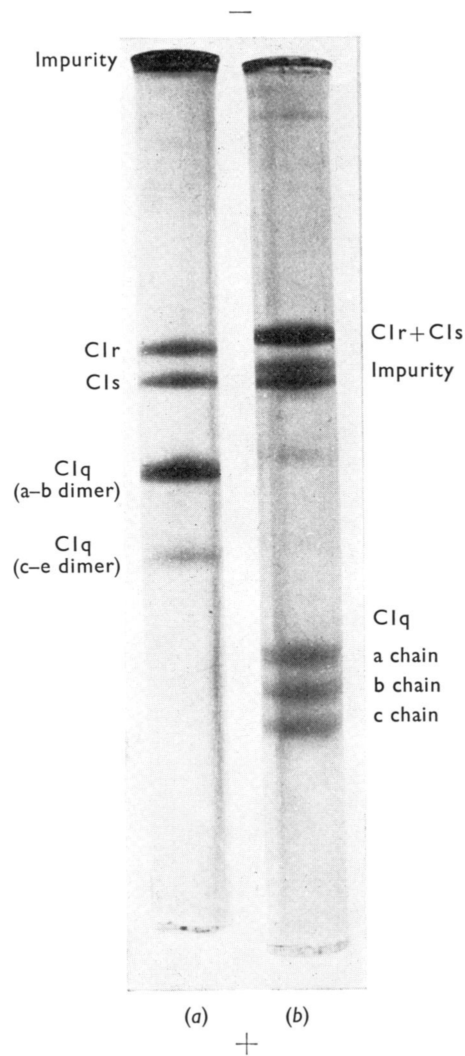
*Preparation of component C*1

Serum (500ml) was thawed, 1ml of 2.5M-Dip-F in propan-2-ol added and then centrifuged at 12500*g* for 30min at 2°C to remove any sediment. The supernatant was added to 2 litres of water at 2°C containing 5mM-$CaCl_2$, 2.5mM-iodoacetamide and 0.2mM-phenanthroline, together with 0.5ml of 2.5mM-Dip-F; 1–2ml of 1M-NaOH was added to re-adjust the pH to 7.4. The suspension was stirred in the cold-room for 2h and centrifuged at 12500*g* for 30min. The small precipitate of euglobulin was resuspended in 30ml of 0.04M-sodium acetate buffer (pH 5.5)/5mM-$CaCl_2$, 0.1ml of 2.5M-Dip-F was added and the mixture centrifuged. The precipitate was taken up in 15ml of 0.2M-NaCl/0.05M-sodium acetate buffer (pH 5.5)/5mM-$CaCl_2$, and 0.05ml of 2.5M-Dip-F added. This solution was centrifuged for 30min at 75000*g*, the film of fat and the sediment were discarded and the slightly opalescent supernatant was fractionated on a column (90cm × 5cm) of Sepharose 6B in the same buffer without Dip-F (Fig. 1). The haemolytically active fractions were pooled as shown and concentrated to 15ml by ultrafiltration after the addition of 0.1ml of 2.5M-Dip-F. The C1 preparations were stable and remained unactivated at 2°C over several weeks, though some precipitation occurred.

Separation of subcomponents C1q, C1r and C1s after the addition of EDTA to 10mM was by gradient elution on a column (30cm × 1.2cm) of DEAE-cellulose (Whatman DE32) with a starting buffer containing 10mM-NaH_2PO_4, 47mM-Na_2HPO_4, 5mM-EDTA, pH 7.4 (500ml), and a final buffer of the same composition (500ml), but containing 0.5M-NaCl (Lepow *et al.*, 1963). Subcomponent C1r was purified further by chromatography on a column (90cm × 2.5cm) of Sephadex G-200 in 0.05M-sodium acetate buffer (pH 5.5)/0.1M-NaCl/1mM-EDTA. This purification step removed the contaminant associated with the whole component C1, which was eluted from the DE32 cellulose column close to subcomponent C1r. Dip-F (2.5M) was added to the solutions of subcomponents C1q, C1r and C1s both before and after concentration to give approx. 5mM. All the subcomponents appeared to be pure as judged by electrophoresis in polyacrylamide gel in buffers containing sodium dodecyl sulphate (see Plate 2*a*).

Preparation of component $C\bar{1}$

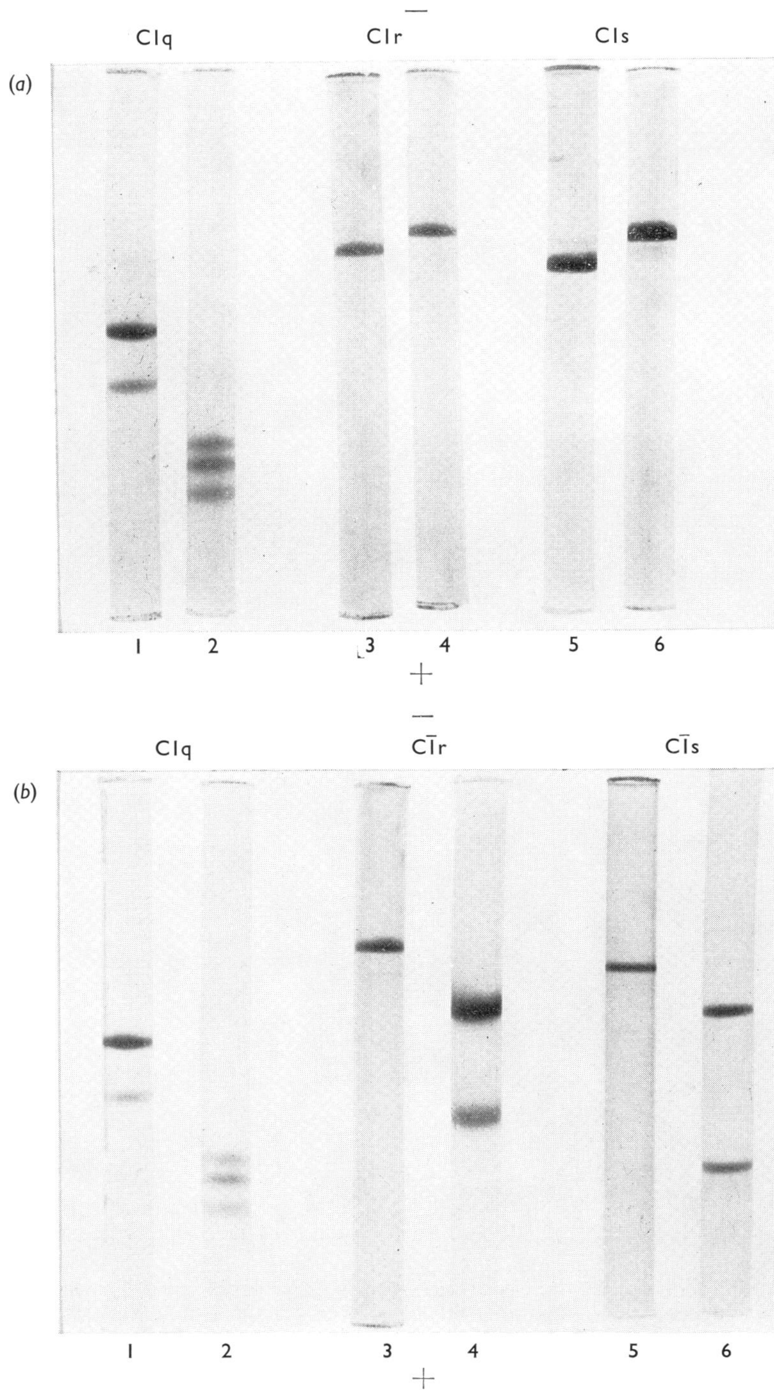
This could be prepared from component C1 by allowing activation to occur by adjusting the pH of the concentrated pool from the Sepharose 6B column to 7.4 without prior addition of Dip-F. Alternatively the C1 component from the Sepharose 6B column was adjusted to pH 7.4 in 0.08M-Tris/HCl/5mM-$CaCl_2$ after the addition of Dip-F to 5mM. After 2h at 0°C to allow the Dip-F to hydrolyse, preformed washed aggregates of ovalbumin and rabbit anti-ovalbumin (at a ratio of 1mg of antibody aggregate/5×10^4 C1 haemolytic units) were added and the



EXPLANATION OF PLATE I

*Electrophoresis of the first component of complement, C*1, *in* 5.6% *polyacrylamide gel containing sodium dodecyl sulphate*

Active pool from column of Sepharose 6B (Fig. 1) was electrophoresed: (*a*) unreduced; (*b*) reduced. Contaminants not entering the gel when unreduced move ahead of components C1r and C1s after reduction. Reduced C1r and C1s components have the same mobility, and reduced component C1q gives three peptide chains.



EXPLANATION OF PLATE 2

Electrophoresis in sodium dodecyl sulphate/5.6% polyacrylamide gel of activated and unactivated subcomponents of component C1

Unactivated (*a*) and activated (*b*) components C1q, C1r and C1s were electrophoresed: 1, C1q; 2, C1q reduced; 3, C1r; 4, C1r reduced; 5, C1s; 6, C1s reduced.

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suspension was incubated at 30°C for 15min. The suspension was cooled in ice, centrifuged, and the precipitate was washed twice with cold 0.1 M-NaCl. Component C$\bar{1}$ was eluted from the aggregates in 1 M-NaCl/50mM-EDTA/50mM-Tris/HCl, pH5.5. All the subcomponents C$\bar{1}$r and C$\bar{1}$s and 80–90% of the subcomponent C1q were eluted under these conditions and could be separated as described above for the unactivated subcomponents and appeared to be pure (see Plate 2*b*).

Preparation of component C4

Component C4 was separated from human serum by techniques developed from those of Schrieber & Müller-Eberhard (1974).

Preparation of rabbit anti-ovalbumin IgG and aggregates of ovalbumin–anti-ovalbumin

Rabbit IgG was obtained by precipitation with 16% (w/v) Na_2SO_4 of rabbit anti-ovalbumin serum. The precipitate was resuspended and dialysed against 0.15M-NaCl. The solution was heated at 56°C for 1h, and any precipitate formed was pelleted by centrifugation and discarded. The antibody content of the solution was estimated by precipitation with increasing concentrations of ovalbumin (Kabat & Mayer, 1961), and the aggregate was prepared by the addition of the optimum concentration of ovalbumin at 2°C. The precipitate was washed twice with cold 0.9% NaCl, resuspended in NaCl at 10mg/ml, then Dip-F was added to 1mM and the mixture stored at 2°C. It appeared to be stable for months.

Assays of components C1, C1q, C1r, C1s and C4

Haemolytic assays. Veronal/NaCl buffers were used as described by Nelson *et al.* (1966). Haemolytic assays for components C1, C1q, C1r, C1s and C4, and the cellular intermediates used in the titrations, were as described (Borsos & Rapp, 1963, 1967; Lepow *et al.*, 1963; Nelson *et al.*, 1966). Results were expressed in H_{50} units, i.e. the dilution of test material giving 50% haemolysis of 1.5×10^8 sensitized erythrocytes.

Component C$\bar{1}$s activity was measured by following the rate of inactivation of component C4 haemolytic activity (Gigli & Austen, 1969). Component C$\bar{1}$r activity was assayed by measuring the rate of conversion of component C1s into C$\bar{1}$s form.

Extinction coefficient of components C1q, C1r and C1s

The values taken for calculation of weight ratios of components were C1q E_{280} 0.68 (Reid *et al.*, 1972), C1r and C$\bar{1}$r E_{280} 1.15, and C1s and C$\bar{1}$s E_{280} 0.95 (R. B. Sim, unpublished work) at concentrations of 1mg/ml. For calculation of molar ratios the mol.wt. of component C1q was taken as 410000 (Reid *et al.*, 1972) and for components C1r, C1s, C$\bar{1}$r and C$\bar{1}$s 83000 (Sim & Porter, 1976).

Sodium dodecyl sulphate/polyacrylamide gel-electrophoresis

This was carried out as described by Fairbanks *et al.* (1971). When reduction of the samples was necessary they were incubated with 50mM-dithiothreitol/4M-urea/1% (w/v) sodium dodecyl sulphate/0.1M-Tris/HCl, pH8.0, at 37°C for 60min and then with 120mM-iodoacetamide for a further 30min at the same temperature. Unreduced samples were incubated at 37°C for 60min in 20mM-iodoacetamide/4M-urea/1% sodium dodecyl sulphate/0.1M-Tris/HCl, pH8.0. Coomassie Blue was used for staining protein (Weber & Osborne, 1969).

Results

Preparation of component C1

Repeated addition of Dip-F at all stages of the preparation of component C1 was essential to prevent spontaneous activation. Iodoacetamide, to inactivate thiol-dependent enzymes, and phenanthroline, to inactivate the zinc-dependent tissue collagenase, were also added to the serum on dilution for euglobulin precipitation. The recovery of component C1 activity in the euglobulin precipitate ranged from 50 to 80%, with 10–20% remaining in the supernatant. The overall recovery from the Sepharose column was high, often up to 90%, but the later active fractions were discarded (because a contaminant of apparent mol.wt. about 160000 was present), giving a final recovery of about 50% overall. The recoveries of activity in one experiment are shown in Table 1. When the material pooled from the Sepharose-column eluate was examined by electrophoresis in polyacrylamide gel in buffers containing sodium dodecyl sulphate, only subcomponents C1r, C1s and the two subunits of C1q were apparent (Plate 1*a*), but some material did not enter the gel. After reduction, the subcomponent C1r and C1s bands moved slightly more slowly and now had the same mobility, whereas component C1q gave the A, B and C chains and the contaminant was apparent moving a little ahead of the C1r and C1s bands (Plate 2*b*). The identification is apparent from the mobilities of the isolated subcomponent (Plate 2).

No activation of component C1 occurred during isolation, as judged by sensitivity of the haemolytic activity to inactivation by Dip-F nor by behaviour of the reduced complex on polyacrylamide-gel electrophoresis. After reduction, subcomponents C$\bar{1}$r and C$\bar{1}$s each separate into two components of lower

Table 1. *Recoveries of C1 activity during isolation*

	Volume (ml)	Haemolytic activity (units/ml)	(1×10^{-6} total units)	(%)
Serum	500	45000	22.5	100
Supernatant	2500	1200	3	13
Euglobulin solution	15.5	1100000	17	75
Pooled fractions from Sepharose 6B column	12	850000	10.1	45

Table 2. *Relative haemolytic activity of component C1 in a euglobulin precipitate and in a mixture of purified C1q, C1r and C1s subcomponents*

'Content' is expressed in arbitrary units by scanning of polyacrylamide-gel-electrophoresis tubes after staining with Coomassie Blue.

	Content of C1q	C1r	C1s	Component C1 haemolytic activity (units/ml)
Euglobulin	650	210	280	700000
Mixture	100	36	44	107000
Ratios $\frac{\text{euglobulin}}{\text{mixture}}$	6.5	5.8	6.4	6.5

Table 3. *Haemolytic activity of components C1q, C1r and C1s alone and in mixtures*

The concentration of each of the components was 1 mg/ml, i.e. approximate molar proportions C1q/C1r/C1s = 1:4:4. Activity is expressed as percentage of that of subcomponents C1q+C1r+C1s.

Subcomponents present	C1 haemolytic activity (units/ml)	(%)
C1q, or C1r, or C1s alone	<10000	0
C1q+C1r	60000	4
C1q+C1s	250000	18
C1r+C1s	<10000	0
C1q+C1r+C1s	1400000	100

molecular weight, whereas the unactivated subcomponents behave as single components of apparently slightly higher molecular weight (Plates 2*a* and 2*b*).

A comparison was made of the relative haemolytic activity of a euglobulin precipitate containing 70% of the original activity of the serum with a mixture of the purified components C1q, C1r and C1s in approximately the same relative concentration as found in the euglobulin. The concentrations were measured directly in both the euglobulin and the mixture by scanning polyacrylamide gels after staining with Coomassie Blue. Although the relative staining properties of components C1q, C1r and C1s are unknown, the proportions found for the content of each in the euglobulin and the artificial mixture is likely to be correct. When the haemolytic assay was also made, it showed that the ratios of haemolytic units were similar to those for the content of each component (Table 2). Although no high degree of accuracy can be claimed for the estimations, duplicate experiments gave similar results. It suggests that subcomponents C1q, C1r and C1s together account for the component C1 haemolytic activity of the euglobulin solution and hence of the original serum.

Estimation by scanning gives a proportion of C1q/C1r/C1s of 2.3:0.76:1 in the euglobulin and therefore presumably in the serum. If this is assumed to be equivalent to the proportions by weight, and as the mol.wts. are 410000, 83000 and 83000 for subcomponents C1q, C1r and C1s, the molar proportions would be 1:1.7:2.2, i.e. approx. 1 mol of C1q to 2 mol each of subcomponents C1r and C1s.

Relative contribution of subcomponents to the haemolytic activity of component C1

When subcomponents C1q, C1r and C1s were assayed alone on sensitized cells no significant component C1 haemolytic activity could be detected (Table 3). Similarly no activity was found in mixtures of subcomponents C1q and C1r nor of C1r and C1s forms, but a mixture of subcomponents C1q and C1s always showed significant haemolytic activity (15–20% of that of C1q mixed with C1r and C1s subcomponents in excess).

If subcomponents C1r and C1s were mixed at equimolar ratio and increasing amounts were added to subcomponent C1q, maximum C1 haemolytic activity was reached at molar ratios of C1q/C1r or C1q/C1s of almost 1:4 (Fig. 2). If subcomponent C1r alone was added to C1q there was no activity at any concentration. If subcomponent C1s alone was added, C1 haemolytic activity was developed to the extent of about one-sixth of that found on addition of equimolar mixtures of subcomponents C1r and C1s, and the maximum value was again at an approximate molar ratio of C1q/C1s of 1:4. This suggested interaction of subcomponent C1s with C1q on the

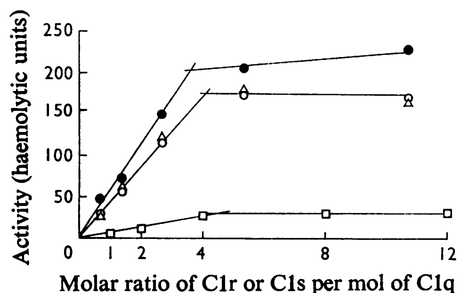

Fig. 2. *Assay of C1 haemolytic activities on the addition of increasing amounts of subcomponent C1r plus C1s to subcomponent C1q and the effect on activity of different ratios of C1r/C1s*

C1q with addition of mixture of C1r/C1s in molar ratio 1:1, △; C1q with addition of mixtures of C1r/C1s in molar ratio 2:1, ○; C1q with addition of mixtures of C1r/C1s in molar ratio 1:2, ●; C1q with addition of C1s alone, □. The abscissa gives the molar ratio of C1r or C1s, whichever is in least concentration, to C1q.

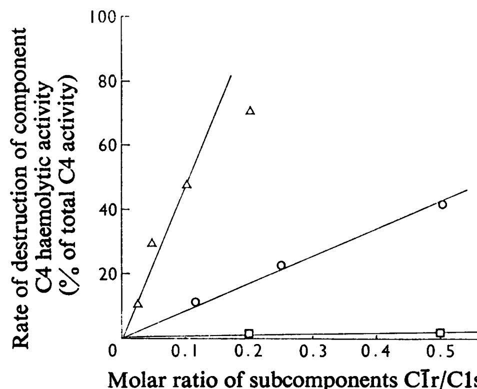

Fig. 3. *Activation of subcomponent C1s by C$\bar{1}$r in solution as measured by development of the capacity to destroy the haemolytic activity of component C4*

Subcomponent C1s was incubated with increasing ratios of subcomponents C$\bar{1}$r or C$\bar{1}$s for 60 min at 37°C, in iso-osmotic gelatine/veronal/NaCl buffer, pH 7.4 (Nelson *et al.*, 1966). Samples were then diluted 1×10^3–1×10^5-fold and incubated with component C4 for 15 min at 37°C and then the remaining activity of component C4 was assayed haemolytically. The results are expressed as the percentage of component C4 destroyed, corrected for the destruction by subcomponent C$\bar{1}$s if added. △, Subcomponents C1s+C$\bar{1}$r in 2 mM-EDTA; ○, subcomponents C1s+C$\bar{1}$r in 5 mM-$CaCl_2$; □, subcomponents C1s+C$\bar{1}$s in 2 mM-EDTA or 5 mM-$CaCl_2$.

sensitized erythrocytes independent of reaction via subcomponent C1r. This was confirmed by doubling first the ratio of subcomponent C1r/C1s in the mixture added. This caused no increase in activity. However, if the ratio C1s/C1r was doubled, 15–20% increased activity was measured at all ratios of C1r/C1q. This increase is comparable with the activity of subcomponent C1s and C1q alone, and confirms that subcomponent C1s can react with subcomponent C1q directly as well as through subcomponent C1r.

Activation of subcomponent C1s by C$\bar{1}$r in solution measured by destruction of component C4

Subcomponent C$\bar{1}$s destroyed the haemolytic activity of component C4 in solution in proportion to the C$\bar{1}$s concentration, but neither subcomponent C$\bar{1}$r nor C1q altered the haemolytic activity of component C4. Hence the activation of subcomponent C1s could be followed by increase in the capacity to destroy component C4. The activation in solution of subcomponent C1s by subcomponent C$\bar{1}$r in 1 h was decreased sixfold in the presence of Ca^{2+}, but Ca^{2+} did not influence the rate of destruction of component C4 by subcomponent C$\bar{1}$s. If subcomponent C$\bar{1}$s was added to subcomponent C1s no activation of the latter was found by measurement of destruction of component C4 after correction for that produced by the added subcomponent C$\bar{1}$s (Fig. 3). When the time of incubation of subcomponent C1s with C$\bar{1}$r was prolonged, complete conversion into subcomponent C$\bar{1}$s occurred whatever the ratio C$\bar{1}$r/C1s in the presence or the absence of Ca^{2+}.

Activation of subcomponent C1s by antigen–antibody aggregates measured by destruction of component C4

When subcomponents C1q, C1r or C1s were each incubated alone with antibody–antigen aggregates no capacity to destroy the haemolytic activity of component C4 was generated. Similarly no inactivation of component C4 occurred in this assay in mixtures containing subcomponents C1q and C1r or C1r and C1s with the aggregates. However, in agreement with the haemolytic data, in the presence of aggregates and subcomponents C1q and C1s, component-C4-destructive capacity developed. This was approx. 20% of the activity developed when subcomponents C1q, C1r and C1s were mixed with aggregates at molar proportions C1q/C1r/C1s of 1:2:2. In both cases, however, activation of subcomponent C1s by antibody–antigen aggregates was followed by loss of the capacity to hydrolyse component C4 (Fig. 4), presumably owing to continued proteolytic action. This finding may explain the variable activity of subcomponent C$\bar{1}$s, whether measured by destruction of component C4 or by direct haemolytic activity, depending on the method of activation. If no inhibitors were added during the preparation, the subcomponent C$\bar{1}$s was isolated

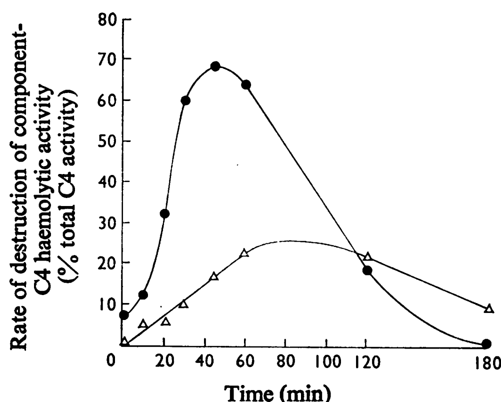

Fig. 4. *Activation of components C1 as measured by the development of capacity to destroy the haemolytic activity of component C4 when the subcomponents are mixed with antibody–antigen aggregates*

Antibody–antigen aggregates, prepared as described in the Materials and Methods section, were mixed with C1q at 1:50 (w/w) ratio of C1q to antibody and incubated at 37°C for 30min in veronal/NaCl iso-osmotic buffer (pH7.4) (Nelson *et al.*, 1966), containing 5mM-$CaCl_2$. The aggregates were centrifuged and resuspended in the same buffer, and subcomponents C1r and C1s or C1s alone were added in molar ratio of C1r/C1q or C1s/C1q of 4:1. Samples were removed at different times, diluted 10000-fold and incubated with component C4 for 30min at 37°C in the gelatine/veronal buffers used for haemolytic assay. The component C4 remaining was assayed haemolytically. Subcomponents C1q+C1r+C1s, ●; subcomponents C1q+C1s, △.

fully activated and had the highest activity. If prepared unactivated, and then activated in solution by the addition of subcomponent C$\bar{1}$r, or particularly by the addition of antibody–antigen aggregates to unactivated component C1, the subcomponent C$\bar{1}$s subsequently isolated showed a threefold range of specific activity (Table 4). The variability in activity of different preparations made by the same method of activation is presumably due to the simultaneous activation and destruction which made precise control difficult. Trace contamination of the subcomponents by other proteolytic enzymes may be another cause of variability.

Inactivation of subcomponents C1r and C1s by Dip-F

When Dip-F was added to subcomponent C$\bar{1}$r or C$\bar{1}$s at different concentrations and their activity assayed by their capacity to activate subcomponent C1s or to destroy component C4, the percentage inhibitions were very similar (Table 5). In spite of the striking differences in specificity of these two enzymes, the reaction with Dip-F is apparently similar.

Table 4. *Subcomponent C$\bar{1}$s specific activities as measured by the destruction of component-C4 haemolytic activity*

In each assay there were 4000 haemolytic units of components C4 and 0.1μg of subcomponent C$\bar{1}$s per ml. After 30min incubation at 37°C, the C4 haemolytic activity remaining was assayed and the loss of activity gave a measure of the enzyme activity of subcomponent C$\bar{1}$s. The results were expressed as a percentage of the enzyme activity of the most active preparation. Results are expressed as percentages of maximum activity.

Method of preparation of subcomponent C$\bar{1}$s	Specific activity (%)
Eluted from antibody–antigen aggregates	53, 60, 34
Activation of subcomponent C1s in solution by subcomponent C$\bar{1}$r	68, 60, 80
From component C$\bar{1}$ activated spontaneously during isolation	100, 92

Table 5. *Inhibition of subcomponents C$\bar{1}$r and C$\bar{1}$s by Dip-F*

Concentrations of subcomponents C$\bar{1}$r and C$\bar{1}$s were 100μg/ml, in 0.1M-Tris/HCl buffer, pH7.4. Incubation with Dip-F was for 60min at 37°C. Subcomponent C$\bar{1}$r activity was measured by activation of subcomponent C1s to C$\bar{1}$s. Subcomponent C$\bar{1}$s activity was measured by the capacity to destroy the haemolytic activity of component C4.

Concn. of Dip-F (μM)	Component	Activity (%)
12.5	C$\bar{1}$s	13
1.25	C$\bar{1}$s	74
0	C$\bar{1}$s	100
12.5	C$\bar{1}$r	19
1.25	C$\bar{1}$r	87
0	C$\bar{1}$r	100

Discussion

The preparation of unactivated component C1 from human serum necessitates the repeated addition of Dip-F throughout all the stages. Dip-F is hydrolysed rapidly in aqueous solution; but it is added in large molar excess over any proteinases likely to be present, the requirement for repeated addition suggesting that continuous slow activation of proteolytic zymogens is occurring. Trace contamination with such zymogens may persist throughout. Iodoacetamide and phenanthroline were also added as inactivators of possible proteolytic contaminants, but there was no certain evidence that they were necessary. The stability of unactivated component C1 in whole serum is probably due to the presence of proteinase inhibitors present in large excess. Their separation during the purification of component C1

increases the vulnerability of the components to proteolysis.

It has been suggested by several authors that component C1 contains a fourth subcomponent in addition to subcomponent C1q, C1r and C1s. Assimeh & Painter (1975) have now identified a component named C1t, which increases the haemolytic activity of mixtures of subcomponents C1q, C1r and C1s by 40%. These subcomponents are isolated by the binding of component C1 from whole serum to an IgG–Sepharose column and subsequent elution, and hence would be expected to be wholly or partly activated. Subcomponent C1t is characterized as binding to subcomponent C1q in the presence of Ca^{2+}, having a sedimentation coefficient of 9S in sucrose density gradients at neutrality and having a high mobility when electrophoresed in starch gel in acidic urea solutions. The latter suggests that subcomponent C1t dissociates under such conditions. Up to 85% of the haemolytic activity of component C1 was found in the euglobulin precipitate, and the remainder was left in the supernatant in the present study. The recovery from the column of Sepharose 6B was nearly 100%, though a part was discarded to avoid an overlapping impurity. The component C1 preparation thus isolated still contained a component in addition to subcomponents C1q, C1r and C1s, but it was a glycoprotein of high molecular weight which did not bind to antibody–antigen aggregates in the presence of components C1 and hence had quite different properties for subcomponent C1t. Further, a reconstituted mixture of electrophoretically homogeneous subcomponents C1q, C1r and C1s in similar proportions to those found in the crude euglobulin had the same C1 haemolytic activity per weight of subcomponents as the euglobulin. The protein concentrations were compared by scanning of electrophoresed gels stained with Coomassie Blue. The error of both haemolytic assay and protein determination may be as high as ±20%, but with that reservation the activity of the three subcomponents appears to account for that found in the euglobulin and presumably in whole serum. Hence no evidence was found for the presence of a fourth subcomponent in component C1.

In agreement with previous work (summarized by Reid & Porter, 1975; Gigli & Austen, 1969) it has been found that the haemolytic activity of component C4 is destroyed readily by subcomponent C$\bar{1}$s but not at all by subcomponent C$\bar{1}$r, and hence the rate of loss of haemolytic activity of component C4 can be used as a measure of activation of subcomponent C1s or of whole C1. Subcomponent C1s cannot be activated in solution by subcomponent C$\bar{1}$s, but only by subcomponent C$\bar{1}$r (contrary with the report by Morgan & Nair, 1975), and in solution the presence of subcomponent C1q has no influence on the rates of activation. When 5mM-$CaCl_2$ is present the rate of activation of subcomponent C1s by subcomponent C$\bar{1}$r decreases to 15% of that in the absence of Ca^{2+}. Subcomponents C1r and C1s form a complex in the presence of Ca^{2+}, and it is possible that this restricts the accessibility of the vulnerable peptide bonds in subcomponent C1s. The similarity in size and composition of subcomponents C1s and C1r (Sim & Porter, 1976) is further emphasized by their same susceptibility to inactivation by Dip-F (Table 5) and is in contrast with their marked difference in enzymic specificity.

It has been assumed that subcomponent C1r becomes auto-activatable owing to conformational changes after adsorption on antibody–antigen aggregates, probably through subcomponent C1q, and that this in turn activates subcomponent C1s. Yet we and others (Assimeh & Painter, 1975) find significant C1 haemolytic activity in mixtures of subcomponents C1q and C1s. No contamination by subcomponent C1r could be detected in either component, and, if the sensitized erythrocytes used for the haemolytic assay were prepared by using component C4 and subcomponent C$\bar{1}$s rather than whole component C1, no difference was found. Further evidence of the independent interaction of subcomponents C1q and C1s to generate component C1 haemolytic activity was given by assays with subcomponents C1q in the presence of increasing amounts of subcomponents C1r and C1s (Fig. 2). The activity of subcomponents C1q and C1s was additive over that of subcomponents C1q+C1r+C1s. This suggests that subcomponent C1s may be activated by direct interaction with subcomponent C1q bound on to the sensitized erythrocytes, independently of activation through subcomponent C$\bar{1}$r. The C1s/C1q molar ratio for optimum component C1 haemolytic activity was about 4:1 whether subcomponent C1r was present or not. Increasing the C1r/C1s ratio did not raise the haemolytic activity, suggesting that, when both are present together with Ca^{2+}, subcomponents C1r and C1s are in an equimolar complex.

By using the destruction of component-C4 haemolytic activity as assay, it was found that, in the presence of subcomponent C1q bound to antibody–antigen aggregates, subcomponent C1s became activated, but again only to about 20% of the activity found if subcomponent C1r was also present. With increasing time the content of activated subcomponent-C1s fell in both cases, though rather faster when subcomponent C1r was also present. It is likely that this loss of activity with time is due to continued proteolysis of subcomponent C$\bar{1}$s and that this must in part be due to autocatalytic destruction of subcomponent C$\bar{1}$s. These results, where the components are adsorbed on aggregates, are in contrast with those in solution, when no action of subcomponent C$\bar{1}$s on C1s could be detected.

The variable activity of isolated subcomponent

$C\bar{1}s$ (Table 4) measured by destruction of component-C4 haemolytic activity (and also by hydrolysis of a peptide substrate; R. B. Sim, unpublished work) may be due to the simultaneous activation and inactivation of subcomponent C1s. The relative rates of the two forms of proteolysis may depend on the condition under which this occurs. No difference between subcomponent $C\bar{1}s$ preparations of different activities was observed when they were compared by electrophoresis in polyacrylamide gel in sodium dodecyl sulphate. This suggests that the proteolysis causing inactivation is confined to the *N*- or *C*-terminal ends of one or both of the two constituent peptide chains, causing little change in molecular weight.

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