*Biochem. J.* (1979) **179**, 449–457
*Printed in Great Britain*



# C$\bar{1}$ Inhibitor-Dependent Dissociation of Human Complement Component C$\bar{1}$ Bound to Immune Complexes

By ROBERT B. SIM,* GÉRARD J. ARLAUD and MAURICE G. COLOMB
*DRF/Biochimie, Centre d'Etudes Nucléaires de Grenoble, 85X, 38041, et Université Scientifique et Médicale de Grenoble, Grenoble Cedex, France*

(*Received* 8 *September* 1978)

The interaction of C$\bar{1}$ Inhibitor with complement component C$\bar{1}$ bound to immune complexes was examined by using $^{125}$I-labelled C$\bar{1}$ subcomponents. The inhibitor binds rapidly to subcomponent C$\bar{1}$s, and more slowly to subcomponent C1$\bar{r}$. Formation of the C1$\bar{r}$–C$\bar{1}$ inhibitor complex causes rapid dissociation of subcomponents C1$\bar{r}$ and C1$\bar{s}$ from the antibody–antigen–component C$\bar{1}$ aggregate. The rate and extent of this release are proportional to C$\bar{1}$ Inhibitor concentration and are also dependent on ionic strength. Results obtained with purified C$\bar{1}$ Inhibitor, plasma or serum as source of C$\bar{1}$ Inhibitor are all closely comparable. Only slight dissociation of subcomponent C1q is observed under the same range of conditions. The implications of the release phenomenon are discussed in relation to the structure of component C$\bar{1}$ and the possibility of differential turnover of C$\bar{1}$ subcomponents.

The complement system in blood plasma is an enzyme cascade system involved in removal and destruction of foreign or antigenically altered substances. The system may be triggered by several different mechanisms, of which the best characterized is the interaction of complement component C1 with antibody–antigen complexes. Component C1 is a multimeric glycoprotein, consisting of three subcomponent types, C1q, C1r and C1s, the two last being serine-proteinase zymogens (Porter, 1977). Intersubcomponent binding forces are principally ionic, and are $Ca^{2+}$-dependent. The binding of subcomponent C1q in component C1 to antibody in immune complexes leads to activation of subcomponent C1r, which then activates subcomponent C1s by limited proteolysis (Dodds *et al.*, 1978). Subcomponent C1$\bar{s}$ in turn cleaves and activates further components in the complement cascade (Porter, 1977).

The active serine proteinases, subcomponents C1$\bar{r}$ and C1$\bar{s}$, are both of mol.wt. 83000, with structure similar to that of plasmin (Sim & Porter, 1976; Sim *et al.*, 1977). In common with other plasma proteinases, the activities of subcomponents C1$\bar{r}$ and C1$\bar{s}$ are regulated by endogenous proteinase inhibitors. Isolated subcomponents C1$\bar{r}$ and C1$\bar{s}$ have been shown to react *in vitro* in the fluid phase with C$\bar{1}$ In (Levy & Lepow, 1959; Ratnoff *et al.*, 1969), a glycoprotein of mol.wt. 100000 (Reboul *et al.*, 1977). Recent studies confirm that C$\bar{1}$ In is the only important inhibitor of subcomponents C1$\bar{r}$ and C1$\bar{s}$ in plasma (Sim *et al.*, 1979). Under physiological conditions, subcomponents C1r and C1s normally become activated when bound, within component C1, to immune complexes, rather than being generated as free independent proteinases in solution. Previous work (Holland *et al.*, 1972; Sumi & Fujii, 1974) has demonstrated that, on addition of ab–ag to serum, component C1 does bind to these aggregates, and is activated, but subsequent binding of C$\bar{1}$ In was not observed. It therefore appeared likely that, if C$\bar{1}$ In did interact with subcomponent C1$\bar{r}$ or C1$\bar{s}$ in ab–ag–C$\bar{1}$, it caused dissociation of the component C$\bar{1}$ molecule, such that the proteinase–(proteinase inhibitor) complex was released into the fluid phase. Recent work in this laboratory has confirmed this dissociation phenomenon (Arlaud *et al.*, 1979*a*), and in the present paper a more detailed analysis of the interaction of C$\bar{1}$ In with component C$\bar{1}$ bound to immune aggregates is presented.

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968). Activated components are indicated by an overbar, e.g. C1$\bar{r}$. iPr$_2$*P*-F, di-isopropyl phosphorofluoridate; iPr$_2$*P*-C1$\bar{r}$, iPr$_2$*P*-C1$\bar{s}$, subcomponents C1$\bar{r}$ and C1$\bar{s}$ respectively, inactivated with iPr$_2$*P*-F; ab–ag, antibody–antigen aggregates; ab–ag–C$\bar{1}$, component C$\bar{1}$ bound to antibody–antigen aggregates; C$\bar{1}$ In, C$\bar{1}$ Inhibitor; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

* Present address: MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OXI 3QU, U.K.

## Materials and Methods

Citrated human plasma was obtained from the Centre de Transfusion Sanguine, Grenoble, France.

Serum was prepared from the plasma as described before (Gigli *et al.*, 1976). $C\bar{1}$ In was isolated as described by Reboul *et al.* (1977). Subcomponent C1q was isolated by the method of Arlaud *et al.* (1979*b*) and labelled with $^{125}I$ as described by Heusser *et al.* (1973). Subcomponents $C1\bar{r}$ and $C1\bar{s}$ were isolated by either of two methods (Sim & Porter, 1976; Arlaud *et al.*, 1979*b*), which yield products equivalent in purity and activity. Labelling of subcomponents $C1\bar{r}$ and $C1\bar{s}$ with $^{125}I$ was by lactoperoxidase catalysis (Sim *et al.*, 1979). This labelling method has no detectable effect on the haemolytic or esterolytic activities of subcomponent $C1\bar{r}$ or $C1\bar{s}$, and does not modify their interaction with $C\bar{1}$ In. The preparation of subcomponents $C1\bar{r}$ and $C1\bar{s}$ inactivated by $iPr_2P$-F ($iPr_2P$-$C1\bar{r}$ and $iPr_2P$-$C1\bar{s}$) was as described by Dodds *et al.* (1978).

The sources of commercial products were as follows: $Na^{125}I$, The Radiochemical Centre, Amersham, Bucks., U.K.; lactoperoxidase (grade B), Calbiochem, San Diego, CA, U.S.A.; $iPr_2P$-F, Serva, Heidelberg, Germany; rabbit anti-($C\bar{1}$ In) antiserum, Behring, Marburg/Lahn, Germany; materials for polyacrylamide gels: sodium dodecyl sulphate and iodoacetamide, Merck, Darmstadt, Germany; gelatin and hen ovalbumin, Sigma, St. Louis, MO, U.S.A.; agarose (indubiose A 37), I.B.F., Clichy, France; *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester, Interchim, Montluçon, France, and flufenamic acid (2-{[3-(trifluoromethyl)-phenyl]amino}benzoic acid), Aldrich-Europe, Beerse, Belgium. Other reagents and chemicals were from Merck, or Prolabo (Rhône-Poulenc Industries), Paris, France.

### *Protein determination*

The isolated proteins $C\bar{1}$ In and subcomponents C1q, $C1\bar{r}$ and $C1\bar{s}$ were quantified from their specific absorption at 280nm, by using respectively $A_{1cm}^{1\%}=$ 4.5, 6.8, 11.7 and 9.4 (Harpel, 1976; Reid *et al.*, 1972; Sim *et al.*, 1977).

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

Preparation of rabbit anti-(hen ovalbumin) antisera, subsequent $Na_2SO_4$ precipitation of the immunoglobulin fraction from these sera and the formation, at equivalence, of anti-ovalbumin IgG–ovalbumin aggregates was as described previously (Gigli *et al.*, 1976; Arlaud *et al.*, 1979*a*).

### *Preparation of anti-($C\bar{1}$ In) coupled to Sepharose 6B, and use in the depletion of $C\bar{1}$ In from serum or plasma*

The total immunoglobulin fraction from 5ml of commercial anti-($C\bar{1}$ In)antiserum was prepared and coupled to 10ml (packed volume) of CNBr-activated Sepharose 6B as described previously (Sim *et al.*, 1979). Serum or plasma depleted of $C\bar{1}$ In antigenicity and activity was prepared by loading 0.5ml samples of serum or plasma on to a column (12cm × 1cm diam.) of the modified Sepharose equilibrated in 5mM-triethanolamine/HCl/145mM-NaCl, pH7.4. Samples were eluted in the same buffer at a rate of 2.5ml/h. The column was regenerated by washing with 1M-$NH_4SCN$. $C\bar{1}$ In in treated and untreated samples was estimated by single radial immunodiffusion (Mancini *et al.*, 1965) for which 4% (v/v) antiserum was incorporated in 1% agarose in 5mM-triethanolamine/HCl/145mM-NaCl/5mM-EDTA, pH7.4. Isolated $C\bar{1}$ In was used as a reference standard. The activity of $C\bar{1}$ In was quantified by measurement of the subcomponent $C1\bar{s}$ *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl esterase activity (Sim *et al.*, 1977) remaining after incubation (15min, 37°C) of isolated subcomponent $C1\bar{s}$ with samples containing $C\bar{1}$ In. Depletion of $C\bar{1}$ In in serum or plasma after passage through the affinity column was shown to be >90% by radial immunodiffusion, whereas the more sensitive activity measurement demonstrated that depletion was >98%.

### *SDS/polyacrylamide-gel electrophoresis*

Electrophoresis in 5.6% (w/v) polyacrylamide gels in buffers containing SDS was carried out as described by Fairbanks *et al.* (1971). Preparation of samples for electrophoresis and staining of gels with Coomassie Blue was as described previously (Sim *et al.*, 1977). Slicing of gels for radioactivity counting was carried out as described previously (Sim *et al.*, 1979).

### *Preparation of component $C\bar{1}$ bound to immune complexes (ab–ag–$C\bar{1}$)*

The component $C\bar{1}$ complex was reconstituted on ovalbumin–anti-ovalbumin complexes by the following general procedure: immune complexes (9mg) + subcomponent C1q (500μg) + subcomponent $C1\bar{r}$ (250μg) were incubated for 30min at 30°C in a total volume of 2.5ml of 5mM-triethanolamine/HCl, 145mM-NaCl/1.67mM-$CaCl_2$/830μg of gelatin/ml, pH7.4. The suspensions were then centrifuged (10min, 1000*g*) and the supernatants discarded. Subcomponent $C1\bar{s}$ (335μg) was then added to the precipitate in a total volume of 650μl of the same buffer, and the resuspended aggregates were incubated for 30min, at 30°C, then centrifuged as above. The precipitate was washed twice in 3ml of 2.5mM-triethanolamine/HCl/72.5mM-NaCl/2.5mM-$CaCl_2$, pH7.4. As summarized in Table 1 (below), modified ab–ag–$C\bar{1}$ complexes of various types were formed by using $^{125}I$-labelled subcomponent C1q in place of unmodified C1q, $iPr_2P$-$C1\bar{r}$, $^{125}I$-labelled $iPr_2P$-$C1\bar{r}$ or $^{125}I$-labelled $C1\bar{r}$ instead of subcomponent $C1\bar{r}$, and $iPr_2P$-$C1\bar{s}$, $^{125}I$-labelled $iPr_2P$-$C1\bar{s}$ or $^{125}I$-labelled $C1\bar{s}$ in place of unmodified subcomponent $C1\bar{s}$. The specific

radioactivity of $^{125}$I-labelled subcomponents was adjusted by dilution with the corresponding unlabelled subcomponent such that 175000–200000 c.p.m. was bound in the precipitate in each experiment.

*Dissociation of subcomponents from ab–ag–$C\bar{1}$*

The various forms of ab–ag–$C\bar{1}$ (see Table 1 below) were reconstituted as described above; in each case, only one subcomponent was $^{125}$I-labelled. The washed pellet was divided into two equal portions by resuspension and centrifugation in the wash buffer. One portion was resuspended in 5ml of a solution containing a known concentration of $C\bar{1}$ In (plasma or serum, diluted or undiluted, or isolated $C\bar{1}$ In in 5mM-triethanolamine/HCl/145mM-NaCl/5mM-$CaCl_2$, pH7.4). The other portion was resuspended in 5ml of a corresponding solution containing no $C\bar{1}$ In. Samples were incubated with constant shaking at 37°C. Aliquots (450$\mu$l) were withdrawn at various times and centrifuged for a minimum of 20s at 11000$g$ in an Eppendorf 3200 centrifuge. The pellet and supernatant were separated, and the percentage of the $^{125}$I-labelled subcomponent released into the supernatant was calculated after counting pellet and supernatant for radioactivity in an Intertechnique CG2000 gamma-counter.

*Complex-formation between $C\bar{1}$ In and $^{125}$I-labelled subcomponents $C1\bar{r}$ or $C1\bar{s}$ in ab–ag–$C\bar{1}$*

Complexes formed between subcomponents $C1\bar{r}$ or $C1\bar{s}$ and $C\bar{1}$ In are stable in the presence of SDS and urea (Harpel & Cooper, 1975; Nagaki, 1975; Arlaud *et al.*, 1979*a*) and may therefore be examined by polyacrylamide-gel electrophoresis in buffers containing SDS. During experiments on the kinetics of dissociation of $^{125}$I-labelled subcomponents $C1\bar{r}$ or $C1\bar{s}$ from ab–ag–$C\bar{1}$ in the presence of isolated $C\bar{1}$ In (see above), 50$\mu$l portions of suspension were withdrawn at intervals during incubation in order to assess the extent of reaction of the $^{125}$I-labelled subcomponent with C1 In. Reaction was stopped in the 50$\mu$l samples by addition of 50$\mu$l of 0.2M-Tris/HCl, 8M-urea/2% (w/v) SDS/40mM-iodoacetamide, pH8.0, at 100°C. Samples were then incubated at 100°C for 4min and examined by SDS/polyacrylamide-gel electrophoresis. Gels were stained with Coomassie Blue, sliced and counted to determine the distribution of $^{125}$I radioactivity. Radioactivity was found only at positions corresponding to the mobilities of free subcomponents $C1\bar{r}$ or $C1\bar{s}$ or of their complexes with $C\bar{1}$ In (Sim *et al.*, 1979), and no proteolytic degradation products were observed. The percentage of total labelled subcomponent present in the form of a complex with $C\bar{1}$ In was calculated.

## Results

*Reconstitution of component $C\bar{1}$ bound to immune complexes*

Incubation of $C\bar{1}$ subcomponents with ab–ag as described in the Materials and Methods section resulted in the binding, per 9mg of ab–ag, of the following quantities of subcomponents: C1q, 411 ± 3$\mu$g; $C1\bar{r}$, 131 ± 4$\mu$g, $C1\bar{s}$, 132 ± 4$\mu$g. Binding was calculated from the known specific radioactivity of $^{125}$I-labelled subcomponents, and the results are the means ± S.D. of 12 determinations. Competition binding studies between each $^{125}$I-labelled subcomponent and the corresponding unlabelled subcomponent showed that $^{125}$I-labelling does not affect binding affinity. Similarly, comparison of the binding of $^{125}$I-labelled subcomponents $C1\bar{r}$ or $C1\bar{s}$ with the binding of $^{125}$I-labelled and $iPr_2P$-F-inactivated subcomponents $C1\bar{r}$ or $C1\bar{s}$ showed that the inactivation does not influence binding. The quantities bound represent a molar ratio of subcomponents C1q/$C1\bar{r}$/$C1\bar{s}$ = 1.0 : 1.6 : 1.6, assuming mol.wts. of 410000, 83000 and 83000 respectively (Reid *et al.*, 1972; Sim & Porter, 1976). It is possible, in different incubation conditions, to increase the amount of subcomponents $C1\bar{r}$ and $C1\bar{s}$ bound, relative to subcomponent C1q. This is in agreement with previous findings (Porter, 1977; Arlaud *et al.*, 1979*b*) that under maximal binding conditions the molar stoicheiometry of component C1 bound to immune complexes is likely to be subcomponents C1q/C1r/C1s = 1 : 2 : 2 or 1 : 4 : 4. For the experiments described below, it is sufficient that the molar quantities of the two subcomponents reactive with $C\bar{1}$ In be equal, and saturation of bound subcomponent C1q with subcomponents $C1\bar{r}$ and $C1\bar{s}$ was unnecessary.

*Dissociation in the presence of serum of component $C\bar{1}$ bound to ab–ag*

Incubation of ab–ag–$C\bar{1}$ with diluted normal serum (Fig. 1*a*) resulted in gradual and parallel release of

Table 1. *Composition of the various forms of ab–ag–$C\bar{1}$ complexes discussed in the text*

An asterisk (*) indicates the $^{125}$I-labelled subcomponent.

| Type | | | |
|---|---|---|---|
| I | ab–ag–C1q*–$C1\bar{r}$ | | –$C1\bar{s}$ |
| II | ab–ag–C1q | –$C1\bar{r}$* | –$C1\bar{s}$ |
| III | ab–ag–C1q | –$C1\bar{r}$ | –$C1\bar{s}$* |
| IV | ab–ag–C1q | –$iPr_2P$-$C1\bar{r}$* | –$C1\bar{s}$ |
| V | ab–ag–C1q | –$iPr_2P$-$C1\bar{r}$ | –$C1\bar{s}$* |
| VI | ab–ag–C1q | –$C1\bar{r}$* | –$iPr_2P$-$C1\bar{s}$ |
| VII | ab–ag–C1q | –$C1\bar{r}$ | –$iPr_2P$-$C1\bar{s}$* |
| VIII | ab–ag–C1q | –$iPr_2P$-$C1\bar{r}$* | –$iPr_2P$-$C1\bar{s}$ |
| IX | ab–ag–C1q | –$iPr_2P$-$C1\bar{r}$ | –$iPr_2P$-$C1\bar{s}$* |

subcomponents C1$\bar{r}$ and C1$\bar{s}$, which reached a maximum of about 60% after 15–20 min incubation at 37°C. In corresponding experiments in which ab–ag–C$\bar{1}$ was incubated either with serum depleted of C$\bar{1}$ In by passage through anti-(C$\bar{1}$ In) linked to Sepharose 6B or with the dilution buffer alone (Fig. 1*a*), release of subcomponents C1$\bar{r}$ and C1$\bar{s}$ was much less, reaching a maximum of 10% after 10–12 min. The small extent of dissociation seen in the two latter cases is likely to be simply an equilibrium effect due to dilution. The behaviour of subcomponent C1q in ab–ag–C$\bar{1}$ under identical incubation conditions is shown in Fig. 1(*b*). In the presence of (C$\bar{1}$ In)-depleted serum, or buffer alone, a slight equilibrium dissociation of 6–7% of the bound subcomponent C1q occurs. In diluted normal serum, release of subcomponent C1q is significantly greater (maximum 9–10%) than in the controls, but is much less than the observed release of subcomponents C1$\bar{r}$ and C1$\bar{s}$ (Fig. 1*a*).

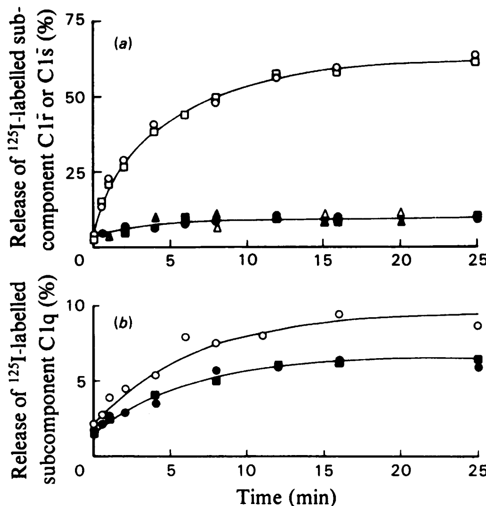


Fig. 1. *Dissociation of immune-complex-bound component C$\bar{1}$ in the presence of serum*

Ab–ag–C$\bar{1}$ complexes of types II and III (Fig. 1*a*) or type I (Fig. 1*b*) were formed, and incubated at 37°C with normal serum, diluted with 1 vol. of 5 mM-triethanolamine / HCl / 145 mM - NaCl / 5 mM - CaCl$_2$, pH 7.4, or with C$\bar{1}$ In-depleted serum, diluted identically, or with the dilution buffer alone. The concentration of bound subcomponent (C1$\bar{r}$ + C1$\bar{s}$) in suspension was 26 $\mu$g/ml in each case. (*a*) Release of subcomponents C1$\bar{r}$ (○) and C1$\bar{s}$ (□) in normal serum; release of subcomponents C1$\bar{r}$ (●) and C1$\bar{s}$ (■) in C$\bar{1}$ In-depleted serum; release of subcomponents C1$\bar{r}$ (△) and C1$\bar{s}$ (▲) in dilution buffer. (*b*) Release of subcomponent C1q in normal serum (○), in C$\bar{1}$ In-depleted serum (●) and in buffer (■). For identification of ab–ag–C$\bar{1}$ complexes, see Table 1. For experimental details, see the text. Values shown are means for four experiments.

Similar results were obtained by using the same dilutions of citrated plasma and (C$\bar{1}$ In)-depleted plasma. Citrate, although a chelating agent, does not itself cause dissociation of component C$\bar{1}$ (Pfueller & Lüscher, 1972). Serum heated at 60°C for 60 min (Haines & Lepow, 1964) or treated with flufenamic acid (Kluft, 1977), both of which represent non-specific methods of destroying serum C$\bar{1}$ In activity, gave results identical with those obtained with serum depleted of C$\bar{1}$ In by the specific immunoaffinity method.

These results suggest that the specific interaction of C$\bar{1}$ In with bound component C$\bar{1}$ causes dissociation of the C1$\bar{r}$ and C1$\bar{s}$ subcomponents, while having only a minor effect on the binding of C1q. The relationship between the release phenomenon and the actual rate of reaction of purified C$\bar{1}$ In with subcomponents C1$\bar{r}$ and C1$\bar{s}$ was investigated as shown in Figs. 2(*a*)–2(*d*). By using ab–ag–C$\bar{1}$ complexes in which both subcomponents C1$\bar{r}$ and C1$\bar{s}$ were fully active (Fig. 2*a*), it was shown that, in the presence of C$\bar{1}$ In, subcomponents C1$\bar{r}$ and C1$\bar{s}$ were both released at the same rate, and their dissociation exactly paralleled the rate of combination of C$\bar{1}$ In with subcomponent C1$\bar{r}$. The initial rate of reaction of subcomponent C1$\bar{s}$ with C$\bar{1}$ In was, in contrast, considerably faster than the release reaction. In separate experiments (results not shown) a very small dissociation of subcomponent C1q was again shown to accompany release of subcomponents C1$\bar{r}$ and C1$\bar{s}$ (as in Fig. 1*b*).

Subcomponents C1$\bar{r}$ and C1$\bar{s}$ treated with iPr$_2$*P*-F do not form stable complexes with C$\bar{1}$ In (Arlaud *et al.*, 1979*a*). When ab–ag–C$\bar{1}$ was reconstituted with iPr$_2$*P*-C1$\bar{r}$ and iPr$_2$*P*-C1$\bar{s}$ and incubated with C$\bar{1}$ In (Fig. 2*b*), no complex-formation between C$\bar{1}$ In and the inactivated subcomponents occurred, and release of iPr$_2$*P*-C1$\bar{r}$ and iPr$_2$*P*-C1$\bar{s}$ was no greater than in the control incubation without C$\bar{1}$ In. If subcomponent C1$\bar{r}$ in the bound component C$\bar{1}$ was inactivated (iPr$_2$*P*-C1$\bar{r}$), but subcomponent C1$\bar{s}$ was unmodified (Fig. 2*c*), release of both subcomponents iPr$_2$*P*-C1$\bar{r}$ and C1$\bar{s}$ did occur, but the rates of release were no longer parallel. Subcomponent C1$\bar{s}$ combined with C$\bar{1}$ In at a rate slightly greater than seen in Fig. 2(*a*), but was released much more slowly and to a final extent of about 27%. iPr$_2$*P*-C1$\bar{r}$ did not form complexes with C$\bar{1}$ In, but underwent slight dissociation. When subcomponent C1$\bar{s}$, but not C1$\bar{r}$, was inactivated with iPr$_2$*P*-F (Fig. 2*d*), subcomponent C1$\bar{r}$ combined with C$\bar{1}$ In slightly faster than seen in Fig. 2*a*, and subcomponents C1$\bar{r}$ and iPr$_2$*P*-C1$\bar{s}$ were released together at a rate exactly parallel to the rate of reaction of C$\bar{1}$ In with subcomponent C1$\bar{r}$.

Results obtained with combinations of iPr$_2$*P*-F-inactivated subcomponents suggest the following

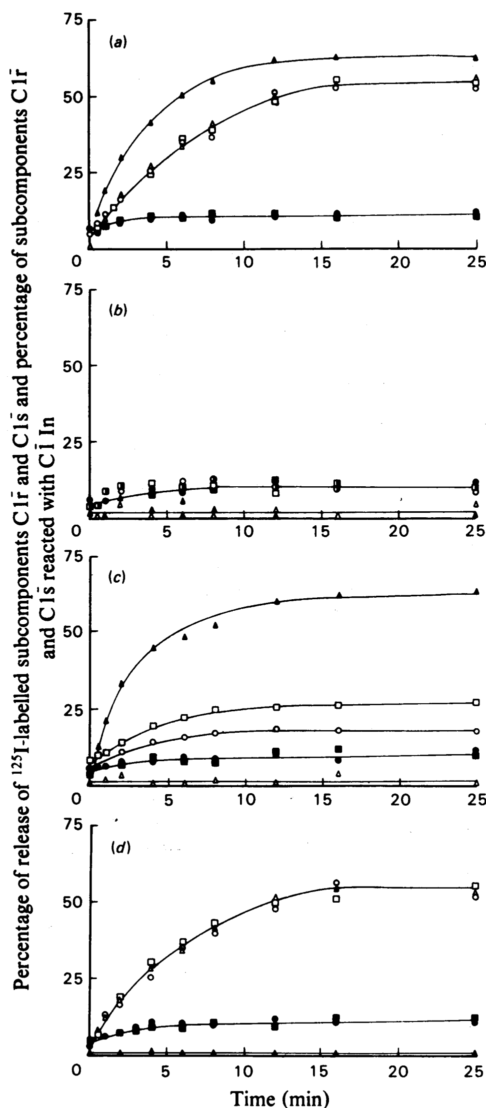


Fig. 2. *Dissociation of immune-complex-bound component C$\bar{1}$ in the presence of purified C$\bar{1}$ In*

Various forms of ab–ag–C$\bar{1}$ (for key, see Table 1) containing in each case only one $^{125}$I-labelled subcomponent, were reconstituted, then resuspended and incubated in buffer in the presence or absence of purified C$\bar{1}$ In as described in the text. The concentration of bound subcomponents (C1$\bar{r}$ + C$\bar{1}$s) in suspension was 26$\mu$g/ml, and C$\bar{1}$ In, where present, was at 118$\mu$g/ml. The release of $^{125}$I-labelled subcomponents into the fluid phase and their combination with C$\bar{1}$ In was measured as described in the Materials and Methods Section. (*a*) ab–ag–C$\bar{1}$ complexes of types II and III. (*b*) ab–ag–C$\bar{1}$ types VIII and XI. (*c*) ab–ag–C$\bar{1}$ types IV and V. (*d*) ab–ag–C$\bar{1}$ types VI and VIII. Symbols: (1) circles: release of $^{125}$I-labelled C1$\bar{r}$ or iPr$_2$*P*-C1$\bar{r}$ in the presence (○) or absence (●) of C$\bar{1}$ In; (2) squares: release of $^{125}$I-labelled C1$\bar{s}$ or iPr$_2$*P*-C1$\bar{s}$ in the presence (□) or absence (■) of C$\bar{1}$ In; (3) triangles: incorporation of either form of subcomponent C1$\bar{r}$ (△) or C1$\bar{s}$ (▲) into proteinase–C$\bar{1}$ In complexes. Values shown are the means for three experiments.

sequence of events: subcomponent C1$\bar{s}$ in ab–ag–C$\bar{1}$ reacts rapidly with C$\bar{1}$ In; this reaction destabilizes the binding of subcomponent C1$\bar{s}$ to ab–ag–C$\bar{1}$ to a small extent, but does not cause rapid dissociation; subcomponent C1$\bar{r}$ forms a complex more slowly with the inhibitor, and this reaction does bring about rapid release of both subcomponents C1$\bar{r}$ and C1$\bar{s}$ from the bound component C$\bar{1}$. The subcomponent C1$\bar{r}$–C$\bar{1}$ In interaction and subsequent dissociation proceeds whether or not subcomponent C1$\bar{s}$ is already complexed with C$\bar{1}$ In (Fig. 2*a*, cf. Fig. 2*c*). The parallel dissociation of subcomponents C1$\bar{r}$ and C1$\bar{s}$ (Figs. 2*a* and 2*d*) suggests that they are released initially as a large (C1$\bar{r}$ + C$\bar{1}\bar{s}$)–C$\bar{1}$ In complex. If, however, subcomponent C1$\bar{r}$ is prevented from binding C$\bar{1}$ In (iPr$_2$*P*-C1$\bar{r}$; Fig. 2*c*), a slower partial dissociation of subcomponent C1$\bar{s}$ occurs, probably in the form of (C$\bar{1}$ In)–C1$\bar{s}$ complexes. In this case much less subcomponent C1$\bar{r}$ than C1$\bar{s}$ is released, showing that the non-covalent binding between subcomponents iPr$_2$*P*-C1$\bar{r}$ and C1$\bar{s}$ is broken.

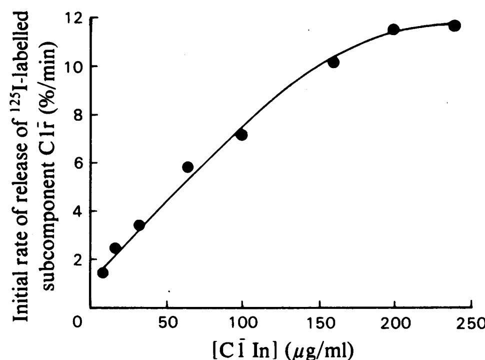


Fig. 3. *Variation in the rate of release of subcomponents C1$\bar{r}$ or C1$\bar{s}$ from ab–ag–C$\bar{1}$ as a function of C$\bar{1}$ In concentration*

ab–ag–C$\bar{1}$ complexes (types II and III) were formed as described in the text, and release of $^{125}$I-labelled subcomponents studied as a function of time at various concentrations of C$\bar{1}$ In. The ab–ag–C$\bar{1}$ was resuspended in a final volume of 15ml for these experiments, giving a final subcomponent (C1$\bar{r}$ + C1$\bar{s}$) concentration of 17$\mu$g/ml. The initial rate of release of bound subcomponent was determined from graphs of the type shown in Fig. 2(*a*). The rates of release of $^{125}$I-labelled C1$\bar{r}$ are shown; the rates for subcomponent C1$\bar{s}$ were the same.

Further experiments of the type shown in Fig. 2(*a*) were carried out with various concentrations of purified $C\bar{1}$ In. The initial rate of dissociation of subcomponents $C1\bar{r}$ and $C1\bar{s}$ from ab–ag–$C\bar{1}$ was shown (Fig. 3) to increase linearly with $C\bar{1}$ In concentration up to about 160$\mu$g/ml, and then to reach a plateau. Similarly, the final extent of dissociation of subcomponents $C1\bar{r}$ and $C1\bar{s}$ (i.e. the 'plateau' release value as seen in Figs. 1*a* and 2*a*) increases with increasing inhibitor concentration (Fig. 4). It is to be expected that the values of rate and extent of release shown in Figs. 3 and 4 will be dependent on the aggregation state of the immune complexes, which will in turn affect the accessibility of bound subcomponents $C1\bar{r}$ and $C1\bar{s}$ to $C\bar{1}$ In. Entrapping of subcomponents $C1\bar{r}$ and $C1\bar{s}$ in large aggregates is the most probable explanation for the observation that 100% release was never obtained (Fig. 4). The average physiological concentration of $C\bar{1}$ In is about 240$\mu$g/ml (Heimburger, 1975), and therefore falls in the range where maximal rate and extent of release are observed.

In agreement with previous results (Arlaud *et al.*, 1979*a*,*b*), the dissociation of subcomponents $C1\bar{r}$ and $C1\bar{s}$ from ab–ag–$C\bar{1}$ incubated with $C\bar{1}$ In is strongly dependent on ionic strength, and increases linearly within the range 30–170mM-NaCl (Fig. 5). In the absence of $C\bar{1}$ In, however, subcomponents $C1\bar{r}$ and $C1\bar{s}$ remain stably bound within ab–ag–$C\bar{1}$ up to about 140mM-NaCl. At the ionic strength used in experiments described in Figs. 1–4, the difference between release in the presence and absence of $C\bar{1}$ In is maximum.

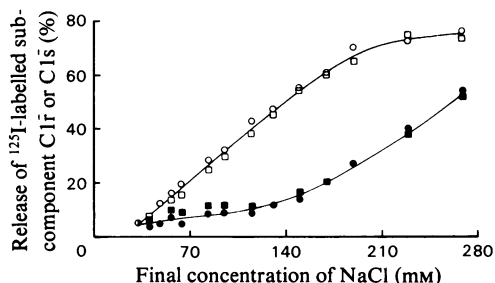


Fig. 5. *Influence of ionic strength on the release of subcomponents $C1\bar{r}$ or $C1\bar{s}$ from ab–ag–$C\bar{1}$ in the presence or absence of $C\bar{1}$ In*

ab–ag–$C\bar{1}$ complexes (types II and III) were prepared and resuspended in 10ml of 5mM-triethanolamine/HCl/145mM-NaCl/5mM-$CaCl_2$, pH7.4. Samples (200$\mu$l) of the suspension were transferred to duplicate tubes containing 750$\mu$l of various concentrations of NaCl (0–300mM). $C\bar{1}$ In (5$\mu$l, 16.7$\mu$g) in the same buffer was added to one series and 5$\mu$l of buffer alone to the other. Samples were incubated for 30min at 37°C, and the release of radio labelled subcomponent was assessed as described in the text. Symbols: release of $^{125}$I-labelled subcomponent $C1\bar{r}$ in the presence (○) and absence (●) of $C\bar{1}$ In; release of $^{125}$I-labelled subcomponent $C1\bar{s}$ in the presence (□) and absence (■) of $C\bar{1}$ In.

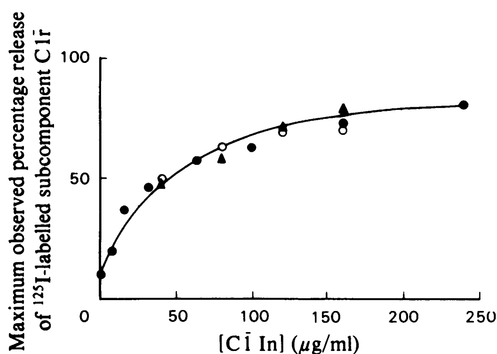


Fig. 4. *Variations in the value of maximum release of subcomponents $C1\bar{r}$ or $C1\bar{s}$ from ab–ag–$C\bar{1}$ as a function of $C\bar{1}$ In concentration*

The maximum (plateau) value (see, e.g., Figs. 1*a* and 2*a*) of release of $^{125}$I-labelled subcomponents $C1\bar{r}$ or $C1\bar{s}$ at various $C\bar{1}$ In concentrations was calculated from the experiments described in Fig. 3. The plateau value was observed to remain constant between 30min and 60min incubation. Further experiments under identical conditions, but with dilutions of plasma or serum in 5mM-triethanolamine/HCl, 145mM-NaCl, pH7.4, as source of $C\bar{1}$ In are also shown. The maximum release of $^{125}$I-labelled subcomponent $C1\bar{r}$ in the presence of purified $C\bar{1}$ In (●), plasma (○) and serum (▲) is shown. Release of subcomponent $C1\bar{s}$ paralleled that of subcomponent $C1\bar{r}$.

## Discussion

The results presented demonstrate that component C1, once bound to immune complexes and activated, undergoes a differential breakdown. Subcomponents $C1\bar{r}$ and $C1\bar{s}$ are released into the fluid phase, whereas subcomponent C1q remains predominantly associated with the immune complexes. The correlation of the rate and extent of subcomponent $C1\bar{r}$ and $C1\bar{s}$ dissociation with $C\bar{1}$ In concentration (Figs. 3–4) and the fact that release does not occur in serum depleted of $C\bar{1}$ In (Fig. 1) or when subcomponents $C1\bar{r}$ and $C1\bar{s}$ are prevented from forming complexes with $C\bar{1}$ In (Fig. 2*b*) indicate that the dissociation of subcomponents $C1\bar{r}$ and $C1\bar{s}$ is due specifically to their interaction with $C\bar{1}$ In.

More detailed studies (Fig. 2) of the dissociation show that it is the combination of subcomponent $C1\bar{r}$ with $C\bar{1}$ In that is most important in causing release. The characteristics of the dissociation of

subcomponents C1r̄ and C1s̄ are very similar whether C1̄ In is supplied in purified form or as plasma or serum (Figs. 1 and 4). This is consistent with the more important role of subcomponent C1r̄ in the dissociation, since in serum or plasma the presence of natural substrates of subcomponent C1s̄ (components C2 and C4) may modify considerably the interaction of subcomponent C1s̄ in ab–ag–C1̄ with C1̄ In. The interaction of C1̄ In with subcomponent C1r̄ in ab–ag–C1̄, however, is unlikely to be modified by other serum or plasma proteins, since the natural substrate of subcomponent C1r̄, namely free subcomponent C1s, not bound within component C1, is not present in significant quantity in plasma or serum (Laurell *et al.*, 1976).

In reconstituting component C1 or C1̄ on immune complexes, subcomponent C1q binds directly and strongly to antibody, subcomponent C1r or C1r̄ binds directly to the collagenous region of subcomponent C1q, and subcomponent C1s or C1s̄ binds to subcomponent C1r (Porter, 1977; Reid *et al.*, 1977; Sim & Colomb, 1977; Ziccardi & Cooper, 1976). The small dissociation of subcomponent C1q that accompanies release of subcomponents C1r̄ and C1s̄ (Fig. 1) suggests that the affinity of subcomponent C1q for antibody may be slightly greater when subcomponents C1r̄ and C1s̄ are present. No enzymic activity is known to be associated with subcomponent C1q, and experiments conducted under the same conditions as shown in Fig. 2 confirmed that subcomponent C1q does not form an SDS- and urea-stable complex with C1̄ In. No evidence for C1̄ In–C1q interactions was obtained by gel filtration in physiological buffers, and it is thus unlikely that the subcomponent C1q dissociation is a result of direct interaction with C1̄ In. The slight change in binding affinity of subcomponent C1q for ab–ag may reflect either a direct effect on subcomponent C1q caused by subcomponents C1r̄ and C1s̄ binding to it, or may suggest that either or both subcomponents C1r̄ and C1s̄ have a weak interaction with ab–ag in addition to the strong bindings discussed above. Weak interactions of this type cannot be demonstrated in the absence of subcomponent C1q (Porter, 1977), but there is some evidence (Allan & Isliker, 1974; Arlaud *et al.*, 1976) for their existence.

During activation of component C1, subcomponent C1r̄ within the ab–ag–C1 complex cleaves subcomponent C1s (Dodds *et al.*, 1978). Thus the active site of subcomponent C1r̄ is likely to be close to the polypeptide chain of subcomponent C1s̄. Despite this, it is clear that subcomponent C1r̄ in ab–ag–C1̄ is accessible for direct interaction with C1̄ In. Prior reaction of subcomponent C1s̄ with C1̄ In, as previously suggested (Arlaud *et al.*, 1979*a*), is not necessary to expose subcomponent C1r̄ (Fig. 2*a*; cf. Fig. 2*d*). Recent results (Reboul *et al.*, 1977) show that, in solution, isolated subcomponent C1r̄ has a very much lower affinity for C1̄ In than does subcomponent C1s̄. In contrast, subcomponent C1r̄ in ab–ag–C1̄ reacts with C1̄ In only 3–4-fold slower than does subcomponent C1s̄ in ab–ag–C1̄ [Fig. 2(*a*) and Arlaud *et al.* (1979*a*)]. As discussed previously (Arlaud *et al.*, 1979*a*), this indicates that the reactivities of subcomponents C1r̄ and C1s̄ with C1̄ In are more closely comparable when the two subcomponents are in ab–ag–C1̄ than when they are free in solution. Preliminary results (R. B. Sim, unpublished work) show that the reactivity of subcomponent C1r̄ towards C1̄ In is increased when subcomponent C1r̄ is incorporated into ab–ag–C1̄. Subcomponent C1s̄ activity is unmodified by incorporation into bound component C1̄. This modulation of subcomponent C1r̄ activity by the rest of ab–ag–C1̄ may be a phenomenon similar to the expression of the inherent activity of proenzymic C1r, which apparently occurs only when subcomponent C1r is in ab–ag–C1 (Dodds *et al.*, 1978).

It is likely that C1 subcomponents in normal serum circulate almost wholly as the component C1 complex, which contains two molecules each of subcomponent C1r and C1s monomers, and one molecule of subcomponent C1q (Ziccardi & Cooper, 1977; Porter, 1977). In this complex, binding between subcomponents C1r and C1s is considerably stronger than their binding to subcomponent C1q (Nagasawa *et al.*, 1974; Porter, 1977). On binding of component C1 to ab–ag, interaction between subcomponents C1q and C1r becomes stronger (Reid *et al.*, 1977), but the strongest binding is still between subcomponents C1r and C1s (Arlaud *et al.*, 1979*b*). The experiments shown in Fig. 2 demonstrate that reaction of subcomponent C1s̄ in ab–ag–C1̄ with C1̄ In weakens slightly the subcomponent iPr$_2$*P*-C1r̄–C1s̄ interaction (Fig. 2*c*). Reaction of subcomponent C1r̄ in ab–ag–C1̄ with C1̄ In, however, appears to break completely the binding between subcomponents C1q and C1r̄ (Figs. 2*a* and 2*d*), and subcomponents C1r̄ and C1s̄ are released together. Subcomponents C1r̄ and C1s̄ are likely to remain associated after release in the form of a complex with C1̄ In. Complexes of $\alpha_2$ electrophoretic mobility, containing subcomponents C1r̄ and C1s̄ and C1̄ In, have been detected in all normal sera (Laurell *et al.*, 1976) and are likely to correspond to the reaction product described here.

Release of subcomponents C1r̄ and C1s̄ from ab–ag–C1̄ as complexes with C1̄ In leaves most of the subcomponent C1q still bound to ab–ag. Binding and activation of component C1 on ab–ag does not appear to modify subcomponent C1q, since haemolytically active subcomponent C1q can be isolated by elution from ab–ag or aggregated IgG (Assimeh *et al.*, 1974; Gigli *et al.*, 1976). As noted above, the affinity of subcomponent C1r–C1s complexes is greater for immune-aggregate-bound C1q than for subcomponent C1q free in solution (Reid *et al.*, 1977). Thus

subcomponent C1q, which remains bound to ab–ag after removal of subcomponents C1$\bar{r}$ and C1$\bar{s}$, is likely to be functional, and may compete with subcomponent C1q in solution for binding of further proenzymic C1r–C1s complexes. Binding of one molecule of subcomponent C1q to ab–ag, therefore, may give rise to a relatively slow 'turnover' of many subcomponent C1r and C1s molecules. This process would leave free subcomponent C1q in solution, in excess of the quantity of proenzymic subcomponents C1r and C1s. Disequilibrium in the concentrations of C1 subcomponents in pathological sera has been reported frequently (Stroud *et al.*, 1970; de Bracco *et al.*, 1974; Laurell & Mårtensson, 1974; Johnson *et al.*, 1977). These reports show in general that the concentrations of subcomponents C1r and C1s correlate closely, but the concentration of subcomponent C1q varies widely relative to that of subcomponent C1s. Either subcomponent C1q or subcomponents (C1r + C1s) may be present in excess. On the basis of the close correlation of subcomponent C1r and C1s concentrations, it has been proposed that the synthesis and catabolism of subcomponent C1q is regulated independently from that of subcomponents (C1r + C1s) (Pickering *et al.*, 1970; Stroud *et al.*, 1970). The results presented here are consistent with a different mode of catabolism of subcomponents (C1$\bar{r}$ + C1$\bar{s}$) from that of subcomponent C1q. As discussed by Laurell *et al.* (1976), subcomponents C1$\bar{r}$ and C1$\bar{s}$, in the form of soluble complexes with C$\bar{1}$ In, are likely to be cleared rapidly from the circulation. Subcomponent C1q remains bound to the component C1-activator system, and its rate and mode of clearance will be strongly dependent on the nature of the activator system. Thus it is suggested that, under pathological conditions in which immune complexes or other component C1-activator systems are not rapidly cleared from the circulation, the differential dissociation of bound component C$\bar{1}$ described here will give rise to a disequilibrium in C1 subcomponent concentrations and catabolism. This may lead in the longer term to an unbalanced rate of synthesis of subcomponents C1q and (C1r + C1s). The recently reported serum inhibitor of subcomponent C1q (Conradie *et al.*, 1975; Ghebrehiwet & Müller-Eberhard, 1978) may function as a regulator of the proposed subcomponent C1r + C1s turnover, by binding to the ab–ag–C1q complexes formed by release of subcomponents C1$\bar{r}$ + C1$\bar{s}$.

This work was supported by the Centre National de la Recherche Scientifique (E.R.A. No. 695), I.N.S.E.R.M. (A.T. 487780), the Délégation Générale à la Recherche Scientifique et Technique (contracts nos. 76.7.1194 and 76.7.1195) and the Fondation pour la Recherche Médicale. R. B. S. thanks the Medical Research Council (U.K.) and I.N.S.E.R.M. for an Exchange Fellowship. We thank Dr. E. Sim for helpful discussion.

## References

Allan, R. & Isliker, H. (1974) *Immunochemistry* **11**, 243–248

Arlaud, G. J., Meyer, C. M. & Colomb, M. G. (1976) *FEBS Lett.* **66**, 132–136

Arlaud, G. J., Reboul, A., Sim. R. B. & Colomb, M. G. (1979*a*) *Biochim. Biophys. Acta* **576**, 151–162

Arlaud, G. J., Sim, R. B., Duplaa, A.-M. & Colomb, M. G. (1979*b*) *Immunochemistry* in the press

Assimeh, S. N., Bing, D. H. & Painter, R. H. (1974) *J. Immunol.* **113**, 225–234

Conradie, J. D., Volanakis, J. E. & Stroud, R. M. (1975) *Immunochemistry* **12**, 967–971

de Bracco, M. M. E., Windhorst, D., Stroud, R. M. & Moncada, B. (1974) *Clin. Exp. Immunol.* **16**, 183–188

Dodds, A. W., Sim, R. B., Porter, R. R. & Kerr, M. A. (1978) *Biochem. J.* **175**, 383–390

Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617

Ghebrehiwet, B. & Müller-Eberhard, H. J. (1978) *J. Immunol.* **120**, 27–32

Gigli, I., Porter, R. R. & Sim, R. B. (1976) *Biochem. J.* **157**, 541–548

Haines, A. L. & Lepow, I. H. (1964) *J. Immunol.* **92**, 479–490

Harpel, P. C. (1976) *Methods Enzymol.* **45**, 751–760

Harpel, P. C. & Cooper, N. R. (1975) *J. Clin. Invest.* **55**, 593–604

Heimburger, N. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B. & Shaw, E., eds.), pp. 367–386, Cold Spring Harbor Laboratory, Cold Spring Harbor

Heusser, C., Boesman, M., Nordin, J. H. & Isliker, H. (1973) *J. Immunol.* **110**, 820–828

Holland, N. C., de Bracco, M. M. E. & Christian C. L. (1972) *Kidney Int.* **1**, 106–114

Johnson, U., Kamme, C., Laurell, A-B. & Nilsson, N. I. (1977) *Acta Pathol. Microbiol. Scand. Sect. C* **85**, 10–16

Kluft, C. (1977) *Haemostasis* **6**, 351–369

Laurell, A.-B. & Mårtensson, U. (1974) *Acta Pathol. Microbiol. Scand. Sect. B* **82**, 585–589

Laurell, A.-B., Mårtensson, U. & Sjöholm, A. G. (1976) *Acta Pathol. Microbiol. Scand. Sect. C* **84**, 455–464

Levy, L. R. & Lepow, I. H. (1959) *Proc. Soc. Exp. Biol. Med.* **101**, 608–611

Mancini, G., Carbonera, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–245

Nagaki, K. (1975) *Nippon Ishikai Zasshi* **73**, 1536–1538

Nagasawa, S., Takahashi, K. & Koyama, J. (1974) *FEBS Lett.* **41**, 280–282

Pfueller, S. L. & Lüscher, E. F. (1972) *Immunochemistry* **9**, 1151–1165

Pickering, R. J., Naff, G. B., Stroud, R. M., Good, R. A. & Gewurz, H. (1970) *J. Exp. Med.* **131**, 803–815

Porter, R. R. (1977) *Biochem. Soc. Trans.* **5**, 1659–1674

Ratnoff, O. D., Pensky, J., Ogston, D. & Naff, G. B. (1969) *J. Exp. Med.* **129**, 315–331

Reboul, A., Arlaud, G. J., Sim, R. B. & Colomb, M. G. (1977) *FEBS Lett.* **79**, 45–50

Reid, K. B. M., Lowe, D. M. & Porter, R. R. (1972) *Biochem. J.* **130**, 749–763

Reid, K. B. M., Sim, R. B. & Faiers, A. P. (1977) *Biochem. J.* **161**, 239–245

Sim, R. B. & Colomb, M. G. (1977) *Ann. Immunol. (Paris)* **128C**, 946–947

Sim, R. B. & Porter, R. R. (1976) *Biochem. Soc. Trans.* **4**, 127–129

Sim, R. B., Porter, R. R., Reid, K. B. M. & Gigli, I. (1977) *Biochem. J.* **163**, 219–227

Sim, R. B., Reboul, A., Arlaud, G. J., Villiers, C. L. & Colomb, M. G. (1979) *FEBS Lett.* **97**, 111–115

Stroud, R. M., Nagaki, K., Pickering, R. J., Gewurz, H., Good, R. A. & Cooper, M. D. (1970) *Clin. Exp. Immunol.* **7**, 133–137

Sumi, H. & Fujii, S. (1974) *J. Biochem. (Tokyo)* **75**, 253–260

World Health Organisation (1968) *Bull. W. H. O.* **39**, 935–936

Ziccardi, R. J. & Cooper, N. R. (1976) *J. Immunol.* **116**, 496–503

Ziccardi, R. J. & Cooper, N. R. (1977) *J. Immunol.* **118**, 2047–2052