

## REQUIREMENTS FOR THE SOLUBILIZATION OF IMMUNE AGGREGATES BY COMPLEMENT

### Assembly of a Factor B-Dependent C3-Convertase on the Immune Complexes\*

By MORINOBU TAKAHASHI, BRIAN F. TACK, AND VICTOR NUSSENZWEIG

(From the Department of Pathology, New York University School of Medicine, New York 10016 and  
The American National Red Cross Blood Research Laboratory, Bethesda, Maryland 20014)

Previous reports from this laboratory showed that antigen (Ag)-antibody (Ab)<sup>1</sup> precipitates could be solubilized by the complement cascade (complement-mediated release activity or CRA) (1, 2). Based on several lines of indirect evidence, we proposed that CRA was due to the incorporation of C3 fragments onto the Ag-Ab lattice rather than to extensive proteolysis of the Ag or Ab (2, 3). We show here that, indeed, during the release reaction, a properdin and factor B-dependent C3-convertase is assembled on the immune precipitates. Its interaction with purified C3 results in the incorporation of large amounts of C3 into the Ag-Ab lattice. The final step of the reaction is a "spontaneous" solubilization of the C3-containing immune precipitates, which takes place in the absence of serum or divalent cations.

### Materials and Methods

*Diluents and Other Reagents.* CRA assays were carried out in phosphate buffered saline (PBS), pH 7.4, containing 0.1% gelatin,  $1.5 \times 10^{-4}$  M  $\text{Ca}^{++}$  and  $5 \times 10^{-4}$  M  $\text{Mg}^{++}$  (GPB<sup>++</sup>). PBS containing 0.01 M EDTA or 0.01 M EGTA plus 0.005 M  $\text{MgCl}_2$ , pH 7.4, and 0.1% gelatin were also used for CRA assay (EDTA-GPB or EGTA-GPB). Isotonic veronal buffer containing 5% glucose, 0.1% gelatin,  $1.5 \times 10^{-4}$  M  $\text{Ca}^{++}$  and  $5 \times 10^{-4}$  M  $\text{Mg}^{++}$  (DGVB<sup>++</sup>) was used for hemolytic assays. Sheep erythrocytes (E) were obtained from the New York City Department of Health. Rabbit antibody (A) to E was purchased from Cordis Laboratories, Miami, Fla.

*Immune Precipitates.* Monomeric bovine serum albumin (BSA) was purified by two successive gel filtrations on Sephadex G-100 columns from BSA (2 × crystallized, Pentex Biochemical, Kankakee, Ill.). IgG was isolated from rabbit antisera to BSA by ammonium sulfate precipitation and DEAE-cellulose column chromatography. A small amount of contaminating gamma globulin aggregates was removed by Sephadex G-100 gel filtration.

Quantitative precipitation reactions were performed to determine the antibody contents of the IgG fraction of the antisera to BSA. Immune complexes were prepared by mixing equal volumes of labeled or unlabeled BSA with anti-BSA in the presence of 0.01 M EDTA. The mixture was incubated at 37°C for 30 min, then at 4°C overnight. The amounts of antigen and antibody were

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<sup>1</sup> *Abbreviations used in this paper:* A or Ab, antibody; Ag, antigen; BSA, bovine serum albumin; C, complement; C3b, C3 fragment; CRA, complement-mediated release activity; DGVB<sup>++</sup>, isotonic veronal buffer containing glucose, gelatin, and  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ ; E, sheep erythrocytes; EAC, erythrocyte antibody complement; GPB<sup>++</sup>, PBS to which 0.1% gelatin was added; Hus, human serum; P, properdin; PBS, phosphate-buffered saline containing  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ; SFU, site-forming units.

calculated to yield complexes at equivalence. The molecular ratio of antibody to antigen was about 2.4.

**Complement Reagents.** Human serum was used as the source of complement. Pooled human serum was stored in small aliquots at  $-70^{\circ}\text{C}$  until use. Human C3 was purified as in (4). After labeling, it showed a single band by SDS-acrylamide gel electrophoresis. Its sp act was  $9.5 \times 10^{12}$  site-forming units (SFU)/mg. Other complement components, guinea pig C1, human C4, C2, C3, C5, C6, C7, C8, and C9 were purchased from Cordis Laboratories. C3b was prepared by treatment of C3 with trypsin for a short time as described in (5). Properdin (P) was purified from pooled human serum by the method of Pensky et al. (6).  $^{125}\text{I}$ -labeled P gave a single symmetrical peak when analyzed in SDS-polyacrylamide gel electrophoresis. The apparent mol wt was 44,000 daltons which corresponds to the monomeric subunit of P (7). Contaminating proteins, as judged by distribution of radioactivity, were less than 1%. The protein concentration of P was adjusted to 0.36 mg/ml. P was stored at  $4^{\circ}\text{C}$  after it had been filtered through a Millipore membrane.

**Antisera.** Rabbit antiserum to human factor B was purchased from Behring Diagnostics, Sommerville, N. J. Rabbit antiserum to human C2 was a gift from Dr. C. Alper. It had been thoroughly absorbed with C2-deficient human serum. Anti-P was raised in rabbits by injecting 0.2 mg of purified P emulsified in Freund's complete adjuvant into their foot pads. Anti-P was absorbed with IgG since it contained small amounts of antibodies to IgG, as determined by double diffusion in agar against normal human serum and purified IgG. The IgG fractions from all antisera were purified by DEAE-cellulose column chromatography.

**CRA Assays.** CRA assays were carried out as already described (1, 2). In kinetic studies, immune precipitates were added to diluted human serum preincubated for 10 min at the indicated temperature. Then, 0.05–0.2-ml aliquots were taken at selected times and immediately mixed with 2–4 vol of cold diluent containing 0.025% of sheep erythrocytes. Supernates and pellets were separated by centrifugation (2,000 g,  $4^{\circ}\text{C}$ ) and assayed for radioactivity in a gamma counter.

**Preparation of Intermediate Complexes of the Release Reaction.** Immune precipitates bearing a C3-convertase were usually prepared by incubating immune precipitates containing about 50–100  $\mu\text{g}$  Ab with 8–16 ml of a 1:2 dilution of human serum at  $37^{\circ}\text{C}$  for 5–10 min. The incubation was terminated by adding 2 vol of cold diluent and then transferring the reaction tube to an ice-water bath kept at  $0^{\circ}\text{C}$ . The precipitates were centrifuged in the cold at 3,500 g for 15 min, and washed three times with a total volume of 32 ml of cold diluent. The resulting precipitates were resuspended in cold GPB<sup>++</sup> and used immediately.

**Binding of  $^{125}\text{I}$ -C3 to Immune Precipitates Incubated with Fresh Serum.** Two experimental protocols were used, in which the ratios of immune precipitate to serum varied widely. In the initial experiments 0.1 ml of immune precipitates containing 80  $\mu\text{g}$  of Ab were added to 0.4 ml of a 1:3 dilution of human serum prewarmed at  $37^{\circ}\text{C}$ , and containing 10  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled C3. Under these conditions, the precipitates are not dissolved even after prolonged incubation at  $37^{\circ}\text{C}$ . 0.1-ml aliquots were taken from the tube at selected times and mixed with 0.2 ml of EDTA-GPB. Pellets were separated from supernates by centrifugation in the cold at 3,500 g for 15 min. Pellets were washed twice with 0.4 ml EDTA-GPB and then counted for radioactivity. In other experiments, immune precipitates formed with 0.97  $\mu\text{g}$  of  $^{131}\text{I}$ -BSA and 5.6  $\mu\text{g}$  anti-BSA were incubated under the same conditions. The kinetics of solubilization and  $^{125}\text{I}$ -C3 uptake were investigated. 67% of the added precipitates were solubilized after 90 min of incubation. The amount of C3 specifically bound at different times was then calculated, that is, C3 bound to immune precipitates incubated with serum minus C3 bound to immune precipitates incubated in EDTA-serum.

**Binding of  $^{125}\text{I}$ -Properdin.** Immune precipitates resuspended in 0.1 ml GPB<sup>++</sup> were incubated at  $37^{\circ}\text{C}$  with 2.0 ml of serum containing 1.2  $\mu\text{g}$  of  $^{125}\text{I}$ -P. 0.2-ml aliquots were taken at selected times and transferred to tubes containing 0.4 ml of cold GPB<sup>++</sup>. The precipitates were isolated by centrifugation at 3,500 g for 15 min and washed once with 0.4 ml cold GPB<sup>++</sup>. The combined supernates and the washed precipitates were assayed for radioactivity.

**Hemolytic Assay of C3.** EAC142 were prepared with guinea pig C1, human C4, and human C2 (8). To assay the hemolytic activity of C3, 0.2 ml of dilutions of the test samples were incubated with 0.2 ml of  $0.9 \times 10^8$ /ml EAC142 at  $30^{\circ}\text{C}$  for 10 min. Then, 0.6 ml of a mixture of 200 U/ml of C5, C6, C7, C8, and C9 were added and the incubation continued at  $37^{\circ}\text{C}$  for 60 min. The degree of hemolysis was determined spectrophotometrically at the wave length of 412 nm.

**Inactivation of C3 by the Ag-Ab Bound C3-Convertase or by EAC142.** 0.1 ml of a suspension of complexes bearing C3-convertase or  $3 \times 10^8$  EAC142 in 0.1 ml was mixed with 0.4 ml of C3 (Cordis

Laboratories, 200 U/ml) at 30°C for 60 min. The supernate was isolated by centrifugation and the residual C3 titer was assayed as indicated above.

*Blocking of C3-Convertase Activity with Antisera to Factor B and C2.* In preliminary experiments we compared the C3-convertase activity of EAC142 and of immune precipitates treated with excess serum for 6–10 min as described above. We found that  $3 \times 10^8$  EAC142 and complexes containing 60  $\mu$ g of Ab had approximately the same activity in our assay, that is, they inactivated 60–70% of C3 added. To determine whether the C3-convertase associated with the immune complexes was C2-dependent or factor B-dependent, the following experiment was performed. 0.1-ml aliquots of immune-complex associated C3-convertase (60  $\mu$ g Ab) were incubated at 0°C for 60 min with 0.2 ml of different dilutions of normal rabbit IgG or antibodies to C2 or factor B. Controls were set up incubating the complexes with diluent, in the absence of antibody. The precipitates were washed twice with cold buffer, and resuspended in 0.1 ml of DGVB<sup>++</sup> and assayed for C3-convertase activity. The specificity of the antisera was controlled by incubating  $3 \times 10^8$  EAC142 cells with different dilutions of the IgG preparations under the same conditions. The erythrocytes were washed twice by centrifugation and assayed for C3-convertase as above.

*Iodination.* Monomeric BSA, C3, and P were labeled with <sup>125</sup>I or <sup>131</sup>I by the chloramine-T method (9), as described in (10) except that the radiolabeling of C3 and P was carried out at 0°C. Radioactive BSA was stored at –20°C and used within 4 wk. <sup>125</sup>I-C3 and <sup>125</sup>I-P were kept at 4°C. The sp act ranged between 10<sup>5</sup> and 10<sup>6</sup> cpm/ $\mu$ g.

*Determination of C3 and Properdin in Serum.* The concentration of C3 in the pooled serum used in the present experiments was 0.84 mg/ml as determined by single radial immunodiffusion by using a reference serum (Behring Diagnostics). The concentration of P was 24  $\mu$ g/ml as measured by the radioimmunoassay described in (11). Purified P was used as the protein standard in this assay.

## Results

*Kinetics of Solubilization.* Fig. 1 illustrates the kinetics of solubilization of the immune precipitates (<sup>125</sup>I-BSA-anti-BSA) in human serum at 25° or 37°C. Solubilization did not occur at 25°C. At 37°C, a time lag of about 10 min was observed before the onset of the solubilization process. By the end of 15 min only 12% of the immune precipitates had been solubilized while more than 50% were dissolved at 30 min. The duration of the lag period varied in different experiments depending on the amount of immune precipitate used, the affinity of the antibody for the antigen, and the dilution and source of complement. Addition of 0.01 M EGTA and Mg<sup>++</sup> to the reaction mixture markedly lengthened the lag period.

*Generation of a C3-Convertase Activity on the Immune Precipitates Precedes Solubilization.* The experiments shown in Fig. 2 demonstrate the presence of a C3-convertase on immune complexes obtained after 10 min of incubation with serum, at a time when no solubilization had occurred. The washed immune precipitates inactivated purified C3 in a dose-dependent fashion. Controls containing the same amounts of immune precipitate but preincubated with EDTA-serum, inactivated less than 5% of the purified C3.

*The C3-Convertase Generated on the Immune Complexes is Factor B- and P-Dependent.* This was demonstrated in the experiments described in the legend of Fig. 3 and in Table I. Fig. 3 shows that the C3-convertase activity associated with the immune complexes is almost completely inhibited by preincubation with antibody to factor B. A comparable amount of anti-C2 or normal rabbit IgG did not inhibit the C3-convertase by more than 5%. In contrast, the antiserum to C2, but not to factor B, inhibited 90% of the C3-convertase activity associated with the EAC142 cells.

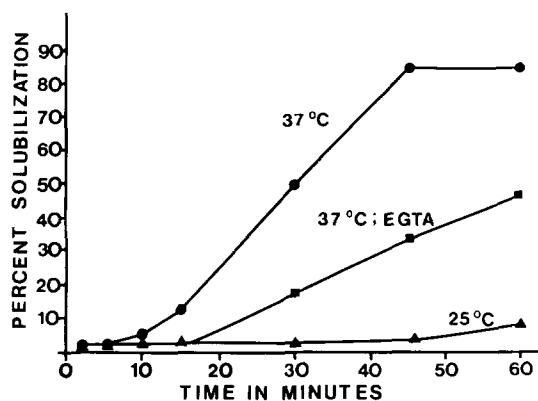


FIG. 1. Kinetics of solubilization. The immune precipitates were prepared at equivalence with  $^{125}\text{I}$ -labeled BSA and the IgG fraction of a rabbit antiserum to BSA. Washed immune precipitates containing  $15\ \mu\text{g}$  of Ab were added to 2 ml of a 1:2 dilution of human serum and incubated at  $4^\circ\text{C}$ ,  $25^\circ\text{C}$ , or  $37^\circ\text{C}$ . 0.2-ml aliquots were taken from the mixture at selected times, diluted with cold buffer, and centrifuged. Supernates and pellets were assayed for radioactivity. In serum containing 0.01M EGTA-5mM  $\text{Mg}^{++}$ , solubilization occurs at a slower rate. No solubilization was observed when the mixture was incubated at  $25^\circ\text{C}$  or  $4^\circ\text{C}$  (not shown in this graph). In control tubes, which contained either heated serum ( $56^\circ\text{C}$ , 30 min) or 0.01M EDTA-serum, 3.2 and 2.7% of the added immune precipitates, respectively, were solubilized after incubation at  $37^\circ\text{C}$  for 60 min.

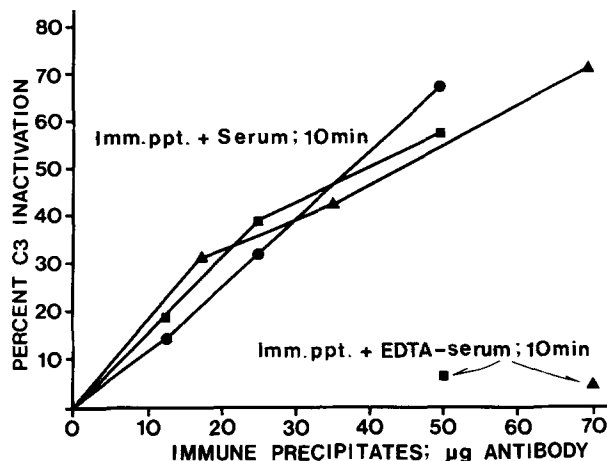


FIG. 2. Demonstration of C3 convertase activity associated with serum-treated immune precipitates. Immune precipitates (Imm. ppt.) containing about  $100\ \mu\text{g}$  of Ab were incubated at  $37^\circ\text{C}$  for 8-10 min with 16 ml of a 1:2 dilution of human serum. At this time, no solubilization was detected. The serum-treated precipitates were washed three times with cold GPB $^{++}$  and resuspended in 0.2 ml of the same buffer. 0.1 ml of the suspension was incubated at  $30^\circ\text{C}$  for 60 min with 0.4 ml of 200 U/ml of C3. Supernates were separated by centrifugation, and the residual C3 was titrated by hemolytic assay. Control immune precipitates were preincubated with EDTA-serum under identical conditions, and then assayed for C3-convertase activity. Results of three separate experiments are represented in this figure.

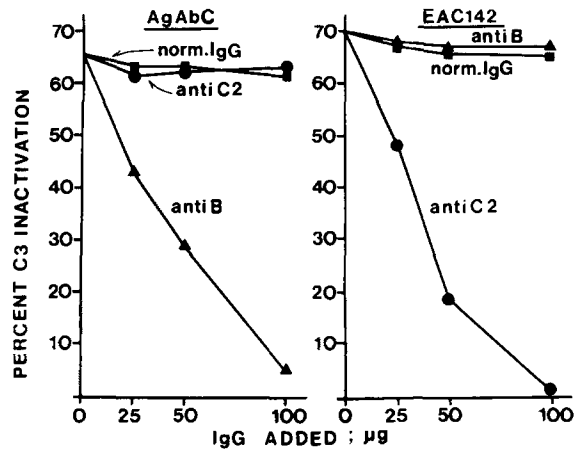


FIG. 3. Blocking of C3-convertase activity associated with the serum-treated immune complexes or with EAC142 by antibodies to factor B or C2. Several portions of immune precipitate were incubated at 37°C for 6 min with a 1:2 dilution of serum (6.0 ml of serum, 60 µg of complexed antibody), washed twice with cold buffer, and resuspended in 0.1 ml of cold GPB<sup>++</sup>. These serum-treated immune complexes (60 µg of Ab) or  $3 \times 10^8$  EAC142 in 0.1 ml of GPB<sup>++</sup> were incubated at 0°C for 60 min with 0.2 ml of dilutions of either anti-factor B, anti-C2, normal rabbit IgG. The precipitates or cells were then washed twice with cold DGVB<sup>++</sup> and resuspended in 0.1 ml of the same buffer and incubated at 30°C for 60 min with 0.4 ml of C3 (200 U/ml). The residual C3 activity in the supernate was assayed by hemolytic titration.

The C3-convertase activity associated with immune precipitates was lost almost completely upon incubation at 37°C for 60 min (Table I). Partial recovery of activity was obtained by the addition of purified factor B. Addition of both P and B, however, restored the C3-convertase activity to the original level.

*Incorporation of Properdin into the Immune Precipitates.* The previous experiments showed that P and factor B restore the C3-convertase activity of immune precipitates after decay at 37°C, and give further support for the finding that the C3-convertase associated with the complexes is alternative pathway-dependent. In Figs. 4 and 5 we show that P is actually bound to the immune aggregates as part of the process which leads to solubilization, and that it is in part released upon reincubation at 37°C.

Immune precipitates were incubated at 37°C with serum to which trace amounts of <sup>125</sup>I-P had been added. Samples were taken at different times, mixed with cold diluent to stop the reaction, and centrifuged. The conditions chosen lead to solubilization of 80% or more of the precipitates after 60 min of incubation. It is clear that P bound rapidly to the precipitates. The peak of P incorporation was reached in 8 min, and at this time solubilization had only started. Solubilization reached completion only at 60 min. A considerable amount of P was added to the precipitates under these conditions. Assuming that <sup>125</sup>I-P and P in serum have the same affinity for the complexes, we calculated that at 8 min close to one molecule of P is bound per molecule of Ab in the complexes.

Next we demonstrated directly the dissociation of P from immune aggregates upon incubation at 37°C. The conditions chosen for the binding of P are described in the legend of Fig. 5. After P had been incorporated, the immune aggregates

TABLE I  
Decay of Immune Complex-Associated C3-Convertase Activity and  
Reconstitution with Factor B and Properdin

Initial treatment of complexes*	Second treatment‡	Addition of§	C3 Inactivation
Serum	GPB <sup>++</sup> , 0°C	—	90.0
EDTA-Serum	GPB <sup>++</sup> , 0°C	—	5.6
Serum	EDTA, 37°C	—	11.7
Serum	EDTA, 37°C	P	24.4
Serum	EDTA, 37°C	B	32.3
Serum	EDTA, 37°C	P, B	91.0

\* Immune complexes (700  $\mu\text{g}$  of Ab) were incubated for 6 min at 37°C with 35 ml of a 1:2 dilution of human serum, and washed in GPB<sup>++</sup>. Then the precipitates were resuspended in GPB<sup>++</sup>, divided in portions, and treated as shown.

‡ 100  $\mu\text{g}$  of washed immune complexes were incubated for 60 min at indicated temperatures either in EDTA-GPB or GPB<sup>++</sup>. After incubation they were washed with DGVB<sup>++</sup>.

§ 10  $\mu\text{g}$  of B, 7.2  $\mu\text{g}$  of P.

|| Treated complexes were incubated at 25°C for 60 min with 0.4 ml of C3 (200 CH50/ml). The residual C3 was assayed by hemolytic titration.

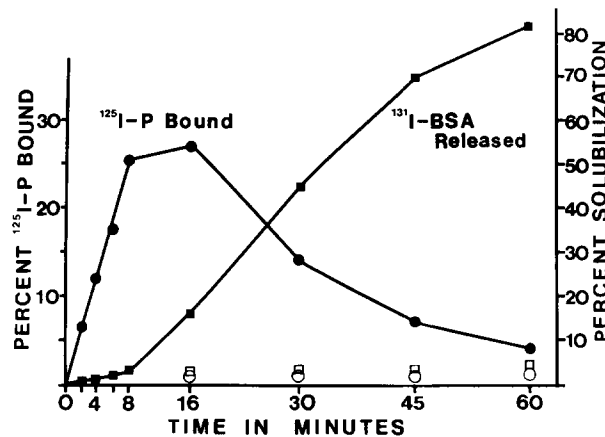


FIG. 4. Incorporation of P into the antigen-antibody lattice. Immune precipitates formed between 1.2  $\mu\text{g}$  of <sup>131</sup>I-BSA and 6.8  $\mu\text{g}$  of Ab to BSA were incubated at 37°C with 2.0 ml of a 1:2 dilution of human serum to which 1.2  $\mu\text{g}$  <sup>125</sup>I-P had been added. 0.2-ml aliquots were taken at indicated times, mixed with 0.4 ml of cold buffer, and centrifuged. The conditions chosen lead to 80% or more solubilization of the precipitates. ●, percent properdin uptake; ■, percent solubilization. Control tubes contain EDTA-serum instead of serum; ○, percent properdin uptake; □, percent solubilization.

were washed at 0°C, resuspended in GPB<sup>++</sup>, and reincubated at different temperatures. After 5 min more than 60% of P had been dissociated into the supernate. The dissociation of P was much less pronounced at lower temperatures. Similar results were obtained when <sup>125</sup>I-P-containing immune precipitates were incubated in the buffer containing 0.01 M EDTA (not shown).

*The Interaction of C3 with Ag-Ab Complexes Bearing C3-Convertase Leads to*

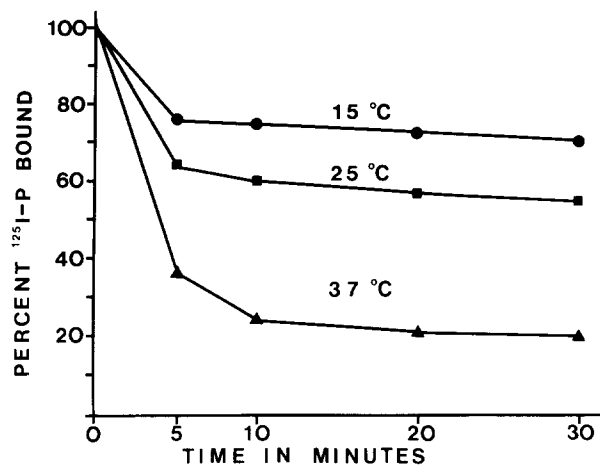


Fig. 5. Dissociation of P from serum-treated immune complexes. Immune precipitates containing 600  $\mu\text{g}$  of complexed antibody were incubated at 37°C for 4 min with 0.4 ml of a 1:4 dilution of human serum plus 1.2  $\mu\text{g}$   $^{125}\text{I}$ -P. The precipitates were then separated by centrifugation, washed twice with cold GPB<sup>++</sup>, and resuspended in 0.15 ml of the same buffer. 0.025-ml aliquots of this suspension were added to 0.4 ml of GPB<sup>++</sup> at different temperatures. At different times 0.05-ml aliquots were taken from the different tubes, diluted with 0.3 ml of cold buffer, centrifuged, and counted. Similar results were obtained when serum-treated immune precipitates were incubated in EDTA-GPB.

**Solubilization.** Washed immune-precipitates bearing C3-convertase activity were prepared as described before, by incubating the precipitates in human serum for 10 min at 37°C. As shown in Fig. 6, when these washed precipitates were reincubated at 37°C with pure C3, solubilization occurred. The reaction required no divalent cations, and proceeded in the presence of EDTA. Control precipitates, preincubated in human serum for 4 min or with EDTA-serum for 10 min, and bearing no C3-convertase activity, were not solubilized when incubated with C3. C3 purified to homogeneity (4) was used in all these experiments.

We also attempted to enhance the solubilization of immune complexes by the addition of cobra venom factor, or zymosan, or inulin, to the serum containing the immune precipitates. Addition of these substances, known as activators of the alternative pathway, strongly inhibited CRA of serum. Also, addition to the precipitates of purified C3b (obtained by trypsin treatment of C3), did not cause solubilization. Therefore, it appears that solubilization depends on the interaction of an immune complex-bound C3-convertase and C3.

**Incorporation of C3 into the Immune Precipitates during the Solubilization Process.** Next we studied the kinetics of C3 incorporation into the immune aggregates incubated with serum. As described in the Methods section, these experiments were performed either under conditions in which complement was limiting or in the presence of large excess of complement.

0.1 ml of immune precipitates containing 80  $\mu\text{g}$  of Ab were incubated at 37°C with 0.4 ml of a 1:3 dilution of serum containing 10  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled C3. Under these conditions (complement is limiting) less than 5% of the precipitates are solubilized after 90-min incubation at 37°C. At 10 min, about 1.5% of the label

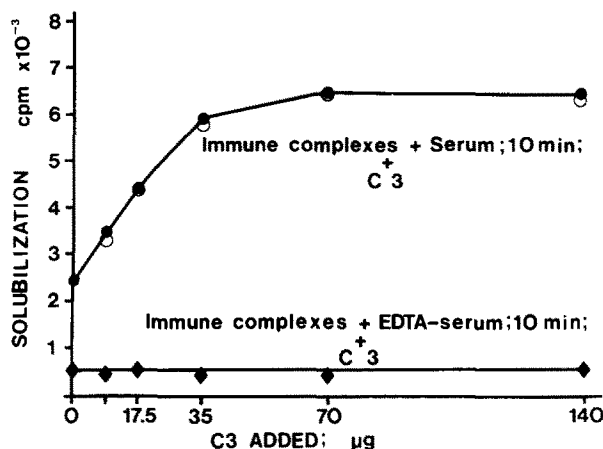


FIG. 6. Solubilization of the serum-treated immune precipitates by C3. Immune precipitates containing 11.2  $\mu\text{g}$  of complexed Ab were incubated with 2.0 ml of a 1:2 dilution of human serum at 37°C for 10 min. The serum-treated precipitates were then washed three times with 2 ml each of cold buffer and resuspended in 0.2 ml of GPB<sup>++</sup>. 0.025-ml aliquots containing 0.2  $\mu\text{g}$  Ag and 1.1  $\mu\text{g}$  of Ab were then incubated for 50 min at 37°C with 0.2 ml of C3 at different concentrations, with GPB<sup>++</sup> (●, ◆) or EDTA-GPB (○) as a diluent. Supernates and pellets were then separated by centrifugation and assayed for radioactivity. Other controls consisted of immune precipitates preincubated with human serum containing 0.01M EDTA under the same conditions, washed, and then reacted with purified C3. The maximum solubilization induced by addition of C3 corresponds to 40% of the immune precipitates added.

had been specifically incorporated into the aggregates (Fig. 7). Assuming that labeled and unlabeled C3 were equally active, the amount of C3 incorporated after 20-min incubation was 0.053  $\mu\text{g}/\mu\text{g}$  of antibody. These values correspond to about one molecule of C3 per 18 antibody molecules, if we assume the mol wt for the C3 fragment bound to the complexes to be 180,000 daltons (C3b).

Much larger amounts of C3 were incorporated into the immune precipitates under conditions which lead to solubilization, as shown in the following experiment. 0.1 ml of immune complexes (<sup>131</sup>I-BSA-anti-BSA, containing 5.6  $\mu\text{g}$  Ab) were incubated at 37°C with 0.4 ml of a 1:2 dilution of human serum, and 20  $\mu\text{g}$  of <sup>125</sup>I-C3. The kinetics of <sup>125</sup>I-C3 uptake and solubilization of the precipitates is shown in Fig. 8. Under these conditions, 67% of the immune precipitates were solubilized in 90 min. At 30 min, 0.21  $\mu\text{g}$  of C3 had been specifically bound to the aggregates containing 0.50  $\mu\text{g}$  of complexed Ab, and the molar ratio of Ab/C3 was 2.5. As will be shown in the next section, the amounts of C3 incorporated into the *solubilized* complexes is even higher, that is, the Ab/C3 ratio is approximately one.

*The Final Step of the Solubilization Process: "Spontaneous" Release.* The preceding experiments demonstrate that different Ag-Ab-C intermediates are generated when Ag-Ab precipitates are incubated in serum at 37°C, and that after 6-10 min of incubation, the Ag-Ab bear C3-convertase activity. Fig. 9 shows the result of an experiment in which the immune precipitates were preincubated with serum for a longer period of time (15 min). They were then washed three times with cold buffer and reincubated at different temperatures



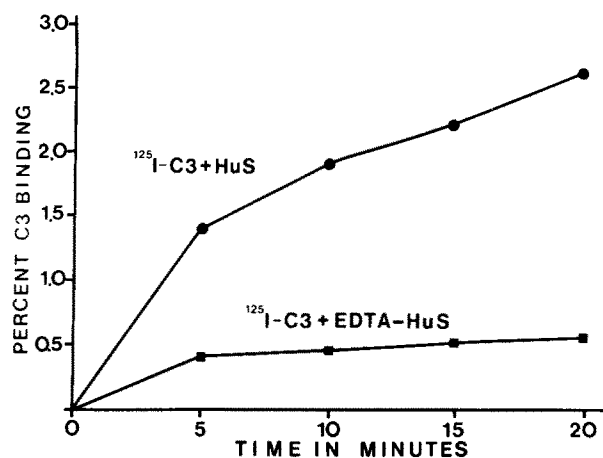


FIG. 7. Kinetics of C3-binding to immune precipitates incubated with limited amounts of complement. Immune precipitates formed with  $13.6 \mu\text{g}$  of  $^{131}\text{I-BSA}$  and  $80 \mu\text{g}$  of Ab were incubated at  $37^\circ\text{C}$  with  $0.4 \text{ ml}$  of a 1:3 dilution of human serum plus  $10 \mu\text{g}$  of  $^{125}\text{I-C3}$ . Controls consisted of EDTA-serum containing the same amount of  $^{125}\text{I-C3}$ .  $0.1\text{-ml}$  aliquots were taken at selected times, diluted, and centrifuged. Pellets were washed twice and counted for radioactivity. Under the conditions used less than 5% of the immune precipitates were solubilized after 60 min of incubation.

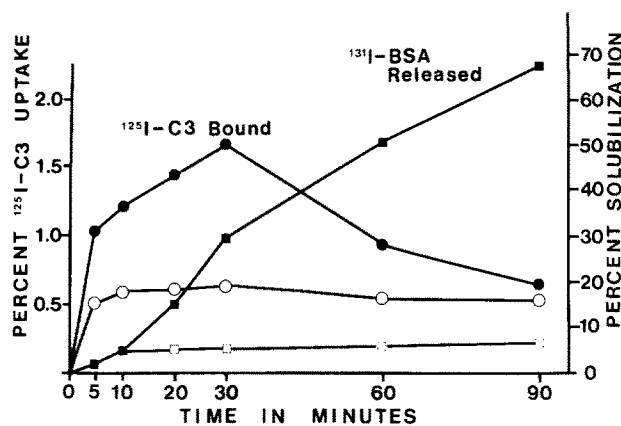


FIG. 8. C3-binding during solubilization. Immune precipitates formed with  $0.97 \mu\text{g}$  of  $^{131}\text{I-BSA}$  and  $5.6 \mu\text{g}$  of anti-BSA were incubated at  $37^\circ\text{C}$  with  $0.4 \text{ ml}$  of a 1:2 dilution of human serum plus  $20 \mu\text{g}$  of  $^{125}\text{I-C3}$ . Samples were taken at indicated times, diluted, and centrifuged. Precipitates were washed three times with cold buffer.  $^{131}\text{I-BSA}$  and  $^{125}\text{I-C3}$  in the supernates and pellets were counted. The control tubes contained EDTA-serum plus  $^{125}\text{I-C3}$ . Symbols used: percent of C3 total counts found in precipitate incubated in human serum ( $\bullet$ ) or EDTA-human serum ( $\circ$ ); percent of solubilization of precipitates incubated in human serum ( $\blacksquare$ ) or EDTA-human serum ( $\square$ ).

for various lengths of time. In contrast with the solubilization of immune precipitates by serum (Fig. 1), this intermediate underwent solubilization without a lag phase upon reincubation at  $37^\circ\text{C}$ , and even at  $25^\circ\text{C}$ . This process, designated as spontaneous release, was almost completed within 20 min at  $37^\circ\text{C}$ . Spontaneous release requires no divalent cations. It is a specific process, since

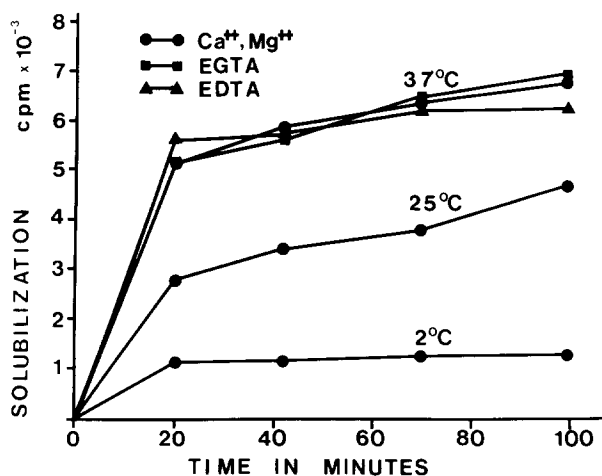


FIG. 9. "Spontaneous" release of the immune precipitates which had been preincubated with human serum. The immune precipitates (15  $\mu\text{g}$  of Ab), prepared as explained in the legend for Fig. 1, were incubated at 37°C for 15 min with 2 ml of a 1:2 dilution of human serum. The serum-treated precipitates were washed three times with cold buffer and resuspended in either GPB<sup>++</sup> or EGTA-GPB or EDTA-GPB. The mixture was then incubated at 2°, 25°, or 37°C. 0.2-ml aliquots were taken at selected times, diluted with cold buffer, and centrifuged. Supernate and pellets were assayed for radioactivity. The maximum solubilization attained by spontaneous release corresponds to about 50% of the immune precipitates added. Controls, in which the same complexes had been preincubated at 25°C in serum or at 37°C in EDTA-serum showed no spontaneous release upon reincubation at 37°C (880 and 1,090 cpm, respectively).

immune precipitates which had been preincubated with fresh serum at 25°C, or at 37°C with serum containing 0.01 M EDTA were not solubilized upon reincubation at 37°C in buffer.

The Ab/C3 ratio among spontaneously solubilized complexes was determined in the following experiment. Immune precipitates prepared with 13.8  $\mu\text{g}$  of <sup>131</sup>I-BSA and 80.3  $\mu\text{g}$  Ab were incubated with 16 ml of a 1:2 dilution of human serum containing <sup>125</sup>I-C3 (10  $\mu\text{g}$ ). The conditions were chosen on the basis of preliminary experiments which showed that more than 85% of these immune precipitates could be dissolved after 60 min of incubation with the serum at 37°C. After 12 min, when solubilization had started, the reaction was stopped by addition of cold EDTA-GPB, and pellets were washed three times with cold buffer. The washed precipitates were incubated with 2.0 ml of EDTA-GPB at 37°C for 40 min. Supernates were assayed for <sup>125</sup>I and <sup>131</sup>I and we calculated that they contained 27.5  $\mu\text{g}$  Ab and 33.5  $\mu\text{g}$  of C3. Precipitation with specific antisera to Ig showed that 80% or more of the C3 molecules in these supernates were bound to Ig (1). The molar ratio of Ab to C3 is close to 1 assuming again that the mol wt of the fragments of C3 was 180,000 daltons. Very similar results were obtained in several other experiments.

### Discussion

We show here that, as in the case of complement mediated cell lysis (12), the solubilization of immune aggregates by complement can be conveniently divided in sequential stages.

*Generation of a Solid-Phase C3-Convertase.* As shown in Fig. 1, immune precipitates are solubilized when incubated with serum at 37°C. This process starts after a lag of a few minutes and does not occur or is very inefficient at temperatures below 25°C. Solubilization is dependent on divalent cations. It is completely inhibited when both Ca<sup>++</sup> and Mg<sup>++</sup> are chelated with EDTA, and proceeds at a slower rate in the absence of Ca<sup>++</sup>. During the lag period, a C3-convertase is generated on the complexes (Fig. 2). This was directly demonstrated by incubation of purified C3 with the washed precipitates obtained after 10 min of interaction with serum at 37°C. This incubation led to a considerable reduction of the hemolytic activity of C3 as compared to controls, in which C3 had been exposed to immune aggregates treated with EDTA-serum for 10 min.

A key finding was that the C3-convertase activity associated with the complexes was dependent on enzymes of the alternative pathway. C3 consumption by the complexes was completely inhibited if they had been previously incubated with an antibody to factor B, but not by an antibody to C2. Controls showed that the C3-convertase assembled on the erythrocyte membranes (EAC142) could be inhibited by anti-C2 but not by anti-B.

Assembly of the enzyme on the complexes depended on divalent cations and occurred after incubation with serum for a few minutes. During this short time, factor B (Fig. 3), P (Fig. 4), and C3 (Fig. 8) were bound to the immune aggregates.

When the P, B, and C3-containing aggregates were separated from serum by centrifugation and reincubated at 37°C, the C3-convertase activity decayed (Table I). This decay did not occur at 0°C, and was much delayed at 15°C. With labeled P, we found that at 37°C P dissociated from the aggregates. After 5 min at 37°C, at a time when solubilization could not be detected, more than 60% of the labeled P was found in the supernate (Fig. 5). However, a considerable proportion of P did not dissociate from the immune complexes even after prolonged incubation. The reasons for this observation are not known at present. Others have found that P has binding affinity for C3, C3b, and C3c in the fluid phase (13) or on the cell membrane (14, 15). It has also been suggested that since P is multivalent, at least two C3 molecules may be required to provide stable bonds between C3 and P (14, 16). Perhaps the fraction of P which is strongly associated with the immune complexes formed multiple bonds with the acceptor molecules.

The activity of the decayed complexes could be fully restored by purified P and B but not by either of them alone. These findings support the idea that this C3-convertase, as found in other systems (16-19) may be a multi-unit structure consisting of B, P, and probably C3b. The finding that under conditions which lead to solubilization, the molecular ratios of P, C3, and Ig on the complexes was close to one, supports this concept.

Since factor D is essential for solubilization (Takahashi, Brade, and Nussen-zweig, Manuscript in preparation), it is not clear why factor D was not required for the reconstitution of the C3-convertase activity after decay at 37°C (Table I). Perhaps, as in the case of the C3-convertase assembled on zymosan (20), some factor D may remain bound to the immune complexes after decay of factor B. Alternatively, our preparations of factor B or properdin may contain trace amounts of factor D.

The observation that an alternative pathway-dependent (not C2-dependent) C3-convertase is assembled on the immune complexes explains our observations that the solubilization of immune precipitates is strictly  $Mg^{++}$ , C3, B (1, 2), P and D dependent and that it cannot occur through the activity of the classical pathway alone (Takahashi, Brade, and Nussenzweig, Manuscript in preparation). Since the activation of the classical pathway by cell-associated immune complexes leads to the formation of a membrane-associated C3-convertase ( $C4\bar{2}$ ) (21, 22), it is not clear why this enzyme could not be detected on the immune aggregates.<sup>2</sup> The reasons for this may be trivial, that is, that the concentrations of factor B and C3 in serum are much higher than those of C2 and C4 respectively (12). Therefore, the number of  $C4\bar{2}$  sites on the complexes may be smaller as compared to the  $C3b\bar{P}\bar{B}$  sites. Another possibility is that P stabilizes the factor B-dependent convertase (15, 25), while the C2-associated enzyme decays more quickly since it is not provided with a comparable stabilizing mechanism.

*Amplification.* During this stage, which is not dependent on divalent cations, the C3-convertase bound to the complexes interacts with C3 and additional fragments of C3 are bound to the complexes (Fig. 8). After a certain time lag solubilization occurs. We emphasize that the assembly of the C3-convertase on the complexes is necessary, but is not sufficient for solubilization. For example, in the experiments shown in Figs. 4 and 6, less than 5% of the immune precipitates taken at 4–10 min were solubilized upon reincubation in the buffer after washing. However, these immune precipitates already contained a considerable amount of P, and had C3-convertase activity.

It appears that the progressive accumulation of C3 fragments onto the lattice causes its disruption and the subsequent release into the fluid phase of soluble material containing Ag, Ab, and several C-derived peptides. The role of C3 as an effector molecule in solubilization is supported by several other observations: (a) solubilization does not occur in the absence of C3 and it does not require C5 (1, 2); (b) CRA in the plasma of mice is closely correlated with its C3 concentration (3); and (c) a large proportion of the C3 moieties in the precipitate were found to be very tightly bound to the Ab. C3-Ab remained associated even after treatment with 8 M urea in 1% SDS for 1 h at 60°C. The aggregates were resolved, however, by treatment with denaturing agents in the presence of dithiothreitol (Czop, Tack, and Nussenzweig, Manuscript in preparation).

Another important point is that solubilization of immune precipitates does not occur if a C3-convertase is generated simultaneously in the fluid phase by the addition of cobra-venom factor, inulin, or zymosan. Also purified C3b is ineffective. Therefore, it appears that only nascent C3b, generated by the *lattice-associated* convertase can effectively bind to and solubilize complexes.

*Spontaneous Release.* The final step of the solubilization process is a secondary reaction which takes place after a large amount of C3 fragments has already accumulated on the complexes. It occurs in medium devoid of serum proteins, and does not require free divalent cations. It proceeds at 25°C but is more rapid at 37°C (Fig. 9). These findings demonstrate that the fixation of C3 is not

<sup>2</sup> However, as will be shown elsewhere (Takahashi, Brade, and Nussenzweig, Manuscript in preparation) the presence of C4 on the complexes could be directly demonstrated by the use of specific antisera. Müller-Eberhard and Lepow (23), as well as Willoughby and Mayer (24) also showed that C4b binds to Ab during complement fixation.

sufficient by itself for solubilization. Some further rearrangement of the lattice, whose nature is not clear, appears necessary. One simple explanation for the spontaneous release of complexes from the aggregate is that it is caused by the dissociation of a few primary Ag-Ab bonds. As shown before (2), primary Ag-Ab bonds markedly affect solubilization rates, which are much higher for precipitates prepared with antibodies of lower affinity for the antigen. It is conceivable that the tight binding of bulky C-peptides to the antibody molecules interferes with Ag-Ab bonds, either by steric interference with the binding of antigen and/or by inducing allosteric changes in the combining site. In addition, these C components may interfere with the movement of the Fab arms around the hinge region and thereby prevent cross-linking of the Ag molecules.

In summary, it appears that solubilization is mediated through the binding of C3b to the immune complex. After spontaneous dissociation of antigen from antibody, the presence of C3 peptides on the antibody may prevent reassociation and cross-linking with antigen. It is important to determine which domains of the antibody molecule are associated with C3 fragments. If they are bound to the Fab portion of the molecule, in proximity to the combining site, they could directly interfere with binding of antigen. However, it should be pointed out that C3b fragments may *also* interfere with lattice formation by inhibiting Fc-Fc nonspecific interactions which occur during precipitation (26). It is also conceivable that the binding of a relatively large number of complement peptides on to the immune aggregates modifies their overall solubility and favors dissociation. Therefore, it is still unresolved whether primary Ag-Ab bonds are affected during the solubilization process.

Finally, it should be emphasized that although the process of solubilization cannot be mediated through the classical pathway alone, it is a combined function of both the classical and properdin pathways. In the absence of  $\text{Ca}^{++}$ , C4, or C2, solubilization is much less effective. As will be shown in a succeeding paper, the role of the classical pathway may be to prime the complexes by depositing some C3b molecules on the antibody, which will then become nuclei for the assembly of the alternative pathway C3-convertase (Takahashi, Brade, and Nussenzweig, Manuscript in preparation). Therefore, deficiencies in either the classical or alternative pathway will affect the process of solubilization of immune aggregates.

### Summary

During the solubilization of immune precipitates BSA-rabbit antibodies to BSA by human complement, at least three stages can be distinguished. (A) Generation of alternative pathway C3-convertase sites associated with the immune complexes. During the first minutes of interaction between the immune aggregates and serum, before any solubilization has taken place, properdin (P), factor B, and C3 moieties are incorporated into the lattice. The washed precipitates have C3-convertase activity, which can be completely inhibited by antibodies to factor B, but not to C2. The assembly of the convertase is temperature-dependent, and does not take place in the absence of  $\text{Mg}^{++}$ . The immune complex-associated C3-convertase activity decays rapidly at 37°C, but it can be restored by addition of purified factor B and properdin. (B) Amplification. When

the aggregates bearing C3-convertase are incubated with purified C3, solubilization takes place. It appears that solubilization is caused by the accumulation of a large number of C3 fragments on the Ag-Ab lattice. In solubilized complexes, the molar ratios of Ab/C3 are close to one. (C) Spontaneous release. The final step in the solubilization process is a secondary reaction, during which some rearrangement of the lattice takes place. It occurs in medium devoid of serum and does not require divalent cations.

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