Requirements for the Solubilization of Immune Aggregates by Complement

THE ROLE OF THE CLASSICAL PATHWAY

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ABSTRACT In this paper we examine the role of the classical pathway in the complement-mediated solubilization of immune precipitates (CRA). Serum reagents were depleted of the alternative pathway components properdin and factor D. Both depleted reagents lack CRA although they have almost intact hemolytic activity. Also, immune complexes were not solubilized when incubated with high concentrations of the classical pathway components C1, C4, C2, and C3. We conclude that CRA is not mediated by the classical pathway alone.

Activation of the classical pathway by the immune aggregates greatly enhances CRA. The effect of the classical pathway is to deposit C3b on the antigen-antibody lattice and promote the assembly of a lattice-associated, properdin-dependent C3-convertase.

Although C3, C4, and properdin were detected on complexes solubilized by serum in the presence of Ca^{++} and Mg^{++}, only C3 and properdin were found on the complexes when Ca^{++} had been chelated by ethylene glycol-bis-(β-aminoethyl ether), N,N'-tetra-acetic acid. In both situations the aggregates were capable of converting C5 in the fluid phase. However, no C5 was found on the solubilized complexes. These findings suggest that in contrast to nascent C3b and C4b, nascent C5-9 lacks binding affinity for immune aggregates.

INTRODUCTION

The interaction of a soluble antigen (Ag)[1] and antibody (Ab) in blood or interstitial fluids leads in most circumstances to complement (C) activation and to the incorporation of certain C components into the lattice formed between immunoglobulin (Ig) molecules and the Ag. It is therefore likely that every biological and pathological effect of immune complexes is modulated by the C components which are part of the aggregates. In fact, those cells whose function is to recognize and interact with immune complexes bear receptors for C3, which is the C component most commonly found in association with them (1).

The understanding of the nature, sites of attachment, and molecular arrangement of the C components which are bound to the Ag-Ab lattice is, therefore, of relevance to immunopathology, and, in particular, to the understanding of the mechanisms which lead to the deposition of immune complexes in tissues.

Previous studies from this laboratory showed that the interaction between fresh serum and different kinds of immune precipitates, prepared with various Ag and Ab, lead to the formation of soluble Ag-Ab-C products. This reaction was found to be a function of the C cascade and denoted complement-mediated release activity, or CRA (2–5).

CRA has since been used as a convenient assay to study the interaction between Ag-Ab complexes, prepared with soluble Ag, and the C system. We found that during the process of disaggregation, several C en-

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[1] *Abbreviations used in this paper:* Ab, antibody; Ag, antigen; B, factor B; BSA, bovine serum albumin; C, complement; C2D, C2-deficient serum; CoF, cobra venom factor; CRA, complement-mediated release activity; D, factor D; $DGVB^{++}$, isotonic veronal buffer containing glucose, Ca^{++}, Mg^{++}, and gelatin; Ea, egg albumin; EDTA-GPB, GPB containing 0.01 M EDTA; EGTA, ethylene glycol-bis-(β-aminoethyl ether), N, N'-tetraacetic acid; EGTA-Mg^{++}-GPB, GPB containing 0.01 M EGTA, 0.005 M Mg^{++}; GPB, PBS plus gelatin; GPB^{++}, PBS containing gelatin, Ca^{++}, and Mg^{++}; HuS, human serum; P, properdin; $\bar{P}$, purified P; RD, factor D-depleted serum; RP, $\bar{P}$-depleted serum; Z, average number of effective hemolytic sites per cell; also, the bar over complement components denotes an activated form.

zymes were formed on the lattice and that the alternative pathway components were essential for CRA.

Indeed, solubilization required the assembly of a factor B-dependent (but not C2-dependent) C3-convertase on the immune aggregates. The interaction of this C3-convertase with C3 leads to solubilization (5). On the other hand, the classical pathway was not essential for CRA because it occurred in C4-deficient or C2-deficient sera and in the absence of Ca^{++}. However, CRA was much less efficient under these conditions (2, 3).

In this report we try to clarify the role of the classical pathway in CRA. Specifically, two questions are asked: (*a*) can the classical pathway alone, in the absence of the alternative pathway, induce effective solubilization? and (*b*) what is the mechanism through which the classical pathway enhances solubilization?

METHODS

Diluents and reagents. Phosphate-buffered saline (PBS), pH 7.4, containing 0.1% gelatin, 150 μM $CaCl_2$, and 0.5 mM $MgCl_2$ (GPB^{++}); GPB containing 0.01 M ethylene glycol-bis-(β-aminoethyl ether),N,N'-tetraacetic acid (EGTA) and 0.005 M Mg^{++} (EGTA-Mg^{++}-GPB); and GPB containing 0.01 M EDTA (EDTA-GPB) were used as diluents for the CRA assay (5). Gelatin veronal buffer, pH 7.5, containing 5% dextrose, 0.1% gelatin, 150 μM $CaCl_2$, and 0.5 mM $MgCl_2$ ($DGVB^{++}$), and gelatin veronal buffer containing 0.01 M EDTA (EDTA-GVB) were used for the hemolytic assay of complement (6). Sheep erythrocytes were obtained from the New York City Department of Health. Rabbit 19S (IgM) antibody to sheep erythrocytes was purchased from Cordis Laboratories, Inc., Miami, Fla. Zymosan and crystallized egg albumin (Ea) were obtained from Sigma Chemical Co., St. Louis, Mo. Crystallized bovine serum albumin (BSA) was obtained from Miles Laboratories, Kankakee, Ill.

Fresh human serum was used as the source of complement. Pooled human serum was stored in small aliquots at −70°C until used. C2-deficient human sera were kindly supplied by Dr. M. M. Glovsky, Kaiser Permanente Medical Group, Los Angeles, Calif. Human C3 (7) and C4 (8) were purified to homogeneity as described. Properdin ($\bar{P}$) was purified from pooled human serum by the method of Pensky et al. (9). Factor B was purified by the method of Götze et al. (10), but contaminating factor D was removed by two successive passages through a Sephadex G-100 column (5.0 × 55 cm, Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated with 0.005 M phosphate buffer and 0.001 M EDTA, pH 7.5. Human factor D was purified according to Fearon et al. (11) and macromolecular C1 was purified from human serum (12). The early C components, C1, C3, and C4, did not contain β1H, C3b-inactivator, or $\bar{P}$ when analyzed by crossed-immunoelectrophoresis with monospecific antisera. Human C2, C5, C6, C7, C8, C9, and cobra venom factor (CoF) were purchased from Cordis Laboratories. All the purified components, except C1 and $\bar{P}$, were stored in small aliquots at −70°C. C1 and $\bar{P}$ were stored at 4°C. $\bar{P}$ was passed through a Millipore membrane (Millipore Corp., Bedford, Mass.) before distribution into small aliquots in sterile plastic tubes.

Hemolytic assays for C. Total hemolytic titers were assayed after a standard 50% lysis method (13) with slight modifications. The total volume of the reaction mixture was reduced to one fifth of the original volume, the total number of sensitized sheep erythrocytes added was 5×10^7, and the incubation was carried out in small, disposable culture tubes (Pyrex, Corning Glass Works, Corning, N. Y., 12 × 75 mm). CoF-induced indirect lysis was carried out as described by Brai and Osler (14). Briefly, 0.2 ml of serum diluted in GVB^{++} was incubated at 37°C for 45 min with 0.2 ml of 50 U/ml CoF. Then, 0.5 ml of washed, unsensitized guinea pig erythrocytes at a concentration of 5×10^7/ml in EDTA-GVB and 0.3 ml of guinea pig serum made 0.01 M with EDTA, pH 7.4, were added to each tube. After incubation at 37°C for 60 min, tubes were centrifuged, and the extent of hemolysis was measured at 412 μm in a spectrophotometer. The average number of effective hemolytic sites per cell, that is, Z, was computed from the degree of hemolysis (15). Hemolytic titers of individual components, C1, C4, C2, C3, and C5 were assayed by using appropriate erythrocyte intermediates and purified C components (6). Purified B and D were assayed according to Fearon et al. by using EAC4,3 cells (16). With the same assay, no factor B activity was detected in the C2 preparation obtained from Cordis Laboratories. The D titer in D-depleted serum (RD) was measured by CoF-induced indirect lysis. Protein concentration of pure D was determined by the Folin method (17) with monomeric BSA as a standard.

Preparation of RD. Human serum was depleted of D by a combination of gel filtration through a Sephadex column and ultrafiltration. 6 ml of human serum, containing 10 mM EDTA, pH 7.4, was passed through a Sephadex G-100 column (5 × 55 cm) equilibrated with 0.005 M phosphate buffer plus 0.002 M EDTA, and 0.15 M NaCl, pH 7.5. The fractions from the exclusion peak and the ascending part of the second peak were pooled and concentrated by ultrafiltration through an Amicon XM-50 membrane (Amicon Corp., Lexington, Mass.) to 6 ml. The retained protein solution was centrifuged and dialyzed against cold phosphate buffer, pH 7.5, containing 0.15 M NaCl. RD was reconstituted with Ca^{++} and Mg^{++} and adjusted to the original volume before use. The degree of the depletion of D in RD was evaluated by measuring the ability of RD to induce indirect lysis of guinea pig erythrocytes in the presence of CoF, as described above. The Z values for 1/4, 1/6, 1/9, and 1/12 dilutions of normal serum were 1.121, 0.787, 0.386, and 0.227, respectively. The Z values for Rd (1/2 dilution) and buffer controls were 0.074 and 0.077, respectively. Therefore RD contained less than 5% of the D concentration of the original serum.

Preparation of $\bar{P}$-depleted serum (RP). RP was prepared (18) by incubating pooled human serum with washed zymosan (4 mg/ml of serum) at 17°C for 60 min. Zymosan was removed by centrifugation in the cold. The supernate was stored in small aliquots at −70°C until use. The efficiency of depletion was greater than 98% as demonstrated by measuring $\bar{P}$ with a solid-phase radioimmune assay as described by Minta et al. (19). Purified $\bar{P}$ with a known protein concentration served as the protein standard. An absorption coefficient of 14.8 (20) was used for the conversion of optical density units to protein concentration.

Immune precipitates. Immune precipitates were formed with monomeric bovine serum albumin (BSA) and rabbit IgG anti-BSA at equivalence. Monomeric BSA was obtained by passage of BSA through a Sephadex G-100 column to remove aggregates. The molar ratio of Ab to Ag in the washed precipitates was 3.4. For the CRA assay, immune precipitates formed with ^{125}I-BSA were used (5).

^{125}I-$\bar{P}$ uptake by immune complexes. Washed immune precipitates containing 100 μg Ab were suspended in 0.1 ml of GPB^{++}. 2.0 ml of dilutions of serum containing 1.2 μg of ^{125}I-$\bar{P}$ was added to the precipitates. Both reagents had been preincubated for 10 min at 37°C. 0.2-ml samples were taken

from the mixture at selected times and diluted with 0.4 ml of cold EDTA-GPB. The precipitates were separated by centrifugation at 3,500 *g* for 15 min and washed once with 0.4 ml cold EDTA-GPB. The pellets and the combined supernates were assayed for radioactivity (5).

Preparation of Ag-Ab precipitates bearing early components of the classical pathway. Immune precipitates containing 100 μg Ab were incubated with 1.0 ml of Cl (45,000 CH_{50}/ml) diluted in $DGVB^{++}$ at 30°C for 10 min. The precipitates were washed twice with 1.0 ml of cold $DGVB^{++}$, and then resuspended in the same buffer containing C2 (2,000 CH_{50}/ml) and purified C4 (0.6 mg/ml). The mixture was incubated at 30°C for 30 min. 0.5 ml of C3 (1.0 mg/ml) was added to the mixture and incubation was continued at 30°C for another 20 min. The precipitates were washed three times with 1.0 ml of cold $DGVB^{++}$, and resuspended in 0.1 ml of the same buffer.

For preparation of Ag-Ab $\overline{C1,4,2,3}$, bearing various amounts of C3, Ag-Ab $\overline{C1,4}$ formed 10 μg of ^{125}I-BSA, and 57 μg of Ab were incubated with 0.25 ml of C3 (2.0 mg/ml) plus 10 μg of ^{131}I-C3 in the presence of various concentrations of C2 (200–2,000 CH_{50}/ml) at 30°C for 60 min. The total volume was 0.5 ml. The precipitates were centrifuged and washed three times with 1.0 ml each of cold $DGVB^{++}$. The ratio of bound C3 vs. Ab was calculated from the radioactivity for ^{131}I and ^{125}I in the immune precipitates.

For preparation of Ag-Ab C4,3 and Ag-Ab C4, intermediate complexes Ag-Ab $\overline{C1,4,2,3}$ or Ag-Ab $\overline{C1,4}$ were incubated at 37°C for 120 min in 0.01 M EDTA-GVB. Precipitates were then washed three times in the same buffer and resuspended in a small amount of GPB^{++}.

The "primed" immune precipitates were kept at 0°C and used immediately.

Assay for C3- and C5-convertase generated on the immune precipitates. Immune precipitates bearing C3- and C5-convertase were prepared by incubating washed immune precipitates formed from 100 μg Ab, at 37°C for 8–12 min with 20 ml of a 1:2 dilution of human serum in GPB^{++} or EGTA-Mg^{++}-GPB. The incubation was terminated by addition of 20 ml of cold buffer and the precipitates were separated by centrifugation. Precipitates were then washed three times and resuspended in 0.2 ml of GVB^{++}. 0.1 ml was added to 0.4 ml of 200 CH_{50} U/ml of C3, and the other half was mixed with 0.4 ml of 80 CH_{50} U/ml of C5. The mixtures were incubated at 30°C for 30 min. The pellets were then separated by centrifugation at 3,500 *g* for 15 min. The supernates were assayed for residual C3 and C5 hemolytic activities. As controls, precipitates were incubated with dilutions of human serum in EDTA-GPB and then assayed for C3- and C5-convertase activities.

CRA assay. This was performed as previously described (5) with precipitates formed with ^{125}I-BSA.

Iodination. BSA and $\bar{P}$ were labeled with ^{125}I or ^{131}I by the chloramine-T method (21) as described by McConohey and Dixon (22). Radiolabeled BSA was stored at −20°C and used within 4 wk. Radiolabeled $\bar{P}$ was stored at 4°C and used within 2 wk.

Coprecipitation experiments. These were carried out to detect complement components bound to the solubilized complexes. The general procedure was as follows. Solubilized complexes were prepared by incubating ^{131}I-BSA-anti-BSA precipitates with human serum (5). After centrifugation at 3,000 *g* for 30 min, small aliquots of the solubilized complexes were mixed with specific antisera to complement components. To insure complete precipitation of the C component bound to the the complexes, the reaction was carried out in slight Ab excess as previously established by quantitative precipitation reactions. Furthermore, in view of the relatively small amounts of C components contained in the aliquots of the solubilized immune complexes, an adequate carrier had to be added. When C3, C4, C5, factor B had to be precipitated, an EDTA human serum with known amounts of these C components served as a source of carrier. However, in the case of $\bar{P}$, present only in trace amounts in serum, a preparation of purified protein had to be added as a carrier to insure the formation of a precipitate with the antiserum to $\bar{P}$. Control tubes included (*a*) samples of solubilized complexes which had not been mixed with antiserum and (*b*) samples of solubilized complexes mixed with Ea followed by rabbit Ab to Ea at equivalence. In this way we evaluated the nonspecific coprecipitation of the solubilized complexes by an unrelated Ag-Ab immune aggregate. Both controls were included in every experiment. Furthermore, the amounts of Ea-anti-Ea precipitates were close to those obtained between C components and the corresponding Ab. All precipitation reactions were carried out by incubating the Ag-Ab mixtures at 37°C for 10 min followed by 18 h at 4°C. The precipitates were separated by centrifugation at 3,000 *g* for 20 min, washed three times with cold PBS, and counted for radioactivity. Results are expressed as the percentage of the ^{131}I-BSA contained in solubilized complexes and which was coprecipitated by the antisera to the C components. It should be pointed out that the values obtained only depict the distribution of different C components among the aggregates, but are not necessarily parallel to the absolute amounts of C components present in these aggregates.

The following antisera were used in these experiments: rabbit antisera monospecific for human C3, C4, and factor B (Behring Diagnostics, Somerville, N. J.) and goat anti-human C5 (Meloy Laboratories, Inc., Springfield, Va.). Anti-$\bar{P}$ was prepared in our laboratory by injecting purified $\bar{P}$ into rabbits. All of these antisera showed only a single precipitin line against human serum in immunoelectrophoresis and Ouchterlony tests. Rabbit anti-Ea was obtained from Dr. Z. Ovary, New York University School of Medicine, New York.

RESULTS

The classical pathway alone cannot solubilize Ag-Ab aggregates effectively. In the experiments below we show that the serum reagents RD and RP, depleted of factors D or P of the alternative pathway, have almost intact hemolytic activity, but very low or nondetectable CRA.

P depletion was achieved by treatment of serum with zymosan. As expected, RP retained 75–85% of the hemolytic activity of the original serum. However, CRA was not detectable even at the highest serum concentration. The addition of physiological amounts of $\bar{P}$ (20 μg per ml of serum) restored CRA (Fig. 1). The effect of smaller doses of $\bar{P}$ was not increased by prolonging the times of incubation at 37°C of the mixtures of RP, $\bar{P}$, and aggregates.

Factor D depletion from serum was achieved by a combination of chromatography on Sephadex G-100 followed by ultrafiltration through an Amicon XM-50 membrane. In Table I we show the results of titrations of the total hemolytic activity, as well as of the individual components C1, C4, C2, and C3 of two dif-

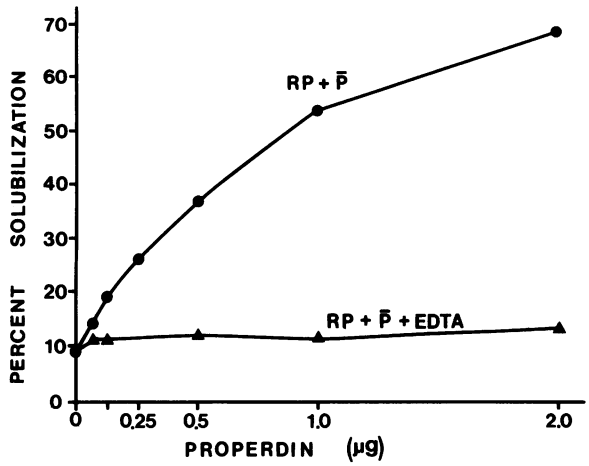

FIGURE 1 Reconstitution of CRA by RP and pure P̄. Various amounts of pure P̄ were added to 0.2 ml of a 1:2 dilution of RP. After the mixtures were prewarmed at 37°C for 10 min, immune precipitates of ^{125}I-BSA and anti-BSA containing 0.5 μg of Ab were added. Tubes were incubated for another 60 min at 37°C, then diluted with 0.4 ml of cold EDTA-GPB, and centrifuged. Supernates and pellets were assayed for radioactivity. The controls contained RP, P̄, and 0.01 M EDTA.

ferent RD reagents. The late components (C5–C9) were not assayed because they do not participate in CRA (2). Except for a diminution of C1 titer in one of the samples, the hemolytic activities of RD and normal serum were not different. In contrast, CRA was barely detectable at the highest serum concentration.

As mentioned in the Methods section and shown in Fig. 2, RD also lost the ability to induce hemolysis of unsensitized guinea pig erythrocytes in the presence of CoF. Addition of 300 ng of purified factor D to 0.2 ml of a 1/6 dilution of RD restored this activity to normal levels. Significant activity was detected even after addition of 16 ng of D. In contrast, our initial attempts to restore CRA by adding purified D to RD failed. Subsequently, after titration of P̄ in RD by solid phase radioimmunoassay, we found that about 75% of P had been nonspecifically depleted from RD. As shown above, P is essential for CRA. For this reason, in subsequent experiments we first restored RD with physiological amounts of pure P̄, and then assayed the effect of addition of D to CRA activity (Fig. 3). Although RD + P̄ or RD + D showed no CRA activity, RD + P̄ + D containing physiological concentrations of D showed full CRA activity. In Fig. 4 we study the dose-response relationship between D and CRA. In this case 600 ng of D were needed to achieve complete restoration of activity of 0.4 ml of a 1/2 dilution of RD + P̄.

In short, these experiments demonstrate that serum reagents in which the classical pathway of C activation was fully active could not solubilize immunoaggregates efficiently when factors D or P̄ of the alternative pathway had been removed.

The role of the classical pathway in CRA. We demonstrated above that the classical pathway alone is not sufficient to solubilize complexes, and we have previously shown that the classical pathway also is not essential for CRA. However, CRA is sluggish in the absence of classical pathway components C2, C4, or Ca^{++} (2, 3).

In the following experiments we study the role of the classical pathway in CRA, and provide evidence that its function is to deposit C3b fragments onto the aggregates, thereby initiating the assembly of the alternative pathway C3-convertase ($\overline{C3bPB}$). This conclusion is based on the following observations.

(*a*) The deposition of C3b fragments on the immune aggregates by the classical pathway enhances CRA.

TABLE I
*CRA and Hemolytic Titers of Human Sera Depleted of Factor D**

Serum tested	Total hemolytic titer	CH_{50} C1	CH_{50} C4	CH_{50} C2	CH_{50} C3	CRA
		U/ml‡				%§
Normal serum	170	34,900	52,300	4,500	9.100	85.0 (10.1)
RD, 080775	150	46,400	52,300	4,500	9,100	8.8 (10.1)
Normal serum	150	38,700	40,300	5,200	9,400	81.9 (14.2)
RD, 082775	146	9,300	64,100	5,500	8,800	19.2 (14.2)

* RD was prepared by a combination of gel filtration and ultrafiltration as described in Methods.

‡ Determined by hemolytic assays.

§ Percent of immune precipitates solubilized after incubation with 0.4 ml of a 1:2 dilution of serum at 37°C for 60 min. Given in parentheses are the control values, that is, the percent immune precipitates solubilized in the presence of EDTA serum. In these experiments, precipitates containing 0.5 μg Ab were added to the sera.

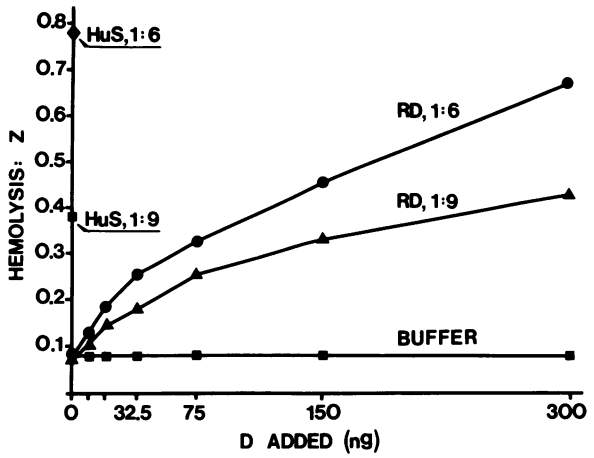

FIGURE 2 Reconstitution of indirect lysis with RD and pure D. 0.2 ml of a 1:6 or a 1:9 dilution of RD and 0.1 ml of various dilutions of pure D were incubated with 0.1 ml of CoF (100 U/ml) at 37°C for 45 min. Controls contained GVB^{++} and D. 0.5 ml of 5×10^7/ml guinea pig erythrocytes suspended in EDTA-GVB and 0.3 ml of guinea pig serum made 0.01 M with EDTA were then added, and the mixtures were incubated at 37°C for 60 min. Z is the average number of effective hemolytic sites per cell computed from the degree of lysis. Z values observed with a 1:6 or a 1:9 dilution of normal serum are indicated on the ordinate by horizontal arrows.

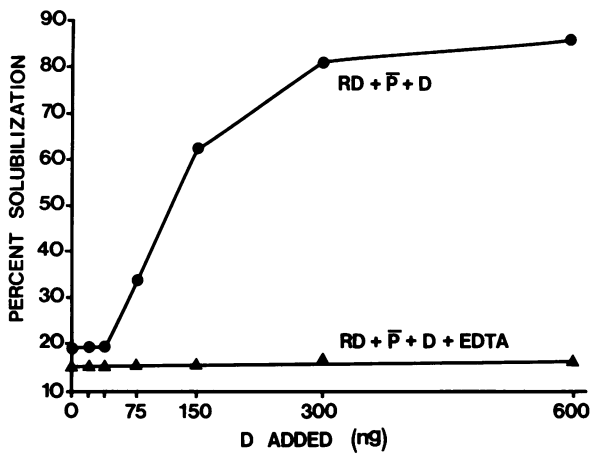

FIGURE 4 Dose-response curve of the reconstitution of RD with various amounts of D. 0.2 ml of RD, supplemented with 0.45 μg of P̄, was mixed with various amounts of purified D. The total volume was adjusted to 0.4 ml with GPB^{++}. Immune precipitates containing 0.5 μg of Ab were incubated with the mixture at 37°C for 60 min. The pellets and the supernates were separated by centrifugation and assayed for radioactivity. Controls consisted of immune precipitates incubated in the presence of 0.01 M EDTA.

In the experiment shown in Table II, ^{131}I-C3 and C2 at various concentrations were incubated with the immune complexes previously treated with C1 and C4. Up to 3.7 μg of C3b per 100 μg of Ab were incorporated into the immune aggregates at the highest concentrations of C2. Very little C3b was bound to immune complexes in the absence of C2. Next, the intermediate complexes Ag-Ab $C\overline{1,4b,2a,3b}$, Ag-Ab $C\overline{1,4b}$, or Ag-Ab were incubated in C2-deficient serum (C2D) at 37°C. As expected (4), solubilization of Ag-Ab in C2D was much less efficient than in normal serum. Solubilization was enhanced in intermediates preincubated with C1, C4, C2, and C3, but not with C1 and C4 alone (Fig. 5). Moreover, the degree of enhancement was greater in complexes containing more

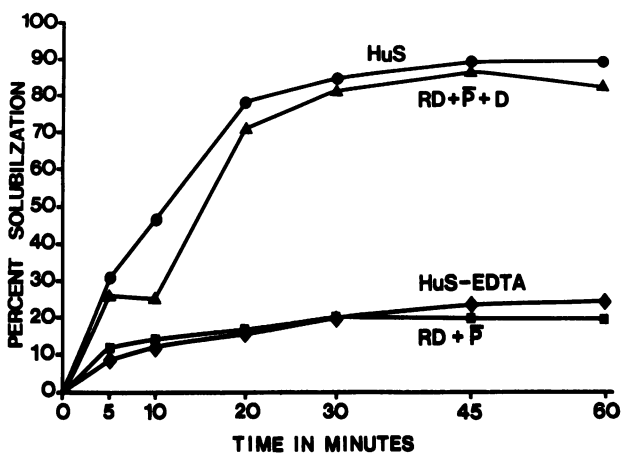

FIGURE 3 Reconstitution of CRA with RD and purified D. Kinetics of solubilization of Ag-Ab complexes by RD plus D. 0.2 ml of RD was mixed with 0.5 μg P̄ and 300 ng D. The total volume was brought to 0.4 ml with GPB^{++}. Immune precipitates containing 0.5 μg Ab were added to the prewarmed reaction mixtures at time 0. 0.05-ml samples were taken at indicated times, diluted, and centrifuged. The supernate and the pellets were assayed for radioactivity.

TABLE II

Incorporation of C3 into the Immune Complexes Treated with Early Components of the Classical Pathway

Reagents	C3 Incorporated*
	μg per 100 μg Ab
Ag-Ab $C\overline{1,4}$ + C3 + C2 (2,000 CH_{50}/ml)	3.7
Ag-Ab $C\overline{1,4}$ + C3 + C2 (400 CH_{50}/ml)	1.5
Ag-Ab $C\overline{1,4}$ + C3 + C2 (80 CH_{50}/ml)	0.7
Ag-Ab $C\overline{1,4}$ + C3 + $DGVB^{++}$	0.2

* Immune complexes Ag-Ab $C\overline{1,4}$ were prepared from ^{125}I-BSA, Ab, and purified components C1 and C4 as described in the Methods section. Intermediate immune complexes containing 57 μg Ab were incubated with C2 and C3 (1.0 mg/ml) plus ^{131}I-C3 at 30°C for 60 min. The total volume of the reaction mixture was 0.5 ml. The pellets were separated by centrifugation and washed. The amounts of C3 and Ab in the precipitates were calculated after measuring the radioactivity in the pellets. Under the conditions of this experiment more than 95% of the immune complexes were recovered in the pellets.

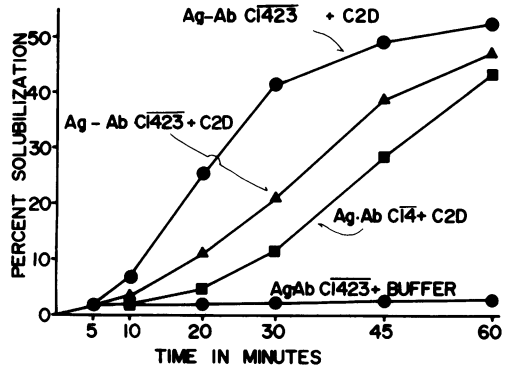

FIGURE 5 Enhanced solubilization of immune precipitates after treatment with the early components of the classical pathway. Immune complexes treated with C1, C4 (Ag-Ab $C\overline{1,4}$) were prepared as described in the Methods section. Ag-Ab $C\overline{1,4,2,3}$ bearing various amounts of C3b were prepared as described in the legend of Table II. Ag-Ab $C\overline{1,4,2,3}$ contained either 3.7 μg of C3b per 100 μg Ab (●) or 1.5 μg of C3b per 100 μg Ab (▲). The various complexes containing 0.1 μg of ^{125}I-BSA and 0.57 μg Ab were incubated with 0.4 ml of C2D or GPB^{++}. 0.05-ml samples were taken at selected times, diluted, and centrifuged. Pellets and supernate were assayed for radioactivity. The kinetics of solubilization in C2D of Ag-Ab alone did not differ from that of Ag-Ab $C\overline{1,4}$ (■).

C3b. In subsequent experiments these primed Ag-Ab $C\overline{1,4b,2a,3b}$ complexes, as well as the Ag-Ab $C\overline{1,4b}$ controls, were incubated at 37°C for 120 min in the presence of EDTA to decay C2 and remove C1s. These precipitates, presumably Ag-Ab C4b,3b and Ag-Ab C4b, were incubated with C2D (Fig. 6). Again, CRA was enhanced only when the complexes contained C3b.

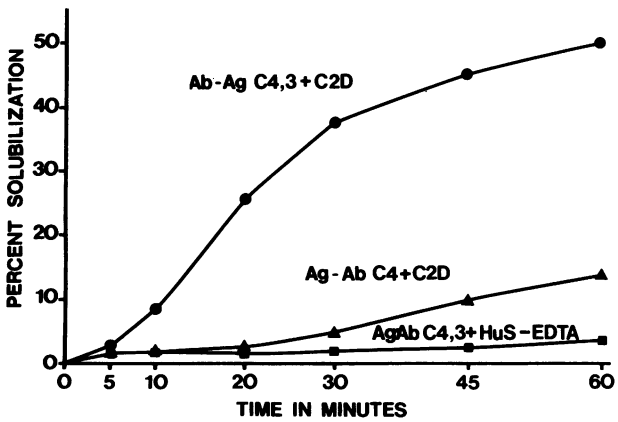

FIGURE 6 Enhanced solubilization of immune complexes bearing C3. Preparation of immune complexes with C4 (Ag-Ab C4) or with C4 and C3 (Ag-Ab C4, 3) is described in the Methods section. These primed immune complexes containing 0.1 μg ^{125}I-BSA and 0.57 μg of Ab were incubated at 37°C with 0.4 ml of a 1:2 dilution of either C2D or EDTA serum. 0.05-ml samples were taken at selected times, diluted, and centrifuged. Supernates and pellets were assayed for radioactivity.

(*b*) The deposition of C3b on immunoaggregates by the classical pathway promotes the assembly of an alternative pathway C3-convertase.

The experiments in Table III demonstrate very substantial C3-convertase activity in mixtures containing B, D, C3, and Ag-Ab $C\overline{1,4b,2a,3b}$. In contrast, incubation of B, D, and C3 with Ag-Ab $C\overline{1,4b}$ or C3 alone with Ag-Ab $C\overline{1,4b,2a,3b}$ resulted in very little inactivation of C3.

Additional direct evidence that the alternative pathway C3-convertase associated with the immunoaggregates is modulated by the classical pathway is shown in Fig. 7. Immunoprecipitates formed with ^{131}I-BSA and Ab were mixed with normal human serum or C2D. Trace amounts of ^{125}I-$\bar{P}$ were added and the mixtures incubated at 37°C for different periods of time. The kinetics of uptake and absolute amounts of ^{125}I-$\bar{P}$ incorporated into the immunoprecipitates were strikingly diminished in C2D as compared to normal serum. The addition to C2D of physiological amounts of C2 corrected the defect.

Analogous results were obtained when we compared the uptake of ^{125}I-$\bar{P}$ by immunoprecipitates incubated with normal serum in the presence or absence of free Ca^{++}. The incorporation of ^{125}I-$\bar{P}$ was greatly diminished after chelation of Ca^{++} with EGTA and inhibition of C1 activation, and addition of Ca^{++} to overcome the effect of EGTA restored the incorporation of ^{125}I-$\bar{P}$ to normal levels (not shown). In short, the deposition on immunoaggregates of $\bar{P}$ was markedly enhanced through the activity of classical pathway components C2 and C1.

Complement components incorporated into immune

TABLE III

Inactivation of C3 by Immune Complexes Pretreated with the Early Components of the Classical Pathway

Reagents*	C3 Inactivation‡
	%
Ag-Ab $C\overline{1,4,2,3}$ + B + D + C3	47.3
Ag-Ab $C\overline{1,4,2,3}$ + C3	5.1
Ag-Ab $C\overline{1,4}$ + B + D + C3	5.1
B + D + C3	4.7

* Immune complexes treated with either C1,C4 (Ag-Ab $C\overline{1,4}$) or C1,4,2 and 3 (Ag-Ab $C\overline{1,4,2,3}$) were prepared as described in the Methods section. The concentrations of B, $\bar{P}$, and C3 were 2.5 μg/ml, 0.25 μg/ml, and 50 μg/ml, respectively.

‡ The various immune complex intermediates containing 34 μg BSA and 200 μg Ab were resuspended in 0.2 ml DGVB^{++}. 0.1 ml of the suspension was incubated with B,D, and C3 or with C3 alone at 37°C for 60 min. The total volume of the reaction mixture was 0.5 ml. At the end of the incubation, the supernate was separated by centrifugation and the residual C3 was titrated by hemolytic assay.

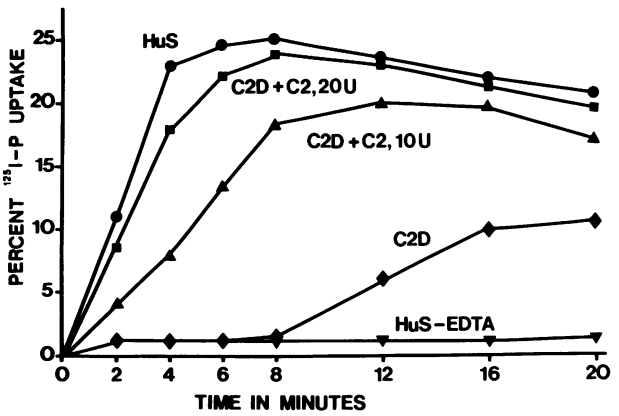

FIGURE 7 ^{125}I-$\bar{P}$ uptake of immune precipitates incubated with C2D. 2.4 μg of ^{125}I-$\bar{P}$ were added to 0.4 ml of a 1:2 dilution of either normal serum, C2D, C2D supplemented with 10 or 20 U of C2, or EDTA serum, and the mixtures were incubated at 37°C for 10 min. Immune precipitates containing 50 μg of ^{131}I-BSA and 280 μg of Ab were added to the mixtures at time 0. 0.05-ml samples were taken at selected times, diluted, and centrifuged. The pellets were washed once with 0.4 ml of cold EDTA-GPB^{++}. Pellets and combined supernates were assayed for radioactivity. Less than 5% of the added precipitates were solubilized at the end of incubation.

complexes during the solubilization process. Table IV summarizes the results of experiments designed to identify C components associated with solubilized immune complexes. Immune aggregates were incubated at 37°C for 60 min in human serum diluted in GPB^{++} or in EGTA-Mg^{++}-GPB, and centrifuged at 3,000 *g* for 20 min. Under the conditions chosen, more than 80% of the aggregates were recovered in the supernate. The solubilized complexes were then divided into 0.025-ml aliquots and reacted with antisera monospecific for various C components. As mentioned in the Methods section, in every experiment we included controls for the nonspecific incorporation of the solubilized complexes into a nonrelated immune precipitate. For this purpose we added Ea and anti-Ea to an aliquot of the solubilized complexes.

TABLE IV

C Components Detected on Solubilized Ag-Ab Complexes by Coprecipitation Experiments

Immune complexes solubilized by	Radioactivity (^{131}I-BSA) coprecipitated by antibody against*					
	C4	C3	C5	B	$\bar{P}$	Ea
	%					
Human serum in GPB^{++}	20.4	80.9	1.4	1.8	46.3	3.7
Human serum in EGTA-Mg^{++}-GPB	2.7	69.7	1.9	3.2	44.4	3.6

* Immune complexes formed with ^{131}I-BSA and Ab were solubilized by incubation in human serum diluted either in GPB^{++} or EGTA-Mg^{++}-GPB. After centrifugation, 0.025-ml aliquots of the supernate were mixed with carrier proteins. EDTA human serum served as a carrier for C4, C3, C5, and factor B. Purified $\bar{P}$ was a carrier for P. As a control, Ea was added to the solubilized complexes. The mixtures were made 0.01 M with EDTA and mixed with the appropriate monospecific antiserum. The amount of antiserum to be added was determined in preliminary experiments, so that Ab was in slight excess in the Ag-Ab reaction. After incubation at 37°C for 10 min, and at 4°C overnight, the precipitates were separated by centrifugation, washed three times with cold buffer, and counted. The results represent means of duplicate determinations.

A large proportion of the solubilized complexes (70–80%) were precipitated by Ab to C3. About 20% of the solubilized complexes were brought down by Ab to C4 if solubilization had been carried out in the presence of Ca^{++} and Mg^{++}. More than 40% of the solubilized complexes were precipitated by anti-$\bar{P}$. In contrast, we did not detect factor B or C5 on the complexes by this method.

It should be pointed out that because the precipitation reaction mixtures contained 0.01 M EDTA, it is very unlikely that C3 or C4 which were added as carriers could have been incorporated secondarily into the solubilized immune complexes. That this was not the case is also shown by the absence of C4 in complexes solubilized in serum containing EGTA-Mg^{++}-GPB. However, it is conceivable that the added purified $\bar{P}$ could bind to the solubilized complexes during coprecipitation because $\bar{P}$ has a binding site for C3b (23, 24). This could happen either by an exchange reaction between the added $\bar{P}$ and previously bound $\bar{P}$, or by the capture of the added $\bar{P}$ by some free C3b sites on the complexes. Whatever the case, the fact that 40% of the solubilized complexes coprecipitated with the $\bar{P}$-anti-P complexes demonstrates the presence of a large number of $\bar{P}$ receptors on the solubilized complexes.

Assembly of a C5-convertase on immune complexes during solubilization. The findings above indicate that C5, in contrast to C3 and C4, is not incorporated in large amounts onto complexes during solubilization. For this reason we assayed for the presence of a C5-convertase on the aggregates incubated with serum. The results of Table III demonstrate that a C5-convertase, as well as a C3-convertase (5) is formed on the immune aggregates incubated in fresh serum. Moreover, both enzymes can be formed via the alternative pathway alone as shown by the experiments in which Ca^{++} had been chelated from serum by the addition of EGTA.

DISCUSSION

These experiments demonstrate that factors D and $\bar{P}$ play an essential role in solubilization of immune-aggregates in vitro. Serum depleted of P had no detect-

able CRA activity when tested at a 1:2 dilution, and addition of $\bar{P}$ at physiological concentrations (20 μg/ml of serum) restored CRA to normal levels. The dose-response curve obtained by plotting the amounts of $\bar{P}$ added vs. CRA, and shown in Fig. 1, suggests that $\bar{P}$ is utilized stoichiometrically in the reaction. The effect of small doses of $\bar{P}$ could not be enhanced by prolonging the times of incubation at 37°C. This result is in agreement with our previous observations showing that $\bar{P}$ was part of the factor B-dependent C3-convertase associated with the immune complexes. Although the nature of the $\bar{P}$ receptor on the immune aggregates is unknown, C3 moieties become associated with the complexes at the same time that the binding of $\bar{P}$ takes place; that is, in the first minutes after incubation with serum (5). Because C3b binds $\bar{P}$, (23–25) it is likely that the C3 molecules on the aggregates are $\bar{P}$ receptors.

RD also had very little or no CRA. Reconstitution of RD with D only partially restored CRA. Subsequently, we found that RD had also been depleted of $\bar{P}$ during its preparation.[2] Addition of a mixture of D and $\bar{P}$ in physiological amounts completely restored CRA, whereas addition of $\bar{P}$ alone had no effect.

In short, these results, in conjunction with our previous finding, establish the essential role in CRA of all components of the alternative pathway, that is, factor B, C3, Mg^{++} (2, 3), $\bar{P}$, and D.

The experiments in which RD and RP were shown to lack CRA also indicate that the classical pathway alone cannot solubilize complexes. Both reagents had almost intact hemolytic activity, and the concentrations of C1, C4, C2, and C3 in RD were close to normal. The ineffectiveness of the classical pathway in CRA is also shown by the experiment depicted in Fig. 5, in which immune aggregates were treated sequentially with high concentrations of C1, C4, C2, and C3. These C-treated complexes showed no tendency to solubilize when reincubated in buffers or in EDTA serum. These observations are in agreement with our previous finding that a lattice-associated factor B-dependent (but not C2-dependent) C3-convertase is essential for CRA (5).

It is not clear why activation of C3 through the classical pathway is quite inefficient in achieving solubilization. One possibility is that a small number of $\overline{C4,2}$ sites, and consequently of C3, become associated with the aggregates after their incubation with the purified classical complement components, or with whole serum depleted of factors D or $\bar{P}$. It is well known that the concentration of C2 in serum is much lower than that of factor B (26) and, in addition, $\overline{C4,2}$ has a much shorter half-life at 37°C (23, 27) than $\overline{C3b,P,B}$. In other words, the number of C3b molecules generated by Ag-Ab $\overline{C1,4,2}$, and which are bound to the immune complexes, may not be sufficient to disrupt the Ag-Ab lattice and solubilize the precipitates.

Although the classical pathway cannot solubilize aggregates by itself, it greatly enhances the activity of the alternative pathway (4). The results of Table II and Figs. 5 and 6 demonstrate that the role of the classical pathway is to deposit C3b onto the Ag-Ab lattice and thereby enhance subsequent solubilization by the alternative pathway. Table III shows the formation of the alternative pathway C3-convertase activity by C3b deposited onto the immunoprecipitates through the activity of the classical pathway.

These results are remarkably similar to those published by Brade et al. (28) in which the formation of a factor B-dependent C3-convertase on zymosan was greatly enhanced by C4. They subsequently provided direct evidence that the assembly of the $\bar{P}$-system enzymes was accelerated by the presence of C3b on zymosan (29). These findings further emphasize the important role of the positive feedback effect of C3b on the alternative pathway (30, 31) and show a similarity of events occurring on the surface of zymosan particles and on small amounts of immune aggregates incubated in a high concentration of serum.

Similar observations were made in 1958 by Nelson (32) in a paper in which he critically reexamined the concepts of Pillemer and his collaborators about the recently discovered P pathway of C activation. In one of his detailed protocols, Nelson clearly showed that, similar to zymosan particles, small amounts of BSA-anti-BSA-washed aggregates consumed a much larger proportion of C3 than C1,4 from guinea pig serum. Recently, with more defined C reagents, Hiramatsu et al. (33) obtained identical results. The present observations support Nelson's contention that the mechanisms of activation of C by zymosan and immune complexes were in some situations not fundamentally different. The key point, however, is that in those situations, the role of the classical pathway appears to be that of an accelerator of the assembly of a membrane, or lattice-associated factor B-dependent C3-convertase.

Finally, we here examine the composition of solubilized complexes (Table IV). In agreement with previous findings (2) most complexes contained C3. The lattice-associated C3 is very firmly bound to the Ig molecules (4). The complete separation of C3 from Ig required treatment both with denaturing and reducing agents.[3] As discussed in detail elsewhere (3–5), these findings

[2] The concentration of P in human serum is only $\cong$20 μg/ml. Depletion of P from RD may have been caused by the binding of P to the small amounts of C3b which remain nonspecifically associated with the Sephadex beads during filtration of serum. In addition, P may have been denatured during the ultrafiltration step of the purification procedure.

[3] Czop, J., B. F. Tack, and V. Nussenzweig. Unpublished observations.

support the concept that it is the accumulation of C3 on the aggregates that causes solubilization.

The presence of C4 on the aggregates solubilized in GPB^{++} was not surprising in light of previous findings. Müller-Eberhard and Lepow (34) as well as Willoughby and Mayer (35) had already pointed out that activation of C4 in the presence of Ig leads to the formation of Ig-C4 complexes. When disaggregation occurred in EGTA-Mg^{++}, most solubilized complexes still contained C3 and $\bar{P}$ in the absence of C4, providing additional evidence that a factor B-dependent C3-convertase can be assembled on the Ag-Ab lattice without participation of the classical pathway (5).

We had previously observed that CRA occurred very effectively in the presence or absence of C5 and C6 (2). We show here that during the process of solubilization of complexes, a C5-convertase is assembled on the aggregates and that C5 can be converted in the fluid phase (Table V). However, we failed to detect firmly bound C5 antigenic determinants on the solubilized complexes. Nilsson and Müller-Eberhard also did not find C5 on immune precipitates incubated with serum and redissolved in excess of Ag (36). These findings suggest that, in contrast with nascent C3b and C4b, nascent C5-9 lacks binding affinity for immune aggregates. In addition, these findings are of relevance to the question of the origin of the terminal C components (C5, C6, and C8) frequently detected in the kidney of patients with nephritis (37). In light of the present observations, it appears more likely that these terminal C components were deposited as a consequence of local C consumption and direct binding to the glomeruli, and not passively in association with circulating immune complexes.

TABLE V

*C3 and C5-Convertase Activities Generated on Immune Aggregates**

Immune complexes reacted with	Incubation time	Inactivation‡ C3	Inactivation‡ C5
	min	%	
Human serum	8	80.1	76.5
Human serum in EGTA-Mg^{++}-GPB	8	55.0	40.3
	12	67.5	60.1
Human serum in EDTA	8	1.3	2.1

* Immune precipitates containing 17.2 μg ^{125}I-BSA and 100 μg Ab were incubated with 20 ml of a 1:2 dilution of human serum, diluted either in GPB^{++} or EGTA-Mg^{++}-GPB or EDTA, at 37°C for the indicated times. The precipitates were then washed three times with cold GVB^{++} and tested for C3- and C5-convertase activities. No solubilization occurred during this time.

‡ Percent inactivation of added C3 (80 CH_{50} U) or C5 (32 CH_{50} U) after incubation at 30°C for 30 min with the immune precipitates.

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