SOLUBILIZATION OF IMMUNE PRECIPITATES BY SIX ISOLATED ALTERNATIVE PATHWAY PROTEINS*

By TEIZO FUJITA, YUKO TAKATA, and NOBORU TAMURA

From the Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba, Niihari-gun, Ibaraki-ken 305, Japan

Antigen-antibody precipitates can be dissolved by the complement cascade (1). The complement-mediated solubilization of immune precipitates requires activation of C3 via the alternative pathway (2) but not participation of the late-acting components, C5 through C9 (1, 3). The classical pathway alone, in the absence of the alternative pathway, is not sufficient to solubilize immune complexes (3).

It has recently been demonstrated that the alternative pathway can be assembled from six isolated plasma proteins: C3, factors B and D, properdin, C3b inactivator (C3bINA),[1] and β1H (4, 5). Cells such as rabbit erythrocytes (6), human lymphoblastoid (Raji) cells (7), and *Escherichia coli* (8) have been shown to be lysed by the isolated alternative pathway proteins and membrane attack pathway proteins. The question therefore arose as to whether the composite mixture of the isolated alternative pathway proteins is capable of solubilizing immune precipitates. In this communication we report that, indeed, the six isolated alternative pathway proteins can solubilize immune precipitates. In addition, we report on the role of the three regulatory factors (C3bINA, β1H, and properdin) in the solubilization process.

Materials and Methods

Buffers. PB: isotonic phosphate-buffered saline, pH 7.4; Mg-GPB: PB containing 0.1% gelatin and 1.2 mM $MgCl_2$; EDTA-GPB: PB containing 0.1% gelatin and 10 mM EDTA; DGVB: isotonic veronal buffered saline, pH 7.4, containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2.5% dextrose.

Immune Precipitates. Egg albumin (grade V) was purchased from Sigma Chemical Company, St. Louis, Mo. Rabbit antiserum to egg albumin was prepared by injecting a rabbit with egg albumin incorporated in complete Freund's adjuvant. IgG antibody to egg albumin was isolated by DEAE-cellulose column chromatography and by affinity chromatography on Sepharose 4B-egg albumin. The IgG was labeled with ^{125}I by the chloramin T method (9). The specific activity was 5.5×10^6 cpm/μg protein.

Immune complexes were prepared at equivalence with egg albumin and labeled IgG antibody to egg albumin. The mixture was incubated at 37°C for 30 min, then at 4°C overnight. The resulting precipitates were washed three times by centrifugation in the cold and resuspended in Mg-GPB.

* Supported, in part, by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, the Government of Japan.

[1] *Abbreviations used in this paper:* Ab, antibody; C3bINA, C3b inactivator; DGVB, isotonic veronal buffered saline, pH 7.4, containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 2.5% dextrose; EDTA-GPB, PB containing 0.1% gelatin and 10 mM EDTA; Mg-GPB, PB containing 0.1% gelatin and 1.2 mM $MgCl_2$; PB, isotonic phosphate-buffered saline, pH 7.4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Purified Complement Components. Human C3 (10) factor B (11, 12), factor D (13), properdin (14), β1H (15), and C3bINA (16) were purified as previously described. The purified properdin did not contain detectable amounts of activated properdin, and properdin did not induce C3 cleavage upon addition to normal serum (17). The C3bINA was found to be contaminated with trace amounts of IgG and transferrin, which were eliminated by passage through specific immunoabsorbents.

To verify purity of the components, 10–20 μg of each alternative pathway protein was reduced with 5% 2-mercaptoethanol in 3 M urea and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). Each protein preparation was homologous and exhibited the appropriate molecular weight for its polypetide chains. Factors B and D, properdin, and β1H each demonstrated a single stained band, and C3 and C3b1NA, which consist of two covalently linked polypeptide chains revealed two bands (Fig. 1). The protein concentrations were calculated from their optimal densities at 280 nm, under the assumption that an absorption coefficient value of 1.0 equals 1 mg/ml.

C3 was trace labeled with ^{131}I by insolubilized lactoperoxidase (Worthington Biochemical Corp., Freehold, N. J.) (19) without loss of functional activity. The specific activity was 6.4×10^5 cpm/μg protein.

For titration of C3, guinea pig C1 (20), human C4 (21), C2 (22), and C5 (10), and guinea pig C6, C7, C8, and C9 (20) were partially purified by previously described techniques.

Preparation of the Isolated Component Mixture. The alternative complement pathway was reconstructed with one-fourth the physiological concentrations of purified C3 (300 μg/ml), factor B (50 μg/ml), factor D (1 μg/ml), properdin (5 μg/ml), C3bINA (10 μg/ml), and β1H (127.5 μg/ml). The buffer was Mg-GPB.

Serum Lacking in Classical Pathway Function (EGTA-Serum). Fresh human serum containing 10

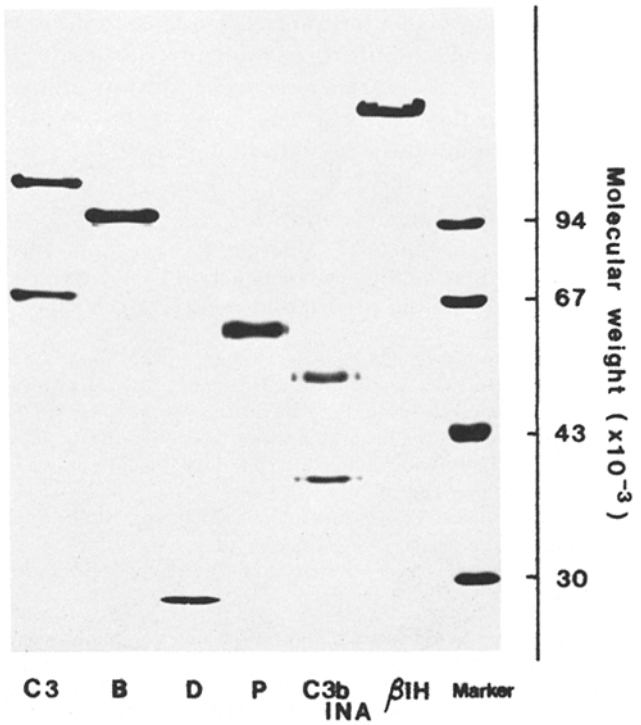

FIG. 1. Preparations of the purified alternative pathway components analysed by SDS-PAGE under reducing conditions. The marker proteins were 94,000 phosphorylase B, 68,000 bovine serum albumin, 43,000 ovalbumin, and 30,000 carbonic anhydrase.

mM EGTA and 5 mM $MgCl_2$ was used as a reagent, allowing complement activation by the alternative pathway alone.

Solubilization Assays. Solubilization assays were carried out in Mg-GPB as described (1). In kinetic studies, immune precipitates were added to a mixture of the six alternative pathway proteins. Then, aliquots were taken at selected times and immediately mixed with 2 ml of cold EDTA-GPB. Supernates and pellets were separated by centrifugation at 5,000 *g* for 20 min and assayed for radioactivity in a gamma counter.

Binding of ^{131}I-C3 to Immune Precipitates Incubated with the Six Isolated Alternative Pathway Components. Immune precipitates containing 1.75 μg of complexed antibody (Ab) were incubated at 37°C in a total volume of 400 μl, with the six isolated proteins at one-fourth their serum concentrations, to which 0.02 μg of ^{131}I-C3 had been added. 40-μl aliquots were taken at selected times and mixed with 2 ml of EDTA-GPB. Precipitates were separated from supernates by centrifugation in the cold at 5,000 *g* for 20 min and washed three times with cold EDTA-GPB. ^{125}I-Ab and ^{131}I-C3 in the supernates and precipitates were counted. The amount of Ab and C3 in nonsolubilized precipitates at different times was calculated, and then the number of C3 molecules bound to one molecule of IgG Ab was calculated, under the assumption that the molecular weights of IgG and the C3 fragment bound to the precipitates were 150,000 and 180,000, respectively.

Molar Ratio of C3/Ab in the Solubulized Complexes. Immune precipitates were incubated with the six isolated proteins under the conditions described above. 70-μl aliquots were taken at 0, 20, 30, and 60 min, mixed with 500 μl of EDTA-GPB, and centrifuged. ^{125}I-Ab in the supernates and precipitates was counted. The supernates were reacted at 37°C for 30 min with anti-rabbit IgG (N. L. Cappel Laboratories Inc. Cochranville, Pa.). The resulting immune complexes were removed by the addition of *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), followed by a 30-min incubation at 0°C with shaking. The amounts of antisera and *Staphylococcus aureus* were determined in preliminary experiments. After washing three times with 2 ml of EDTA-GPB, ^{125}I-Ab and ^{131}I-C3 bound to the bacteria were counted. The amount of C3 specifically bound was then calculated, that is, the amount of C3 bound at 20, 30, and 60 min minus that of C3 at time 0.

Hemolytic Assay of C3. EAC142 were prepared with guinea pig C1, human C4, and oxidized human C2 (23). 0.2 ml of dilutions of the test samples in DGVB were mixed with 0.2 ml of 1×10^8/ml EAC142 and 0.2 ml of a reagent containing 200 U each/ml of C5, C6, C7, C8, and C9. These mixtures were incubated at 37°C for 60 min, and after the addition of 2.4 ml of DGVB, the degree of hemolysis was determined spectrophotometrically at 412 nm.

Results

Solubilization of Immune Precipitates by the Six Isolated Alternative Pathway Proteins. A reaction mixture was constituted to contain one-fourth the respective serum concentrations of purified C3, factor B, factor D, properdin, C3bINA, and β1H. The mixture was incubated at 37°C with immune precipitates of egg albumin and radiolabeled rabbit IgG antibody to egg albumin, and their solubilization was assayed kinetically. For comparison, solubilization by a 1:4 dilution of human serum or EGTA-serum was examined. As shown in Fig. 2, immune precipitates were solubilized by the six purified components. The kinetic curve of solubilization is virtually identical to that obtained with EGTA-serum. In human serum, solubilization proceeded at a faster rate, indicating that the classical pathway activation enhances solubilization. No solubilization was observed when individual proteins, such as C3, factor B, or factor D, were deleted from the complete mixture.

Role of the Three Regulatory Factors in Solubilization. In the experiments shown in Table I, we evaluated the participation of C3bINA, β1H, and properdin in the process of solubilization. The various mixtures described in Table I were incubated at 37°C for 1 h with immune precipitates containing ^{125}I-Ab. Supernates and pellets were separated by centrifugation and assayed for solubilization and residual C3 activity. In the

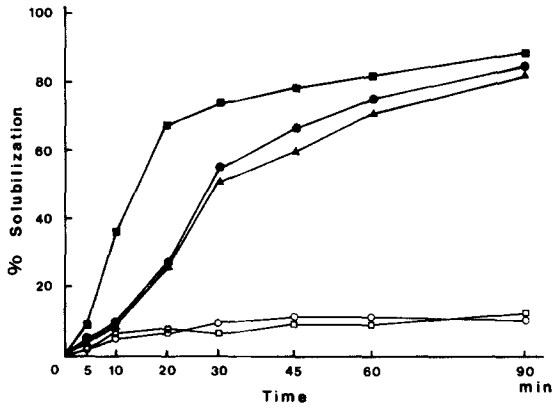

FIG. 2. Kinetics of solubilization in the six isolated alternative pathway proteins. Immune precipitates containing 0.44 μg of Ab were incubated at 37°C with 200 μl of a mixture of the six components (●) at one-fourth their serum levels or with 200 μl of a 1:4 dilution of human serum (■) or of EGTA-serum (▲). 25-μl aliquots were taken from the mixture at selected times, diluted with 2 ml of cold EDTA-GPB, and centrifuged. Supernates and pellets were assayed for radioactivity. When the purified components or the 1:4 dilution of human serum were treated with EDTA, no solubilization was observed. □, EDTA-serum; ○, EDTA-components.

TABLE I

C3 Consumption and Solubilization in Various Mixtures of the Six Isolated Components

Protein mixtures‡	C3 consumption*		Solubilization
	With antigen-antibody§	Without antigen-antibody	
	%	%	%
C3 B D P C3bINA β1H	47.9	0	60.9
C3 B D C3bINA β1H	4.6	0	12.1
C3 B D P C3bINA	99.2	99.0	13.5
C3 B D P β1H	38.5	39.0	10.8
C3 B D P	99.2	99.2	9.8
C3 B D	98.7	99.2	10.2
Buffer	—	—	9.6

* The residual C3 was assayed by hemolytic titration. Another control consisted of antigen or antibody alone incubated with the mixtures. The results were the same as those obtained without antigen-antibody.

‡ The protein concentrations were C3, 300 μg/ml; factor B, 50 μg/ml; factor D, 1 μg/ml; properdin, 5 μg/ml; C3bINA, 10 μg/ml; β1H, 127.5 μg/ml. The buffer was Mg-GPB, and the total volume of the reaction mixtures was 200 μl.

§ Immune complexes (antigen-antibody) contained 0.44 μg Ab.

absence of immune precipitates, the mixture containing all the six components exhibited no loss of hemolytic C3 activity, even after prolonged incubation at 37°C. The addition of immune precipitates to the complete mixture resulted in 60% solubilization of the precipitates and 47.9% consumption of C3, indicating that immune complexes containing rabbit IgG Ab are activators of the alternative pathway and that as a result of the activation, solubilization takes place. In the properdin-deficient reaction mixture, neither solubilization nor C3 consumption was observed.

On the other hand, the absence of β1H allowed total consumption of C3 in the absence of immune precipitates, and no effect on the immune precipitates could be recognized. When C3bINA was deleted from the reaction mixture, solubilization did not occur. The same degree of C3 inactivation was observed in both the presence and the absence of immune precipitates. These results indicated that in the absence of either β1H or C3bINA, cleavage of C3 occurs only in the fluid phase. Therefore, the presence of a regulated fluid-phase reaction is essential for solubilization. This is more clearly shown in the following experiments.

Decreasing amounts of C3bINA and β1H were added to a constant amount of C3, factor B, factor D, and properdin. The mixtures were incubated at 37°C for 1 h with immune precipitates. As shown in Fig. 3, C3 consumption increased in a dose-related manner with decreasing inputs of C3bINA and β1H. The degree of solubilization was proportional to the amounts of C3bINA and β1H in the mixture, and the maximum solubilization was observed in a reaction mixture of the six purified components reconstituted to their relative serum concentrations.

In short, it is likely that C3bINA and β1H function as regulators that retard fluid-phase amplification of C3 cleavage so that the reaction can be directed to the immune precipitates.

Incorporation of C3 into the Immune Precipitates. The experiments described above suggested that solubilization results from efficient fixation of C3 on the immune precipitates. In subsequent experiments, we studied the kinetics of C3 incorporation into the immune aggregates incubated with the isolated component mixture. Immune precipitates containing ^{125}I-labeled Ab were incubated at 37°C with the six purified proteins containing ^{131}I-labeled C3. Fig. 4 shows clearly that C3 bound rapidly to the aggregates. The onset of solubilization followed the binding of one molecule of C3 to one molecule of Ab in the precipitates. After 15 min, 1.4 molecules of C3 were bound to one molecule of Ab, and at that time solubilization had only started. At 30 min,

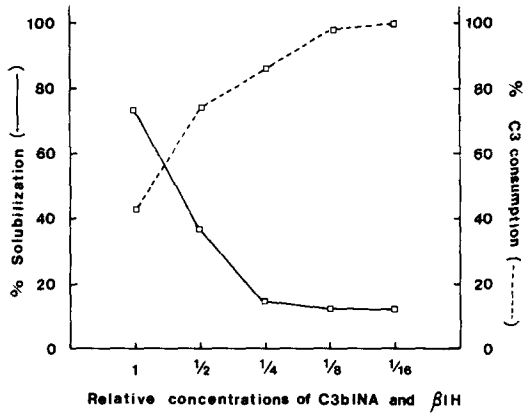

FIG. 3. Role of C3bINA and β1H in solubilization. Various amounts of C3bINA (3.3–0.2 μg/ml) and β1H (42.5–2.7 μg/ml) were added to mixtures of C3 (400 μg/ml), factor B (67 μg/ml), factor D (1.3 μg/ml), and properdin (6.7 μg/ml) constituted to contain one-third serum concentrations. The mixtures were incubated in a total volume of 200 μl at 37°C for 1 h with immune precipitate containing 0.38 μg of Ab. Supernates and pellets were separated by centrifugation and assayed for solubilization (——) and C3 consumption (- - -). When the ratio of C3bINA and β1H to the other components was equal to their physiological concentration in serum, the relative concentrations of the regulators were expressed as 1.

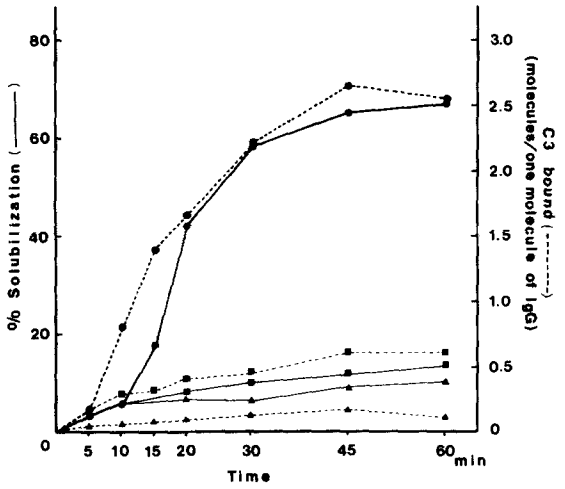

FIG. 4. Incorporation of C3 into the immune precipitates. Immune precipitates containing 1.75 μg of Ab were incubated in a total volume of 400 μl with the six isolated component mixture (●) or a mixture (■) of C3, factor B, factor D, and properdin, to which 0.02 μg of ^{131}I-C3 had been added. The control (▲) contained ^{131}I-C3 and Mg-GPB. 40-μl aliquots were taken at the indicated times, mixed with 2 ml of EDTA-GPB, and centrifuged. Precipitates were washed three times with cold buffer. ^{125}I-Ab and ^{131}I-C3 in the supernates and pellets were counted. The number of C3 molecules bound to one molecule of Ab in the precipitates was calculated.

2.2 molecules of C3 were bound and solubilization exceeded 50%. In contrast, more than one molecule of C3 was never bound to one molecule of Ab in the mixture of C3, factor B, factor D, and properdin, and no solubilization was observed.

Finally, we determined the C3:Ab ratio in the solubilized complexes. Immune precipitates were incubated with the six purified components under the conditions described in the legend of Fig. 4. The solubilized complexes at 20, 30, and 60 min were immunoprecipitated with specific antisera to rabbit IgG. Precipitates were assayed for ^{125}I and ^{131}I. 90% or more ^{125}I-Ab were precipitated. The molar ratio of C3: Ab was 0.99, 1.08, and 0.86 at 20, 30, and 60 min, respectively. These values represent means of duplicate determinations.

Discussion

This is the first reported demonstration of the solubilization of immune precipitates entirely by isolated alternative pathway proteins. That the alternative pathway of complement activation can be assembled from C3, factors B and D, properdin, and the three known regulatory factors has been documented (4, 5). Previous studies (1, 3, 24) have demonstrated that complement-mediated solubilization of immune precipitates proceeds via the alternative activation pathway alone, but this is enhanced by concomitant participation of the classical activation pathway. The late-acting complement components are thought to be nonessential for solubilization (1, 3). However, whether solubilization is mediated solely by the alternative activation pathway has heretofore not been critically evaluated. The results of this study indicate that no factors other than the six isolated alternative pathway proteins are essential for complement-mediated solubilization. The quantitative similarity of the kinetic

curves obtained with the component mixture and with EGTA-serum further supports our conclusion.

We confirmed the previous observation (25) that immune complexes activate the alternative pathway. In addition, our findings should serve to clarify the mechanisms of activation of the alternative pathway by immune complexes. As shown in Table I, no loss of hemolytic C3 activity was observed in a mixture of the six purified components. The introduction of immune complexes to the mixture induced moderate C3 consumption and efficient C3 fixation to the complexes (Table I and Fig. 4). When C3bINA and β1H were deleted from the mixture, complete consumption of C3 occurred in the fluid phase and resulted in a very small amount of C3 fixation. These results agree well with the concept that an activator of the alternative pathway provides sites that protect bound C3b from the action of C3bINA and β1H and permits C3b deposited by the low-grade fluid-phase reaction to assemble a membrane-associated amplification convertase that can deposit additional protected C3b (4). Therefore, it is clear that immune complexes have protected C3b sites, thereby activating the alternative pathway.

Another important finding is that solubilization followed the binding of at least one molecule of C3 to one molecule of Ab in the precipitates (Fig. 4). The molar ratio of C3:Ab in the solubilized complexes is ~1. The precise mechanism of solubilization is not entirely clear. However, our findings strongly support the concept (2, 25, 26) that C3 fragments intercalate into the antigen-antibody lattice and disrupt it sufficiently to generate small, soluble immune complexes. Therefore, it is important to clarify the nature of binding C3 fragments to the immune complexes. Hydrophobic interaction and covalent, esterlike linkage may be involved in this binding (27). This is currently under investigation.

Summary

Immune precipitates were solubilized by the alternative pathway of complement assembled from isolated proteins, i.e., C3, factor B, factor D, properdin, C3b inactivator (C3bINA), and β1H. The kinetic curves of solubilization in the isolated system and in EGTA-serum were virtually indistinguishable. No requirement of other factors was apparent.

Deletion of C3bINA and β1H from the complete mixture caused total consumption of C3 in the fluid phase and resulted in neither C3 binding to the complexes nor solubilization. Thus, the presence of a regulated fluid-phase reaction is essential for efficient fixation of C3 and the consequent solubilization. In addition, properdin plays an essential role in complement-mediated solubilization in the presence of the two regulators.

A large amount of C3 was incorporated into the antigen-antibody lattice. Solubilization of immune complexes started after the binding of one C3 molecule to one antibody molecule in the complexes, and the molar ratio of C3:antibody in the solubilized complexes also is ~1.

The authors wish to thank Dr. Akiko Baba of this laboratory for contributing to the supply of isolated proteins and Miss Harumi Sukegawa for manuscript preparation.

Received for publication 14 April 1981 and in revised form 7 July 1981.

References

1. Miller, G. W., and V. Nussenzweig. 1975. A new complement function: solubilization of antigen-antibody aggregates. *Proc. Natl. Acad. Sci. U. S. A.* **72:**418.
2. Takahashi, M., B. F. Tack, and V. Nussenzweig. 1977. Requirements for the solubilization of immune aggregates by complement. Assembly of a factor B-dependent C3-convertase on the immune complexes. *J. Exp. Med.* **145:**86.
3. Takahashi, M., S. Takahashi, V. Brade, and V. Nussenzweig. 1978. Requirements for the solubilization of immune aggregates by complement. The role of the classical pathway. *J. Clin. Invest.* **62:**349.
4. Fearon, D. T., and K. F. Austen. 1977. Activation of the alternative complement pathway with rabbit erythrocytes by circumvention of the regulatory action of endogenous control proteins. *J. Exp. Med.* **146:**22.
5. Schreiber, R. D., M. K. Pangburn, P. H. Lesavre, and H. J. Müller-Eberhard. 1978. Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3948.
6. Schreiber, R. D., and H. J. Müller-Eberhard. 1978. Assembly of the cytolytic alternative pathway of complement from 11 isolated plasma proteins. *J. Exp. Med.* **148:**1722.
7. Schreiber, R. D., M. K. Pangburn, R. G. Medicus, and H. J. Müller-Eberhard. 1980. Raji cell injury and subsequent lysis by the purified cytolytic alternative pathway of human complement. *Clin. Immunol. Immunopathol.* **15:**384.
8. Schreiber, R. D., D. C. Morrison, E. R. Podack, and H. J. Müller-Eberhard. 1979. Bactericidal activity of the alternative complement pathway generated from 11 isolated plasma proteins. *J. Exp. Med.* **149:**870.
9. McConahey, P., and F. J. Dixon. 1966. A method for trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29:**185.
10. Tack, B. F., and J. W. Prahl. 1976. Third component of human complement: purification from plasma and physicochemical characterization. *Biochemistry.* **15:**4513.
11. Boenisch, T., and C. A. Alper. 1970. Isolation and properties of a glycine-rich β-glycoprotein of human serum. *Biochim. Biophys. Acta.* **221:**529.
12. Hunsicker, L. G., S. Ruddy, C. B. Carpenter, P. H. Schur, J. P. Merrill, H. J. Müller-Eberhard, and K. F. Austen. 1972. Metabolism of third complement component (C3) in nephritis. Involvement of the classical and alternative (properdin) pathways for complement activation. *N. Engl. J. Med.* **287:**835.
13. Lesavre, P. H., T. E. Hugli, A. F. Esser, and H. J. Müller-Eberhard. 1979. The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol.* **123:**529.
14. Medicus, R. G., A. F. Esser, H. N. Fernandez, and H. J. Müller-Eberhard. 1980. Native and activated properdin: interconvertibility and identity of amino- and carboxy-terminal sequences. *J. Immunol.* **124:**602.
15. Whaley, K., and S. Ruddy. 1976. Modulation of the alternative complement pathway by β1H globulin. *J. Exp. Med.* **144:**1147.
16. Fearon, D. T. 1977. Purification of C3b inactivator and demonstration of its two polypeptide chain structure. *J. Immunol.* **119:**1248.
17. Götze, O., and H. J. Müller-Eberhard. 1974. The role of properdin in the alternative pathway of complement activation. *J. Exp. Med.* **139:**44.
18. Laemmli, J. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.
19. Thorell, J. L., and I. Larsson. 1974. Lactoperoxidase coupled to polyacrylamide for radioiodination of proteins to high specific activity. *Immunochemistry.* **11:**203.
20. Nelson, R. A., J. Jensen, I. Gigli, and N. Tamura. 1966. Methods for separation, purification

and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry.* **3:**111.

21. Bolston, C., S. Morris, B. Tack, and J. Parhl. 1977. The purification and structural analysis of the fourth component of human complement. *Biochemistry.* **16:**2008.
22. Tamura, N. 1970. An incompatibility in the reaction of the second component of human complement with the fourth component of guinea-pig complement. *Immunology.* **18:**203.
23. Gigli, I., T. Fujita, and V. Nussenzweig. 1979. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc. Natl. Acad. Sci. U. S. A.* **76:**6596.
24. Czop, J., and V. Nussenzweig. 1976. Studies on the mechanism of solubilization of immune precipitates by serum. *J. Exp. Med.* **143:**615.
25. Fujita, T. 1979. The activation mechanism of human complement system by immune precipitate formed with rabbit IgG antibody. *Microbiol. Immunol.* **23:**1023.
26. Takahashi, M., S. Takakashi, and S. Hirose. 1980. Solubilization of antigen-antibody complexes: a new function of complement as a regulator of immune reactions. *Prog. Allergy.* **27:**134.
27. Law, S. K., and R. P. Levine. 1977. Interaction between the third complement protein and cell surface. *Proc. Natl. Acad. Sci. U. S. A.* **74:**2701.