Complement Activation by a Univalent Hapten-Antibody Complex*

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SUMMARY

The univalent hapten, nonadeca lysyl ε-Dnp-lysine, binds tightly to rabbit anti-2,4-dinitrophenyl antibody, and the complex has a sedimentation coefficient of 6.7, characteristic of a single antibody molecule. In this communication, we show that this complex is a good activator of the serum complement system. For activation to occur, the univalent hapten must contain the specific group which binds to the antibody, and also the polycationic chain. In addition, activation requires a functional complement-binding region on the intact antibody molecule. The classical pathway appears to be involved since the first, fourth, and second components of complement are markedly depleted when the complement system is activated by this univalent hapten-antibody complex.

The serum complement system is composed of over a dozen proteins involved in a sequence of cascading reactions ultimately resulting in the destruction of the cellular membranes and elaboration of biologically active products (1). The complement system may be triggered either through the "classical pathway" by the presence of antibody aggregates, or through the "alternate pathway" by the presence of certain foreign polysaccharides, as well as by antibody aggregates (2). The initial step in the activation of the classical pathway involves an interaction between the first component of complement, Cl, and a number of receptor sites present on the immunoglobulin molecules forming an aggregate. Little is known about this activation process, and its detailed mechanism represents an important problem for further study.

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which two 2,4-dinitrophenyl haptenic groups were attached to ε-amino groups at the two extreme ends of a polylysine peptide composed of 10 lysine residues. When mixed with anti-2,4-dinitrophenyl antibody, this large bivalent hapten formed a 9.7 S complex which we believe to be a dimer composed of 2 antibody molecules and 2 haptenos. This dimer exhibited strong complement-fixing activity.

As a control for these bivalent hapten studies, we also prepared by solid state synthesis a univalent hapten containing a polylysine chain of 20 residues. This hapten has a single 2,4-dinitrophenyl moiety attached to the first ε-amino group at the COOH-terminal end of the polypeptide. The hapten binds firmly to its specific antibody, but no dimer or higher polymer can be detected.

We had expected that the complex formed between antibody and this monovalent hapten would not fix any complement. To our surprise, it turned out to be extremely active. This paper presents the initial experiments we have undertaken in order to describe and explore this phenomenon of complement activation by a single antibody molecule-hapten complex.

**EXPERIMENTAL PROCEDURE**

**Antibody Preparation**—Two hundred milligrams of antigen were prepared from bovine γ-globulin (Pentex, Kankakee, III.) and 2,4-dinitrobenzenesulfonic acid (Eastman, Rochester, N. Y.) as described by Eisen et al. (9). The final solution was extensively dialyzed at 4°C against 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4, to remove unreacted reagents, and finally clarified by centrifugation. Using the extinction coefficients provided by Eisen et al., we calculated that an average of 84 dinitrophenyl groups were coupled to each molecule of bovine γ-globulin.

Freund's adjuvant (5 ml) was mixed with the dinitrophenylated bovine γ-globulin (5 ml at 10 mg/ml). Approximately 0.25 ml of the mixture was injected into each footpad of 12 young adult female New Zealand white rabbits. No booster injections were given. Sera were taken by repeated cardiac puncture between 30 and 70 days after immunization. Pooled sera were stored frozen at −20°C before antibody isolation.

From the sera, anti-Dnp antibody was prepared by the procedure of Eisen et al. (11). To prepare the precipitating antigen, human serum albumin (Pentex) was coupled with dinitrobenzenesulfonic acid as described above. The equivalence point was established by a precipitin curve, and then the sera and antigen mixed in the proper ratio to ensure the maximum yield of antibody. Dnp-glycine (0.1 M) was employed to disassociate the precipitate. The Dnp-haptenated albumin and Dnp-glycine were then removed from the antibody by passing the mixture through a column (4.8 X 40 cm) containing a top bed of DEAE-cellulose (Sigma, St. Louis, Mo.) and a bottom bed of Dowex 1-XS (Bio-Rad) as described by Eisen et al. (11). A more detailed description of the preparation and isolation of Dnp antibody is given by Warner (12).

Antibody recoveries ranged from 1 to 8 mg/ml of starting serum, prepared in batches of 500 to 1000 mg. The final antibody preparations were usually homogeneous in the ultracentrifuge, and possessed a single, symmetrical boundary of 6.3 to 6.7 S, depending upon concentration. Occasionally, a small leading shoulder would be present, representing a contamination of about 5%, probably due to the presence of dimerized antibody. When present, the leading shoulder was removed by gel filtration. Depending upon the preparation, 7 to 15% of the available binding sites were contaminated with Dnp-glycine not removed by the column treatment. Of the remaining sites, 90 to 95% were titratable with ε-Dnp-L-lysine. Antibody was dialyzed against 0.15 M NaCl and stored in lyophilized form at −20°C.

Hapten Synthesis—The Merrifield solid phase synthesis as described by Young and Stewart (13) was employed in this study. The following amino acid derivatives were synthesized using standard procedures: lysine-copper complex (14), ε-Dnp-L-lysine (15), ε-Dnp-α-Nps-L-lysine (16), ε-Dnp-α-Boc-L-lysine (18), ε-Cbz-L-lysine (19), and ε-Cbz-α-Nps-L-lysine (17). All prepared amino acid derivatives migrated as single spots on silica gel thin layer chromatography (butanol/pyridine/acetic acid/water, 15/10/3).

Chloromethylated polylysine (Merrifield peptide resin, Calbiochem) was esterified with ε-Dnp-α-Nps-L-lysine and then deblocked with 4 M HCl-dioxane. Nineteen cycles of the synthesis were performed using dicyclohexylcarbodiimide/ε-Cbz-ε-Nps-L-lysine as the acylating agent and 1 M HCl-acetic acid as the deblocking reagent.

The peptide and the ε-Cbz groups were cleaved from the resin by dissolving anhydrous hydrogen bromide through a suspension of the resin-peptide in trifluoroacetic acid under anhydrous conditions. The crude peptide was then purified on a column (4.8 X 90 cm) of carboxymethylcellulose equilibrated with 0.05 M LiCl, 0.0002 M sodium phosphate, pH 7.4 (16), using an 8-liter gradient of 0.7 to 0.8 M LiCl, 0.0002 M sodium phosphate. The major peak was concentrated by absorption to carboxymethylcellulose and then removed with a small volume of 0.15 M HCl and neutralized. The Dnp-lysine ratio was established in two ways. In the first procedure, Dnp was measured spectrophotometrically and lysine measured by ninhydrin analysis of a HCl-hydrolyzed sample. In the second procedure, the peptide bond absorption at 191 nm was used to determine the concentration of islyal residues, after correction for the absorption of Dnp at 191 nm. Both procedures gave Dnp to lysine ratios of 0.9:20. The hapten was stored frozen in 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4.

Hapten Succinylation—Hapten (0.55 mmol) in 0.25 ml of the phosphate-buffered saline was diluted with 0.5 ml of a saturated carbonate buffer, pH 8.5. Solid succinic anhydride (3.5 mg) was added, and the solution was allowed to stand for 30 min at room temperature. It was then diluted 20-fold with water, the pH adjusted to 7.0 with 1 N HCl, and the succinylated hapten absorbed on DEAE-cellulose. The DEAE-cellulose was washed with water, dried, and eluted with a small volume of 0.15 N NaOH. The eluant containing the succinylated hapten was neutralized with 6 N HCl. Ninhydrin analysis before and after succinylation showed that an average of 16 out of 20 amino groups had been succinylated, forming a net charge of −19.

Sedimentation Velocity—Sedimentation velocity studies were performed with a Beckman-Spinco model E analytical ultracentrifuge. The ultra centrifuge was equipped with a photoelectric scanner as well as a schlieren optical system, enabling simultaneous monitoring of protein and hapten sedimentation. All runs were performed at 60,000 rpm, using 12-mm, double sector, aluminum-filled Epon centerpieces and quartz windows.

**Complement Assay: Buffers**—Five times concentrated Veronal-buffered saline (VBS) was used as a general diluent and diluted with double-distilled deionized H2O prior to use. When appropriate, 1.5 X 10⁻⁴ M CaCl2, 1.0 X 10⁻⁴ M MgCl2, 0.1% gelatin, or 1 X 10⁻⁶ M dihydrostreptomycin was added. All final dilutions required, VBS was diluted with an equal volume of 5% glucose.

Sensitized Erythrocytes (EA)—Ten milliliters of sheep red blood cells in Alsever's solution (Colorado Serum Co. Laboratories, Denver) were centrifuged at 3000 rpm for 15 min, resuspended in cold VBS-gelatin, and repelleted. The cells were washed two more times with VBS-gelatin, and finally suspended to a concentration of about 2 X 10⁸ cells/ml, as estimated from an absorbance at 541 nm of 0.375 for a 1:50 dilution in water. Equal volumes of cells and a 1:200 dilution of anti-sheep hemolysin (Beckman Diagnostics Operations, Fullerton, Calif.) in VBS-gelatin were incubated for 30 min at 37°C. These antibody-sensitized erythrocytes were then chilled to 0°C for 30 min before washing three times with cold VBS-gel. They were stored at 0°C at 1 X 10⁸ cells/ml in low ionic strength VBS up to 5 weeks. Prior to use, they were washed with VBS-gelatin and resuspended to the desired concentration in VBS-gelatin.

**Whole Serum Complement (CH₅₀)**—The activity of freshly frozen (−70°C) serum from man, guinea pigs, and rabbits was determined spectrophotometrically by a method described by Becker (10). This method employed the method in calculating the number of CH₅₀ units per ml of undiluted serum.

**Complement Fixation Assays**—In order to determine the relative activity of test samples to consume complement, a second procedure was employed. Five CH₅₀ units of complement, determined as described above, in 1.0 ml of VBS-gelatin, were incubated with 1.6 ml of VBS-gelatin containing the test sample for 60 min at 37°C. Then, 0.4 ml of 0.8 X 10⁸ cells/ml of antibody-sensitized erythro-
eyes was added and the incubation continued for 60 min at 37° before pelleting the cells and measurement of lysis at 541 nm. The fraction of unlysed cells was plotted as a function of the concentration of the test material. The point at which 50% lysis occurred was used as an indicator of the relative effectiveness of the test material to consume complement.

Complement Component Hemolytic Assays—C1q and C2p were prepared in functionally pure form by the methods of Nelson et al. (20) and Borsos et al. (21). Human serum C1 hemolytic activity was determined using EAC4p, cells (22). Human C2 and C4 hemolytic assays were performed using EAC4p and EAC4e cells, respectively (23, 24).

Components C3, C5, C6, C7, C8, and C9 assays used EAC4p, C4p, C2p prepared by mixing equal volumes of EAC4p, C4p with 100 units/ml of functionally pure human C2 at 30°. The result was EAC42 cells were cooled in a 0° bath and held for a maximum of 2 to 3 hours before use. C3 and C5 were assayed using diluted serum as a source of the component and the remainder of the functionally pure components added in excess. For example, to measure C3, 0.5 ml of EAC42 (1.5 × 10^6/ml) cells was added to 0.5 ml of diluted serum, followed by the addition of human C5, C6, and C7 (0.5 ml of each containing 50 units). This mixture was incubated 20 min at 30°. Then, 0.5 ml of a mixture of guinea pig C8 and C9 (50 units each) and 2.5 ml of glucose VBS was added, and the mixture was incubated for 60 min at 37°. Appropriate controls with no C3 and total lysis controls were included. Following this, 5 ml of ice-cold glucose-VBS was added and the hemoglobin read spectrophotometrically after centrifugation. Hemolytic units/ml were calculated by plotting the negative natural logarithm of the unlysed cells (−n [1 − y]) arithmetically against the negative amount of the test sample. The reciprocal of the dilution of the test-sample giving a hemolysis of unity (18) is recorded as the number of units/0.5 ml. This is multiplied by two to give the units/ml. C5 titrations were determined in a similar manner using diluted serum as the source of C5. Functionally pure human C3, C5, C6, C7, C8, and C9 and guinea pig C8 and C9 were obtained from Cords Labs, Miami, Fla. Microtiter assays of human C6, C7, C8, and C9 were determined using V-shaped microtiter plates (Cooke Engineering, Miami, Fla.) and functionally pure human complement components (as described by Cords Labs, Miami, Fla. 1973).

Reduction and Alkylation of Antibody—Anti-Dnp antibody was reduced and alkylated by a modified procedure of Nisonoff (25). Sixty-five milligrams of dithiotheritol (Cleland’s Reagent, Sigma) were dissolved in 10 ml of 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4. Two milliliters of this reagent were added to 10 ml of the same buffer containing 73 mg of antibody. The pH was adjusted to 7.0, if necessary, and the mixture incubated at 37° for 2 hours. An amount of 0.15 M neutralized iodoacet acid (1.33 M) (recrystallized from hot water and ethanol) was then added to give a final iodoacet acid concentration of 0.015 M, and the mixture was incubated at 37° for an additional 2 hours. The solution was finally dialyzed against five 1-liter changes of 0.025 M NaCl, or 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4. Analytical ultracentrifugation of either reduced and alkylated or unaltered antibody in 0.025 M NaCl, pH 7.4, yielded single symmetrical peaks with a S20,w of 6.7. At pH 2.4 the reduced and alkylated antibody gave a single peak with a S20,w of 3.1, while unaltered antibody sedimented with a S20,w of 5.0. These values are similar to those of Palmer and Nisonoff (29), who obtained S20,w values of 2.9 and 4.1 for reduced and alkylated, and untreated antibody in pH 2.4. 0.025 M NaCl. The reduced and alkylated antibody had 92% of the untreated antibody’s binding activity when titrated with Dnp-glycine.

Preparation of F(ab′)2 Fragments—F(ab′)2 fragment preparation followed closely that of Nisonoff et al. (25). Ten milligrams of pepsin (1:200,000, Sigma) were dissolved in 2 ml of a pH 4.5, 0.1 M acetic buffer. Two milliliters of this reagent were added to 4 ml of pH 4.9 acetic buffer containing 100 mg of antibody. The mixture was incubated for 10 hours at 37°, neutralized with 1 N NaOH, and dialyzed at 4° against PBS, 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4. The solution was then alkylated at room temperature to a 2.5 X 100 cm column of Sephadex G-150 previously equilibrated with 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.4. The eluted peak corresponding to the F(ab′)2 fragments was concentrated by ultrafiltration, dialyzed against the same buffer at 4°, and clarified by centrifugation (10,000 rpm, 30 min). A S20,w of 1.36 ml/gm cm was used for concentration estimation. Analytical ultracentrifugation yielded a single sedimenting peak with a S20,w of 5.0.

RESULTS

Consumption of Complement by Antibody-Hapten Complexes—The complex formed between the univalent hapten, 4-Dnp-polylysine, and Anti-Dnp antibody caused a progressive consumption of complement as measured by the hemolytic assay. The results are shown in Fig. 1, A and B. Samples of the test complex were incubated at 37° with a limiting amount of human complement. At timed intervals, aliquots were added to sensitized sheep red blood cells and incubated for an additional 60 min. The degree of consumption of complement by the test complex was

![Graph](https://www.jbc.org/content/248/3/4920/F1)

**Fig. 1.** Kinetics of complement inhibition by heat-aggregated human γ-globulin and antigen-antibody complexes. Human γ-globulin, Cohn Fraction II, was aggregated by heating a 20 mg/ml solution for 30 min at 65° in 0.15 M NaCl (Hgg). 2, 4-Dinitrophenyl-bovine serum albumin-antibody aggregates (Dnp-BSA-antibody) were made by adding 100 μl of a 1.86 mg/ml solution of Dnp-BSA (substituted with 20 Dnp groups/molecule) to 1 mg of antibody contained in 2.0 ml of Veronal-buffered saline (VBS). This resulted in a Dnp-BSA to antibody ratio of 0.4. One milligram of antibody and 0.07 × 10^−3 ml of 1.4-Dnp-polylysine, were mixed in 2.0 ml of VBS (1.4-Dnp-Lys40-antibody). All solutions were incubated for 15 min at 37°, and centrifuged at 13,000 rpm for 45 min at 4° to pellet any precipitate formed. A, 0.037 mg of Dnp-BSA-antibody (○), in terms of antibody weight, and 0.003 mg of Hgg (△—△) were each incubated with 25 CH3 units of human complement in a total volume of 8.0 ml, and incubated at 37°. At various times, 0.4 ml aliquots of the test mixtures were pipetted into 0.06 ml of 6 × 10^6 EA/ml and incubated for 60 min at 37°. Lysis was read at 452 nm after 0.0 ml of ice-cold VBS was added. Absorbance was read at 452 nm to afford a convenient optical density near 1.0 at the start of the experiment. VBS was used throughout. B, 0.017 mg of Dnp-BSA-antibody (○—○) and 0.011 mg of 1.4-Dnp-polylysine, antibody (□—□) were tested for the kinetics of complement inhibition as described above.
reflected in the degree of inhibition of the lysis of sheep red blood cells. Red blood cell lysis, as measured in terms of released hemoglobin determined at 432 nm after centrifugation, is plotted in Fig. 1, A and B as a function of incubation time. In these experiments, the kinetic behavior of complement consumption was measured for antibody complexes with univalent haptens; as controls, the kinetic behavior was also measured for antibody complexes with Dnp-bovine serum albumin, and with heat-aggregated IgG. It may be seen that the kinetic data of fixation were similar for all three test materials.

**Dependence of Complement Fixation on the Concentration of Antibody-Hapten Complexes**—The efficiency of complement fixation by the antibody-1-ε-Dnp-polylysine20 complex was compared with the efficiency of complexes formed between antibody and Dnp-bovine serum albumin, as well as heat-aggregated IgG. These data are shown in Fig. 2. Five CH50 units of complement were preincubated at 37° with various concentrations of heat-aggregated IgG or of antibody plus 1-ε-Dnp-polylysine20 or Dnp-bovine serum albumin. Sensitized sheep red blood cells were added 60 min later and incubated an additional 60 min at 37°. The red blood cells were lysed by any residual complement not consumed by the presence of the test material.

Heat-aggregated human IgG was the most potent complement-fixing material tested. Antibody complexed with the 1-ε-Dnp-polylysine20 was intermediate in potency, whereas Dnp-bovine serum albumin-antibody complexes were the poorest of the three. As an additional control, antibody by itself was found to be a very poor complement fixer, requiring concentrations two orders of magnitude higher than the other test materials.

**Complement Fixation by Various Antibody Preparations**—Three different preparations of rabbit antibody were used during the course of these studies, and they were tested for complement-fixing ability using the 1-ε-Dnp-polylysine20 hapten, Dnp-bovine serum albumin, and heat-aggregated immunoglobulin, as described above. The results of these studies are tabulated in Table 1.

With antibody preparation I, a slight turbidity developed upon the addition of the 1-ε-Dnp-polylysine20 hapten. The turbidity could be abolished by increasing the ionic strength to 0.5 M, or lowering the pH to 4.0, or adding 0.015 M Mg++. We chose the latter for the complement fixation studies; 0.015 M Mg++ was used with the first antibody preparation. Subsequent antibody preparations required no excess Mg++, since no turbidity appeared upon hapten addition. Centrifugation of hapten-antibody complexes for 1 hour at 200,000 g did not alter their ability to fix complement.

**Ultracentrifugation of Antibody-Hapten Complexes**—When mixtures of antibody and 1-ε-Dnp-polylysine20 were studied in the analytical ultracentrifuge, a single symmetrical boundary was observed with a sedimentation coefficient of 6.7 S. No faster material was detected. As a control, a mixture of antibody and α,ε-di-Dnp-lysine was also studied. With this bivalent hapten, 40% antibody dimer was formed, prominent as a 9.3 S boundary. Mixtures of antibody and α,ε-di-Dnp-lysine did not exhibit any increased fixation of complement in spite of the presence of this antibody dimer (Table II).

**Comparison of the Fixation of Human, Guinea Pig, and Rabbit Complement**—Three different kinds of sera were used as sources for the complement employed in these studies. Human and guinea pig complement seem indistinguishable in our complement fixation experiments using rabbit antibody complexed with 1-ε-Dnp-polylysine20 or with Dnp-bovine serum albumin. Rabbit complement appeared to require higher antibody concentrations for an equivalent degree of complement fixing with 1-ε-Dnp-lysine20. With heat-aggregated IgG, all three sera were similar in behavior. These data are shown in Table III.

**Requirement for Dnp-Binding to Antibody for Complement Fixation**—When nonspecific rabbit IgG was used in place of antibody, no fixation of complement occurred either with 1-ε-Dnp-polylysine20 or with Dnp-bovine serum albumin. Moreover, if the Dnp-specific antibody was mixed with polylysine15, which lacks a dinitrophenyl haptenic group so that no specific binding occurs, then the mixture was unable to promote the fixation of complement. Finally, the addition of an excess of ε-Dnp-lysine to a mixture of antibody and 1-ε-Dnp-polylysine20 resulted in a complete loss of enhanced complement consumption. All of these studies are summarized in Table II.

**Requirement for the Positively Charged, Polylysine Chain for Complement Fixation**—The positively charged polylysine chain

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**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>CH50 units</th>
</tr>
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<tbody>
<tr>
<td>Ab</td>
<td>0.54</td>
</tr>
<tr>
<td>Ab + Dnp-BSA</td>
<td>0.005</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Lys20</td>
<td>0.003</td>
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<tr>
<td>IgG</td>
<td>0.009</td>
</tr>
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</table>

**Table II**

<table>
<thead>
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<th>Sample</th>
<th>Protein to fix 4 of 5 CH50 units of preparation no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Ab</td>
<td>0.54</td>
</tr>
<tr>
<td>Ab + Dnp-BSA</td>
<td>0.005</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Lys20</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein to fix 5 CH50 units of preparation no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Ab</td>
<td>0.54</td>
</tr>
<tr>
<td>Ab + Dnp-BSA</td>
<td>0.005</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Lys20</td>
<td>0.003</td>
</tr>
</tbody>
</table>
were prepared as described under “Experimental Procedure.”

The antibody Cl acceptor site on the Fc portion of the molecule could be demonstrated.

Failure of Hapten Complexes Formed with F(ab')2 Fragments or Reduced and Alkylated Antibody to Fix Complement—Antibody F(ab')2 fragments do not have the C1q acceptor site and are incapable of activating via the classical pathway, although they can activate via the alternate pathway (27, 28). In order to test whether the monovalent 1-ε-Dnp-polylysine18 hapten was capable of fixing complement when complexed to the F(ab')2 fragments, we performed the following experiment. F(ab')2 fragments were prepared as described under “Experimental Procedure.”

TABLE II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein to fix 4 of 5 CH50 units</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG + 1-ε-Dnp-Polylysine18</td>
<td>0.70</td>
</tr>
<tr>
<td>IgG + Dnp-BSA</td>
<td>&gt;3.00</td>
</tr>
<tr>
<td>Ab + Poly-ε-Lysine18</td>
<td>2.00</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18 + ε-Dnp-Polylysine18</td>
<td>&gt;1.00</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18</td>
<td>0.010</td>
</tr>
<tr>
<td>Ab + Dnp-BSA</td>
<td>0.015</td>
</tr>
<tr>
<td>Ab + ε-ε-Dnp-Polylysine18</td>
<td>&gt;1.00</td>
</tr>
</tbody>
</table>

to which the DNP group was attached also appeared to be required for the enhanced consumption of complement. Treatment with succinic anhydride, as described under “Experimental Procedure,” replaced 80% of the positive charges on the hapten.

Spectrophotometric titration with Dnp-glycine showed that these fragments bound equivalent amounts of Dnp-glycine, as did whole antibody. These fragments were then tested for their ability to fix complement in the presence of 1-ε-Dnp-polylysine18, and the results are shown in Table V. No evidence for complement-fixing ability by these complexes could be demonstrated.

The antibody Cl acceptor site on the Fe portion of the molecule is susceptible to mild reduction and alkylation, resulting in the loss of complement-activating ability (29). When antibody was reduced and alkylated, as described under “Experimental Procedure,” it still exhibited 92% of the binding capacity as whole antibody, determined by spectrophotometric titration with Dnp-glycine.

Absorbed human r-globulin (Hgg) in Veronal-buffered saline (VBS) was mixed with antibody and 1-ε-Dnp-polylysine18 (Table IV). Milligrams of antibody required to fix 4 of 5 CH50 units of human complement, determined as in Table I.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antibody to fix 4 of 5 CH50 units</th>
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</thead>
<tbody>
<tr>
<td>Ab ................</td>
<td>0.54</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18</td>
<td>0.063</td>
</tr>
<tr>
<td>Ab + succinylated 1-ε-Dnp-Polylysine18</td>
<td>0.24</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18 + succinate</td>
<td>0.015</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18 + polylysine18</td>
<td>0.150</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18 + protamine chloride</td>
<td>0.030</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antibody to fix 4 of 5 CH50 units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab ................</td>
<td>1.00</td>
</tr>
<tr>
<td>Ab + 1-ε-Dnp-Polylysine18</td>
<td>0.014</td>
</tr>
<tr>
<td>F(ab')2 fragments</td>
<td>&gt;0.40</td>
</tr>
<tr>
<td>F(ab')2 fragments + 1-ε-Dnp-Polylysine18</td>
<td>&gt;0.40</td>
</tr>
</tbody>
</table>
used for the other experiments described above are also activators of the classical pathway. Therefore, we have continued to use the materials for these studies on complement depletion.

We have compared such mixtures of molecules for their abilities to complement with other mixtures containing the same amount of antibody, but an excess of hapten. The second mixture contained only the antibody species having two bound haptens. A fraction of the antibody molecules would have both binding sites occupied with hapten, whereas another fraction would have hapten bound at only one site. Finally, there would be a fraction of antibody which had no hapten bound at all. If all of the antibody-binding sites were independent and had the same affinity for hapten, these three antibody species would be present at a ratio of 1:2:1, assuming all of the hapten to be bound. Actually, since there would be a distribution of binding affinities present, the real ratio would be expected to be biased toward the species with two bound hapten molecules and those with no hapten bound.

We have compared such mixtures of molecules for their abilities to fix complement with other mixtures containing the same amount of antibody, but an excess of hapten. The second mixture contained only the antibody species having two bound haptens.

Complement-fixation experiments were performed with these antibody-hapten mixtures, and the results are shown in Table VIII. It can be seen that the fully saturated antibody-hapten mixture was a much better promoter of complement consumption than was the equivalent mixture. The enhancement was 14- to 10 fold.

Depletion of the Early Components of the Classical Pathway by Hapten-antibody Complexes—Successful activation of the classical pathway requires that the successive components of complement be consumed. These include C1, C4, C2, C3, and C5. As described under “Experimental Procedure,” standard assays have been developed for the analysis of the depletion of these individual components. The control substances which we have used for the other experiments described above are also activators of the classical pathway. Therefore, we have continued to use the heat-aggregated IgG and Dnp-bovine serum albumin as control materials for these studies on complement depletion.

The results of these studies are shown in Fig. 3. Marked reductions in the levels of C1, C4, C2, and C3 occur in serum after incubation with mixtures of antibody and the monovalent hapten, 1-e-Dnp-polylysine. Similar levels of depletion were found for mixtures of antibody and Dnp-bovine serum albumin. Pronounced depletion occurred with the heat-aggregated IgG. As a negative control, antibody was mixed with ε-Dnp-lysine. Approximately 100% activity was found for each component in this case, indicating no activation (not shown).

The later components, C6, C7, C8, and C9, were assayed by the microplate method, as described under “Experimental Procedure.” These components are not cleavage products of enzymic reactions, and depletion is usually less pronounced. However, it may be seen in Fig. 3 that some depletion was measured for C7 and C8.

**Discussion**

The fixation of complement by the antibody-constant hapten complexes, as reported in this communication, is not a general phenomenon; that is, monovalent haptens have not been previously reported to cause the fixation of complement when combined with their specific antibody. Several particular structural features apparently are required for the successful interaction of the monovalent hapten-antibody complex which we have studied.

First, the requirement for the specific haptenic group which binds to the antibody seems established. Thus, the 1-e-Dnp-polylysine does not cause complement consumption when it is mixed with nonspecific immunoglobulin G. Moreover, it will not cause complement consumption in the presence of excess ε-Dnp-lysine, which acts as a competitive inhibitor for the binding to the hapten.
The similarity of behavior of Dnp-bovine serum albumin-antibody complexes with the Dnp-polysine-antibody complexes in the kinetics of fixation of whole serum complement activity provides suggestive evidence of a similar stoichiometry of activation. Moreover, the similar amount of protein required to fix a standard concentration of human complement for both complexes adds credence to this possibility. The stoichiometry of the activation, that is, the number of antibody-hapten complexes required to activate 1 molecule of C1, should be amenable to experimental study using procedures analogous in some respects to those described by Cohen (5). Certainly, further work is required to elucidate the nature of the antibody-hapten complex and its interaction with complement components. It is even possible that a conformational change is induced in the antibody molecule upon binding a large positively charged hapten, resulting in enhanced complement-activating capacity. However, we do not have any evidence for such a conformational change in the antibody, and we prefer the explanation that we are increasing the number of binding sites capable of interacting with Cl upon the addition of our particular monovalent hapten to its specific anti-Dnp antibody.

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