THE FIXATION OF COMPLEMENT AND THE ACTIVATED FIRST COMPONENT ($C\bar{1}$) OF COMPLEMENT BY COMPLEXES FORMED BETWEEN ANTIBODY AND DIVALENT HAPTEN

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Complement is bound when an appropriate antibody reacts with its antigen or when it is nonspecifically aggregated by heat or chemical means; no significant binding occurs with the isolated antibody (1). It is therefore likely that the interaction of the first component of the complement sequence with antibody is due to intramolecular conformational changes within the antibody molecule, or to intermolecular association of several molecules, or to a combination of both phenomena.

In the usual interaction between antibody and complex antigens and in the production of nonspecific aggregates, an ill-defined product is formed in which it is difficult to investigate the physical changes which have occurred in the antibody molecule. The need for a more easily characterized material was recognized by Valentine and Green, who succeeded in reducing the molecular complexity by using a divalent hapten of minimum length to form polymers with specific IgG antibody (2).

By electron microscopy they showed that the product of this reaction was a mixture of dimers, trimers, tetramers, pentamers, and some higher polymers. An attempt has therefore been made to fractionate this mixture and to test the complement-fixing efficiency of the different-sized polymers to determine if the ability to bind complement could be correlated with size. The results suggest that dimers and trimers cannot fix complement or $C\bar{1}$, but higher aggregates can.

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Materials and Methods

Bis-Dinitrophenyl (DNP)[1]*-Octamethylene-Diamine.*—Fluorodinitrobenzene (4 mmoles, 0.76 g) was dissolved in ethanol (20 ml) and added with stirring to octamethylenediamine (2 mmoles in 5 ml 1 M $NaHCO_3$). The mixture was stirred for 2 hr and the resulting precipitate filtered and crystallized from ethyl acetate. mp 134–5°C. The water-insoluble bivalent hapten (calculated for $C_{20}H_{24}N_6O_8$:C, 50.0%; H, 5.0%; N, 17.5%; found C, 50.2%; H, 4.90%; N, 17.9%) was soluble in dimethylformamide.

Epsilon-DNP-aminocaproic acid was prepared by the method of Carsten and Eisen (3).

Preparation of Purified Rabbit Anti-DNP Antibody.—Anti-DNP was prepared using DNP-BGG according to Farah, Kern, and Eisen (4) and the following schedule employed in immunizing normal rabbits: a primary injection subcutaneously distributed among the four legs of 5 mg BGG-DNP in 2 ml of 50% water-in-oil emulsion containing Difco complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) was followed by a booster injection at 6 wk of the same volume of 50% complete adjuvant emulsion containing 1 mg of BGG-DNP. Thereafter, weekly bleedings were made from the marginal vein of the ear. Sera were collected after clotting overnight by centrifugation at 2500 *g* for 1 hr. A washed immune precipitate prepared at equivalence from pooled antisera with dinitrophenylated bovine serum albumin was solubilized in 0.1 M 2,4-dinitrophenol which was then displaced by dialysis against *para*-nitrophenol by the method of Hong and Nisonoff (5). The para-nitrophenol was removed from the final antibody solution by gel filtration on Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 0.20 M Tris HCl, pH 8.0.

The OD of the final antibody solution was 3.0 at 280 mμ and 0.070 at 360 mμ; in estimating the amount of purified antibody, an $E^{1\%}_{280} = 16$ and a molecular weight of 150,000 were assumed. The number of available combining sites per mole for the preparation used throughout these studies was 1.0 as determined in fluorescence titration with episilon-DNP-aminocaproic acid under the conditions specified below. From this standard antibody preparation a concentrated solution containing 9.8 mg/ml antibody protein was made by negative pressure ultrafiltration in Visking dialysis tubing; this solution was used for forming hapten-antibody complexes with the synthetic bivalent hapten *bis*-DNP-octamethylene diamine at "fluorescence equivalence" for gel filtration column chromatography.

Fluorescence Titration of Anti-DNP Antibody with Epsilon-DNP Aminocaproate.—The activity of the purified antibody was determined by fluorescence titration (6) with epsilon-DNP-aminocaproate using the apparatus described by Green (7). All fluoresence readings (at 25°C) were made relative to a standard solution of 10^{-6} M *N*-acetyltryptophanamide. The antibody solution (20 μg/ml) was titrated by addition of 2-μl portions of a 20-μM solution of epsilon-DNP-aminocaproate with continuous magnetic stirring. The maximal quenching observed in the presence of 2×10^{-7} M free hapten was 55%, and the calculated equivalence was 1 combining site per mole of antibody with a dissociation constant of 3×10^{-9} M. The antibody was greater than 90% precipitable by DNP-bovine serum albumin (BSA) despite the relatively low activity found on fluorescence titration.

As fluorescence titration was performed at an antibody concentration of about 10^{-7} M, the discrepancy between calculated available combining sites and the observed precipitable activity of the purified antibody was assumed to be due to partial titration of antibodies of

[1] *Abbreviations used in this article:* BSA, bovine serum albumin; BGG, bovine gamma globulin; C-EDTA, complement ethylenediaminetetraacetate; DNP, dinitrophenyl; EDTA, ethylenediaminetetraacetate; TCB, Tris complement buffer; TCB-EDTA, Tris complement buffer–ethylenediaminetetraacetate; TCB-S, Tris complement buffer with sucrose; TCB-S-A, Tris complement buffer with sucrose and albumin.

dissociation constants in excess of 10^{-7} M which would also account for the relatively reduced maximal quenching value.

Reaction of Bis-DNP-Octamethylene-Diamine with Anti-DNP.—To a 1.5-ml volume of purified anti-DNP antibody in 0.20 M Tris, pH 8.0, containing 14.7 mg of antibody was added with immediate mixing 0.15 ml of a dimethyl-formamide (British Drug Houses, Ltd.) solution containing 54.9 nanomoles of *bis*-DNP-octamethylenediamine which represented reaction at fluorescence equivalence. No precipitate or opalescence was observed. The reagents were allowed to react for 30 min at room temperature and then the entire sample applied to the Sepharose column at 4°C. After chromatography overnight, column fractions were kept in the cold.

Preparation of Antibody-Hapten Complexes for Sedimentation Measurements.—Bis-DNP-octamethylenediamine (30 μl of a 2-mM solution in dimethylformamide) was added slowly from a microsyringe, with constant stirring, to a solution of rabbit high-affinity anti-DNP antibody (12 mg in 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.4). Previous titration of the antibody with epsilon-DNP-aminocaproate had shown that this was sufficient to give 90% saturation of the calculated binding sites. The solution was used on the same day for measurements of sedimentation velocity. Sedimentation velocity was determined in a Spinco Model E analytical ultracentrifuge at 59,780 rpm. Pooled samples of antibody-hapten complexes fractionated in Tris complement buffer (TCB) on Sepharose (see below) were concentrated by ultrafiltration for sedimentation velocity studies.

Agarose Gel Filtration Column Chromatography.—A glass column with adjustable ends was packed under 1 meter hydrostatic pressure head with Sepharose 4B (Pharmacia Fine Chemicals, Inc.) in TCB (see section on buffers) to give final bed dimensions of 143 cm by 1.5 cm diameter; under reverse direction flow and a net pressure of 150 cm of water, a flow rate in the cold of 10 ml/hr was attained. Sample volumes of 1.25 ml were collected. This column retained its flow and separation characteristics for 6 months. (When the column was equilibrated with Tris complement buffer with sucrose (TCB-S) for studies of $C\bar{1}$ fixation, a shift was noted in the elution profile towards larger elution volumes without alteration of the total volume occupied by the fractionated antigen-antibody complexes). All preparations described in the present report were prepared on this column using 14.7 mg of antibody mixed with divalent hapten in 1.6 ml total volume, although equivalent fractionation was successfully obtained on a shorter column (73.5 × 1.5 cm gel bed) of Sepharose 6 B using smaller amounts of antibody-hapten complexes (1.5 mg in 1.0 ml).

Electron microscopy.—The technique of negative staining on carbon grids with sodium silicotungstate developed by Valentine (8, 19) was employed both on samples chosen from individual column fractions and on pooled fractions which had been concentrated by negagive pressure ultrafiltration. An E_{280} of about 0.100 was optimal, since it insured sufficient protein to produce even spreading of the negative stain without overcrowding the images of the molecules. Samples in TCB and TCB-S (see section on buffers) could be used directly without change of buffer. Best results were obtained when samples to be studied were kept cold at all times and examined within a day. The samples were examined in a Phillips E. M. 200 electron microscope at a magnification of 55,000.

Preparation of Hemolysin and Complement.—Rabbit hemolysin was prepared using erythrocyte stromata made according to Mayer from sheep erythrocytes (Wellcome Reagents, Ltd., Wellcome Research Laboratories, Beckenham, England) to immunize normal rabbits by the intravenous route, following the recommended schedule designed to produce antibodies of the IgM class (9, 10). The immune sera were decomplemented at 56°C for 30 min before dispensing in 1.0-ml portions into vials for freezing at −20°C. Whole complement was prepared by bleeding under ether inhalation anaesthesia normal Hartley-strain guinea pigs by cardiac puncture and allowing the blood to clot at 4°C for 2 hr before separation by centrifugation in the cold. The pooled sera were either frozen at −20°C in 1.0-ml portions without further

treatment to be used as a source of whole complement or were processed immediately in preparing functionally pure $C\bar{1}$ and C2 (see below).

Rabbit hemolysin was titered in 5.0-ml reaction volumes with 1×10^9 cells and guinea pig complement in 7.5-ml reaction volumes with 5×10^8 cells according to the methods of Mayer (9).

Buffers Used in Complement Studies.—Erythrocytes were washed in phosphate-buffered saline (0.145 M NaCl, 0.05 M PO_4, pH 7.4), followed by Tris complement buffer [TCB; 0.05 M Tris (hydroxymethyl) methyl amine (AnalaR, British Drug Houses, Ltd., Poole, Dorset, England), 0.04 M HCl, 0.00015 M $CaCl_2$, 0.0005 M $MgCl_2$, 0.098 M NaCl, pH 7.5] which had an ionic strength of 0.139 and an osmotic strength of 286.2 milliosmoles per liter, and was used in place of Veronal-buffered saline for hemolysin and complement titrations. For storage of $C\bar{1}$ and C2 preparations and all reactions requiring low ionic strength-isoosmotic conditions, Tris complement buffer with sucrose and albumin (TCB-S-A) was used [0.05 M Tris, 0.04 M HCl, 0.00015 M $CaCl_2$, 0.0005 M $MgCl_2$, 0.025 M NaCl, 0.146 M sucrose, (AnalaR), pH 7.5, 0.1 g/100 ml bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.); $\mu = 0.066$, 286.3 milliosmoles per liter]. TCB-S is identical to TCB-S-A with albumin omitted. Complement ethylenediaminetetraacetate (C-EDTA) was prepared by appropriate dilutions of whole complement with ice-cold Tris complement buffer–EDTA (TCB-EDTA) which was free of Ca and Mg and contained 0.05 M Tris, 0.04 M HCl, 0.098 M NaCl, and 0.01 M EDTA [ethylenediaminetetraacetic acid, disodium salt (AnalaR); stored as a 0.1 M stock solution which had been adjusted to pH 7.5 with 2 N NaOH]; pH of buffer was 7.5. (For convenience in storage of buffers, five-fold concentrates of TCB, TCB-S, and Ca-Mg–free-TCB were normally prepared). A 0.0955 M solution of sodium carbonate was used in photometric standardization of erythrocyte suspensions (9) and a citrate-saline "stop reagent," 0.015 M in sodium citrate and 0.150 in NaCl, was used in hemolysin titrations. Acetate, phosphate, saline-acetate, and ammonium sulphate buffers used in preparation of C2 followed the directions of Nelson et al. (11) except that ionic strengths were adjusted by conductivity measurements at room temperature against room temperature standard NaCl solutions.

Method of Complement Fixation Assay.—The method of Wasserman and Levine (12) was modified as follows: Two-fold dilutions in TCB of the sample to be studied for complement fixation (C-fixation) were prepared, and 4.0 ml of a complement dilution in TCB containing 0.4 CH_{50} units per ml was added to 1.0 ml volumes of each of the sample dilutions and appropriate controls (C-fixation blank, pro- and anticomplementary effects, and complement color) and allowed to react overnight at 4°C. The following day 2.0 ml of optimally-sensitized sheep erythrocytes (9) in a concentration of 2.75×10^8 cells per ml were added to each tube and to cell fragility and complete hemolysis controls (latter made with distilled water) and the reaction mixtures incubated for 90 min at 37°C. After centrifugation in the cold, the optical density of the supernatants (warmed to room temperature) was read at 541 mμ with a 1 cm light path in a Unicam SP500 spectrophotometer (Unicam Instruments Ltd., Cambridge, England) and the difference calculated in optical density units between the degree of hemolysis in the C-fixation blank and sample tubes. To determine in OD 541 units the amount of complement fixed by an individual sample, the difference values for the tubes showing 30–80% hemolysis (viz. the linear range of partial hemolysis) were multiplied by their dilution factors. On this basis each OD_{541} unit represented approximately 2 CH_{50} units of complement fixed. As shown in the Results section, the validity of this method was confirmed by the dose-response curve shown in Fig. 7, which shows a linear relationship between protein concentration and amount of complement fixed. The slope of the curve obtained reflected the C-fixing efficiency of the individual preparations studied.

Preparation of Partially Purified Complement Components.—$C\bar{1}$ was prepared from whole guinea pig serum according to Linscott's modification of Tamura and Nelson's method

(13, 14) without the gel filtration step; the final precipitate was dissolved in TCB-S-A, clarified by ultracentrifugation, the supernatant dispensed as 1.0-ml portions into vials, and stored at −20°C.

C2 was prepared according to Nelson et al (11) from the fourth $(NH_4)_2SO_4$ precipitate by CM-cellulose column chromatography (Whatman CM 32, H. Reeve Angel and Co., London), using for 100 ml of solubilized and dialyzed material a packed total bed volume of 150 cc contained in a 5 cm diameter column with an effective head of 1 meter. Under these hydrostatic conditions, flow rates of 100–200 ml per hr were achieved. The effluents containing the highest optical density at 280 mμ and representing 110 % of the applied sample volume were pooled and the pH adjusted to 6.0 with 0.15 N NaOH before dispensing into vials in 1.0-ml portions for freezing at −20°C. (A significant decay in titers of both purified $C\bar{1}$ and C2 was observed over a 3-month interval at this storage temperature.) Dilutions of C2 were made in TCB-S-A.

Both $C\bar{1}$ and C2 prepared by these methods were functionally pure.

Molecular Titrations of $C\bar{1}$ and C2.—EAC14 were prepared using sheep erythrocytes which were washed thrice with TCB-EDTA, thrice with TCB, sensitized with a 1:50 dilution in TCB of rabbit hemolysin at room temperature for 15 min, washed and resuspended in TCB-S, cooled and reacted at 0°C for 10 min in a concentration of 1×10^9 cells/ml with 1.0 ml of unabsorbed whole complement for each 40 ml of suspension, washed twice in the cold and resuspended in TCB-S-A, incubated at 37°C for 90 min, washed and finally stored in TCB-S-A at 4°C where suspensions were stable for approximately 1 wk, and exhibited a t_{max} of 4–5 min with functionally pure C2 (15).

EAC4 were prepared after the method of Borsos and Cooper (16) from freshly prepared EAC14 cells of known t_{max} by a 10-min incubation at 37°C in TCB-EDTA buffer containing 0.1 g % BSA followed by washing with the same buffer, two cold washes with TCB containing 0.1 g % BSA, and final storage at 4°C in a suspension of 1×10^9 cells/ml in the same buffer. EAC4 prepared and stored in this manner were stable for at least 2 wk without added antibiotics, showed less than 1 % hemolysis in assay controls for fragility, exhibited a t_{max} of 4–8 min with limiting C2 when converted to high multiplicity SAC14 with excess $C\bar{1}$ (15, 17), and demonstrated at least 40 min of plateau at maximal SAC142 with limiting SAC14 and excess C2 to reproduce conditions used for $C\bar{1}$ titrations according to Borsos and Rapp (18).

C2 titrations were performed according to the method of Borsos, Rapp, and Mayer (15) with EAC14 with the exception that reactions were conducted in TCB-S-A up to the addition of C-EDTA.

$C\bar{1}$ titrations using EAC4 and excess C2 were done as described by Borsos and Rapp (18), substituting TCB-S-A for Veronal-buffered saline–sucrose.

$C\bar{1}$ Fixation Assay.—To 0.5 ml of undiluted column fractions in TCB-S and controls was added 0.5 ml of $C\bar{1}$ diluted in ice-cold TCB-S-A to contain approximately 1.2 CH_{50} units; the conditions studied for optimizing $C\bar{1}$ fixation are described under Results. In the final system adopted, the mixture of sample and $C\bar{1}$ was held at 0°C until all tubes were prepared, then incubated at 30°C for 10 min, followed by the addition at 15 sec intervals (and with immediate mixing) of 0.5 ml containing 7.5×10^7 EAC4 (equilibrated to 30°C). Each tube was incubated with EAC4 for exactly 15 min and then removed from the water bath, mixed immediately with 3.0 ml of ice-cold TCB-S-A, held at 0°C until the remaining tubes were completed, and then centrifuged at 3°C for 5 min at 300 *g*. The supernatants were carefully aspirated as completely as possible without removing cells which were then resuspended in 1.0 ml of cold TCB-S-A and held at 0°C until all tubes were processed. After 10-min equilibration at 30°C, at 15-sec intervals each tube was mixed with 0.5 ml of warm C2 in sufficient molecular excess to sustain maximal SAC142 multiplicity. After a 10-min

incubation with C2, each tube was removed and 6.0 ml ice-cold C-EDTA (1:50) added, and held at 0°C until all tubes completed. The entire set of tubes was then incubated for 90 min at 37°C, followed by centrifugation at 3°C for 5 min at 600 *g*. The supernatants were allowed to warm to room temperature before reading the optical density at 412 mμ.

Controls were included for cell fragility, specificity of hemolysis (EAC4 + $C\bar{1}$ + C2; EAC4 + $C\bar{1}$ + C-EDTA; EAC4 + C2 + C-EDTA; EAC4 + C-EDTA), complement color, and complete hemolysis (EAC4 + distilled water). Specific partial hemolysis (EAC4 + $C\bar{1}$ + C2 + C-EDTA) controls were interspersed among the sample tubes to allow correction for $C\bar{1}$ decay. The amount of $C\bar{1}$ fixed by an individual sample was calculated by subtracting the observed *z* value (18) from appropriate control value which had been corrected for time-dependent $C\bar{1}$ decay.

RESULTS

Size Distribution of Antibody-Hapten Complexes.—The complexes formed when the binding sites were saturated with bivalent hapten were studied by electron microscopy and analytical ultracentrifugation. Electronmicrographs

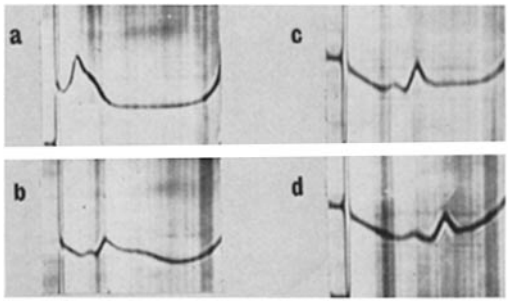

FIG. 1. Sedimentation of complexes of rabbit anti-DNP antibody and bis-DNP-octamethylene-diamine after reaching top speed. a, 11 min; b, 27 min; c, 43 min; d, 59 min. Base angle 60° for a and b, 50° for c and d.

of the unfractionated mixture, published elsewhere (2, 19, 20), showed predominantly cyclic polymers containing from 2 to 12 or more antibody molecules; open chain forms and monomers were also present. The molecules were linked together through the bivalent hapten by their Fab fragments, and the Fc fragments, identified by their susceptibility to pepsin digestion, could be particularly well seen as small projections at the corners of the polygonal shapes. On counting the individual forms in various plates, the most common species were dimers and trimers, their contribution on a weight basis to the total mixture being as much as 30–50%.

The results of a sedimentation velocity experiment on the unfractionated mixture are shown in Fig. 1. The major peak ($s_{20,w}$ 9.9) accounted for about 25% of the total protein and indicated that a dimer composed of hapten linking two 7S molecules was the predominant species. The remaining protein apart from a small 7S peak sedimented as a broad region comprising a mixture of species with sedimentation constants in the region of 12–20S.

Repetition of the analysis after addition of epsilon-DNP-aminocaproate to the bivalent hapten-antibody mixture to a final monovalent hapten concentration of 0.01 M gave a single peak of $s_{20,w}$ 6.7, consistent with complete depolymerization of the polymers in the presence of monovalent hapten.

Separation of Antibody-Hapten Complexes of Varying Size by Agarose Gel Filtration.—When the whole mixture of antibody-hapten complexes formed at fluorescence equivalence was subjected to gel filtration on Sepharose 4B as shown in Fig. 2, an effluent profile of protein concentration was obtained which was similar to the Schlieren pattern seen in the ultracentrifugal analysis of the unfractionated mixture (cf. Fig. 1a). It can be seen from Fig. 2 that two over-

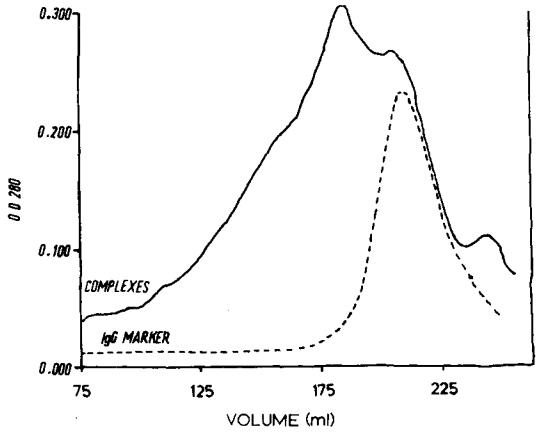

FIG. 2. Chromatography of antibody-hapten complexes on a column of Sepharose 4B. The elution profile of rabbit anti-DNP alone (dotted line) is compared with the elution profile of the same antibody after reaction at fluorescence equivalence with the bivalent hapten. The small terminal peak of the latter profile (solid line) is due to absorption at 280 mμ by the hapten solvent dimethylformamide.

lapping components were present under the main peak, one of which with an elution volume of 205 ml directly overlay the effluent profile of the monomeric IgG marker. When fractions from the 181–185-ml region at the apex of the larger peak were pooled and concentrated, the observed $s_{20,w}$ of 8.7 was interpreted as representing the average sedimentation rate of a mixture of dimers and monomers of IgG. Under the conditions of low speed (31,410 rpm), short duration (2 hr), and low protein concentration used in this analysis, there was no appreciable resolution of this apparently homogenous material into two components.

Fractions 124–138-ml from the middle of the region presumed on the basis of their elution volumes to represent the larger complexes, were concentrated by ultrafiltration. Ultracentrifugation of this material gave a broad, diffuse low profile with a mean $s_{20,w}$ of between 13.5 and 16.0.

Although the different polymeric species had not been completely separated from one another, the entire effluent profile and the elution volume of its individual identifiable major and minor peaks remained remarkably constant from one fractionation to the next. The relative proportions of the different fractions identified by gel filtration could be changed, however, by alteration in the proportions of hapten and antibody used in preparing the hapten-antibody mixture: both hapten excess and antibody excess led to a reduction in the number of larger forms and a corresponding increase in the protein content of the fractions containing the dimer and monomeric forms.

The physical stability of the larger forms was tested by concentrating separately fractions from the middle region of the ascending portion of the effluent profile and fractions from the main peak, and rechromatographing each independently. In both instances there was a shift towards larger elution volumes and, therefore, smaller forms than predominant in the original source. It was concluded that some rearrangement can occur once the complexes have been formed but that any rearrangement leads to production of dimer or monomeric configurations rather than genesis of larger polymeric rings.

Electronmicroscopy of Fractionated Antibody-Hapten Complexes.—Electronmicrographs were made of concentrated pooled fractions as well as of individual fractions. The results were independent of the buffer used in eluting the complexes. As the low molecular weight peak of the elution profile coincided with the IgG marker, it was not examined in the electron microscope.

The main peak (Fig. 3), of $s_{20,w}$ 8.7, showed predominantly (80–90%) rod-shaped dimers composed of IgG molecules doubly-linked by two molecules of bivalent hapten and in which the four Fab fragments appeared either as two parallel bars or as a single denser bar attributed to the effect produced by a lateral view (20). A number of monomers and trimers could also be identified in this fraction, but there were very few larger species.

Material from the high molecular weight end of the chromatogram, such as the individual fraction at 110 ml shown in Fig. 4, was characterized by the prevalence of large rings or chains constructed from hapten and antibody ($n \geq 6$). Tetramers, trimers, and even a few dimers were also present, but in view of the ability of Sepharose to fractionate molecular species according to size, it is likely that most of these smaller forms resulted from slow rearrangement of larger polymers since the smaller rings are favored by entropy factors. This conclusion is supported by experiments in which pooled fractions from the same region of large polymers were concentrated by ultrafiltration and allowed to stand at room temperature for several hours (Fig. 5). Such samples showed a great increase in the proportion of small rings at the expense of the larger ones and explained the observed $s_{20,w}$ of 13.5–16.0 for concentrates of this same region.

Fractions emerging from the column between the dimer peak and the large polymer region shown in Fig. 4 were similar in size distribution to Fig. 5.

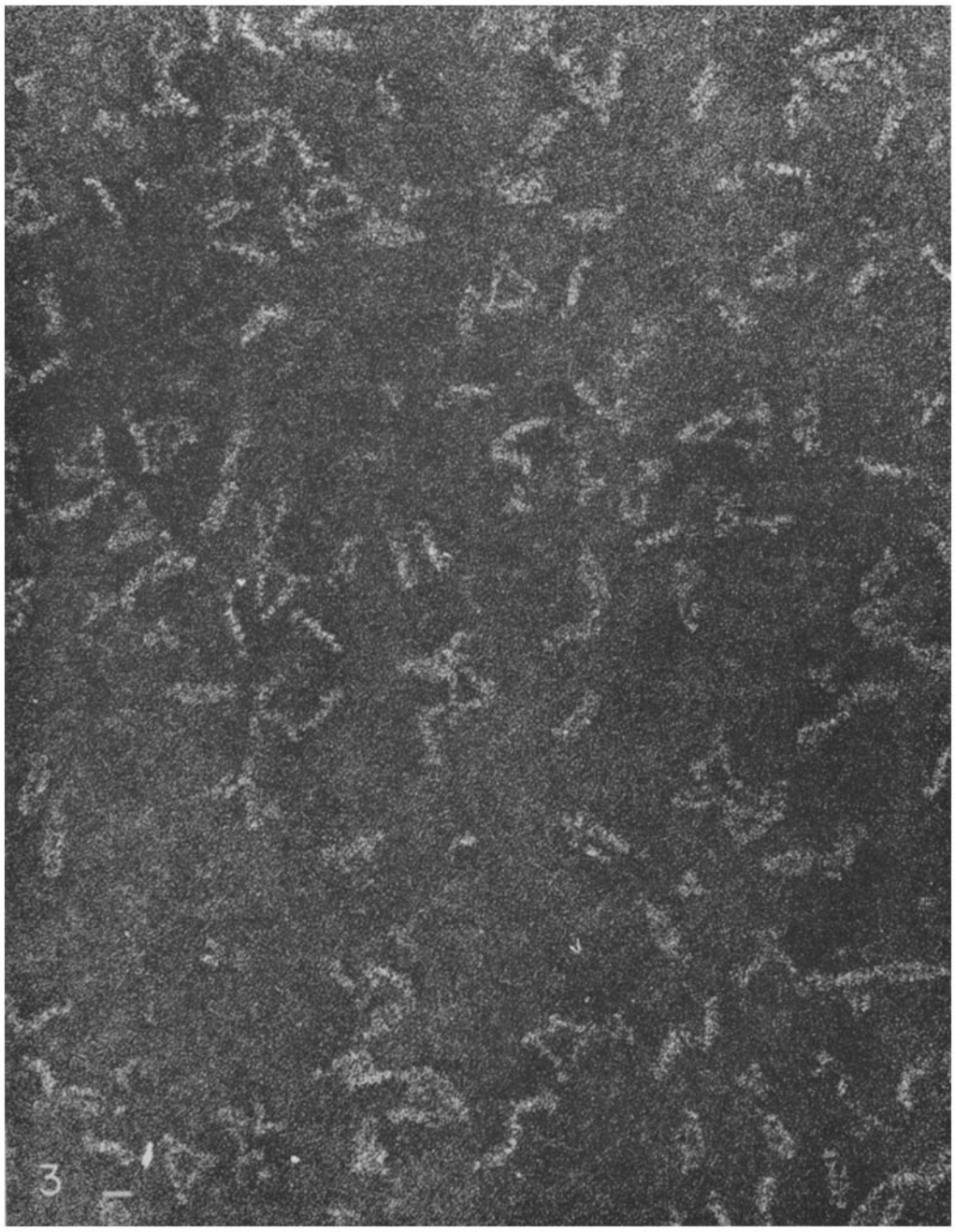

FIG. 3. Antibody-hapten complexes from the main protein peak of Fig. 2, which do not fix complement (× 385,000, scale marker = 100 A). The predominant species is a dimer which shows a number of different profiles depending on the orientation of the two pairs of Fab fragments in the stain. Sometimes both pairs can be seen clearly as two almost parallel bars, with an Fc fragment at each end, but often they are superimposed so that only a single bar is visible. In both orientations the length of the dimer is about 220 A. Monomers and trimers are also present.

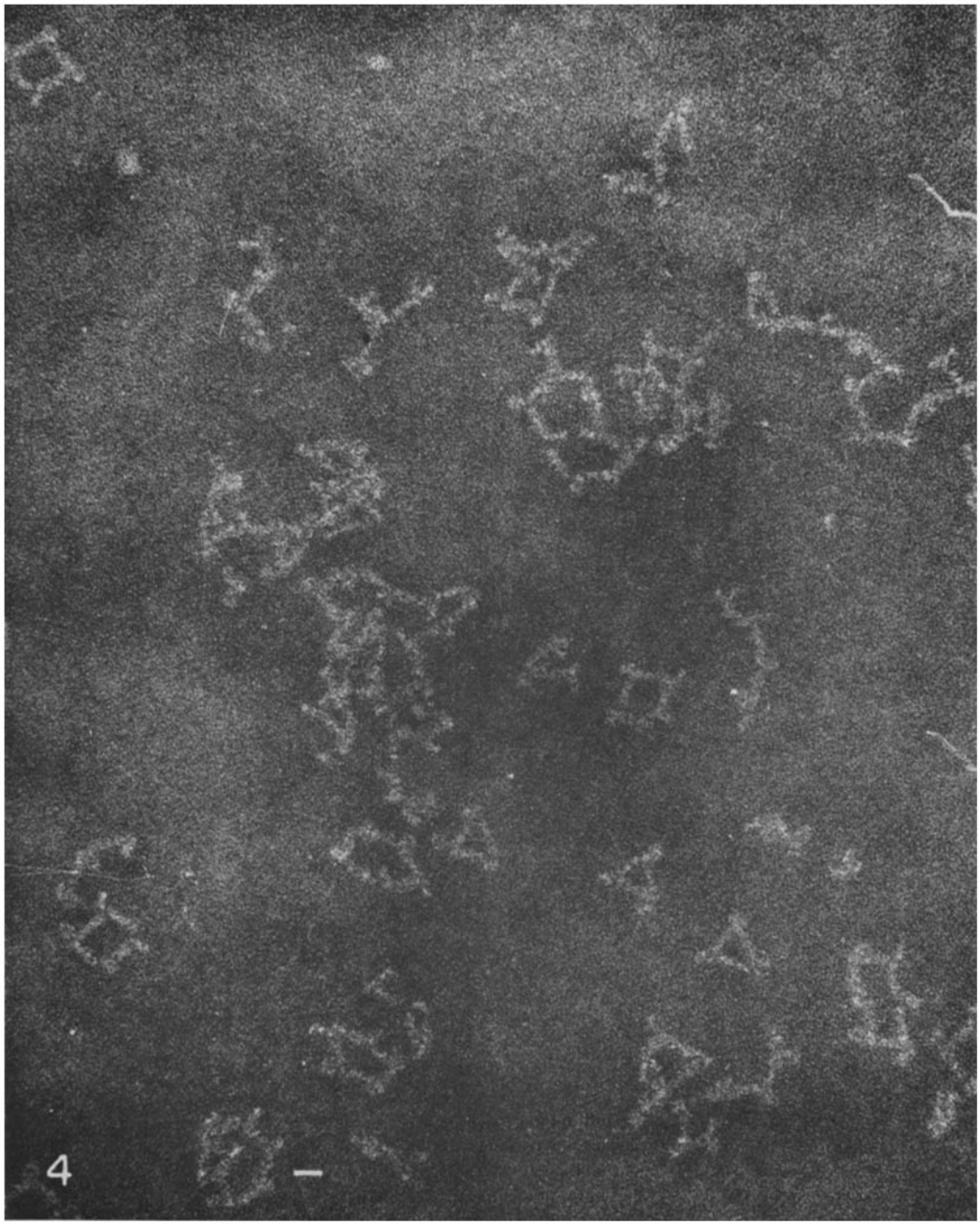

FIG. 4. Antibody-hapten complexes showing maximal complement fixation. Fraction of 110 ml elution volume, Fig. 2 (× 385,000, scale marker = 100 A). The predominant species are large linear or cyclic polymers. A number of trimers, tetramers, and pentamers are also visible.

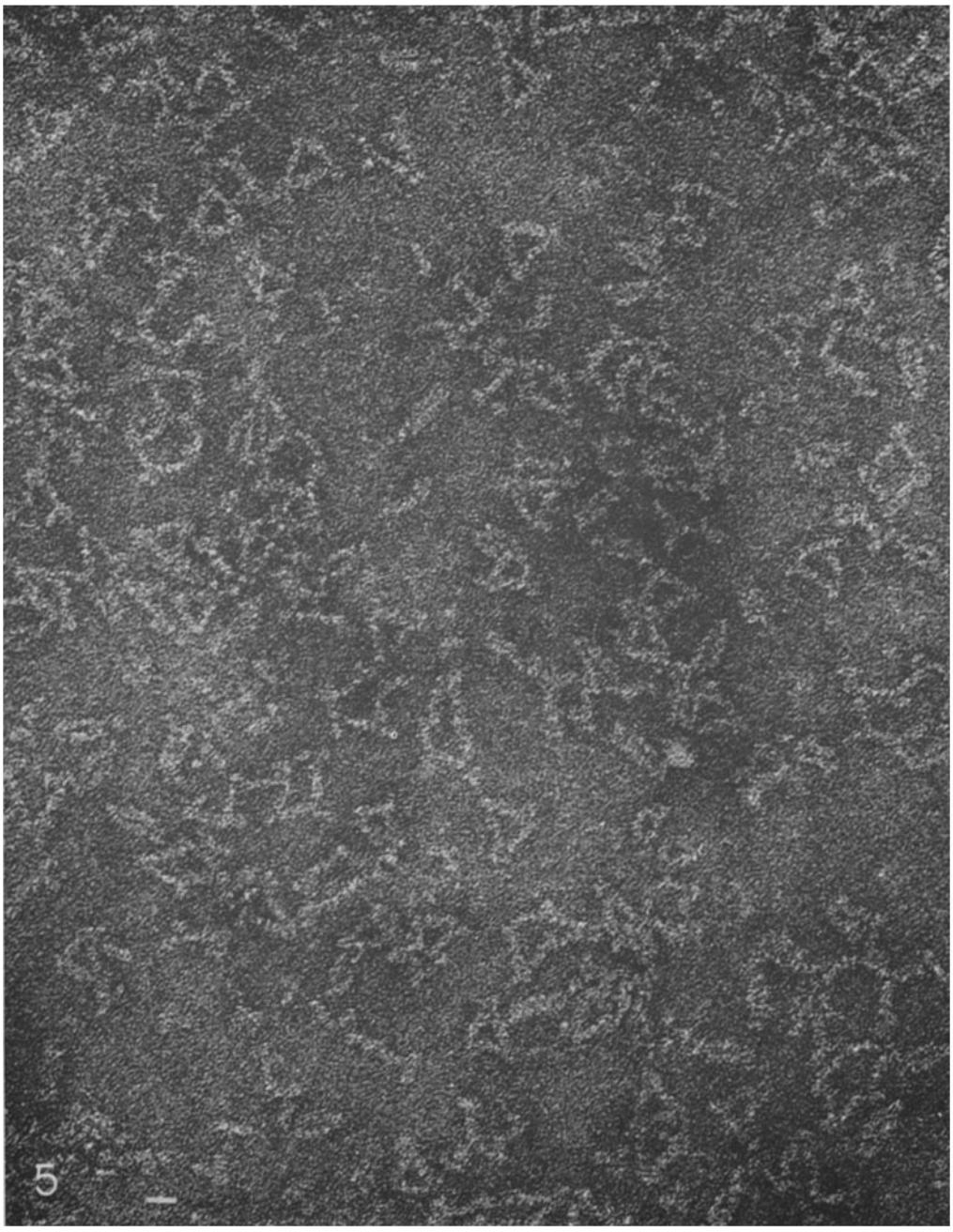

FIG. 5. Antibody-hapten complexes from the region of maximal complement fixation after concentration and storage at room temperature. The proportion of dimers and of small rings has increased at the expense of the larger polymers. (× 385,000, scale marker = 100 A).

Complement-Fixing Properties of Individual Column Fractions.—When individual fractions in Fig. 2 were tested directly for ability to fix whole guinea pig complement, as shown in Fig. 6, it was apparent that efficient complement fixation was confined to a limited range of fractions which eluted from the column in a position consistent with sizes of complexes definitely larger than the dimer form (which itself showed very little absolute C-fixation). In other experiments when fractions from the C-fixing region were concentrated and rechromatographed, the elution position of the highly active C-fixing fractions did not change, although the effluent profile of protein concentrations became more symmetrical and underwent a shift towards a larger elution volume.

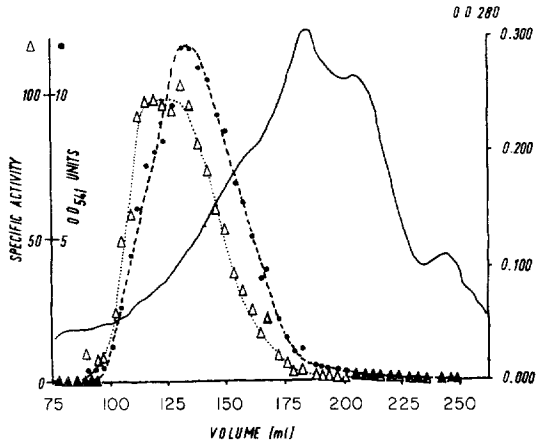

FIG. 6. Complement-fixation profile from Sepharose 4B gel-filtration in TCB of hapten-antibody complexes. Every third fraction assayed. C-fixation values (●) for individual fractions, expressed in OD_{541} units, are compared with the profile of protein concentration of the fractions (solid line). The specific activity (△) was derived by dividing the amount of complement fixed by the OD_{280} of the sample.

Repeated experiments with C-fixation analysis of chromatographed hapten-antibody complexes confirmed the limitation of expression of this type of biological activity to the fractions shown in Fig. 6, and neither isolated dimer region material nor fractions obtained from rechromatography of concentrates of the dimer region showed significant C-fixation.

Dose-Response Studies of C-Fixation by Complexes.—To test independently the validity of the specific activity plot in Fig. 6, dose-response experiments were performed by comparing amounts of complement fixed by varying amounts of isolated complexes from small pools of fractions from the complement-fixing region. The dose-response curve illustrated in Fig. 7 is from one such experiment performed with material from a region of particularly high specific activity. In each instance a linear response which passed through the origin was obtained

with all C-fixing species but the slope for individual preparations varied as one would predict from differences in specific activity.

Specificity of Complement Fixation by Polymeric Forms of Hapten-Antibody Complexes.—Inhibition by monovalent hapten of C-fixation was studied by allowing 1.0-ml portions of a dilution (containing 0.05 nanomoles of antibody and capable of fixing approximately 1 CH_{50} unit of C) of the isolated C-fixing complexes used to construct the dose-response curve in Fig. 7 to react at room temperature for 20 min with 0.1 ml of various dilutions of the water-soluble hapten epsilon-DNP-aminocaproic acid to produce a variety of molar

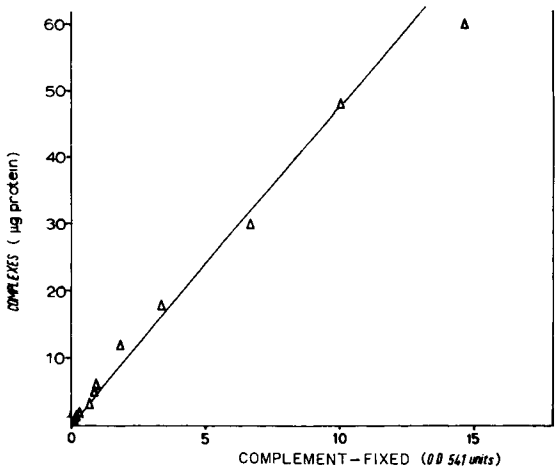

FIG. 7. Dose-response analysis of C-fixation by varying amounts of C-fixing polymers pooled from the region of high specific activity. On the ordinate is the amount of polymer expressed in µg protein; the abscissa is the number of OD_{541} units of complement fixed. (An OD_{541} unit is approximately equal to 2 CH_{50} units.)

excess ratios of monovalent hapten. After the initial reaction of monovalent hapten with complexes, the usual dilution of complement was added and the entire mixture incubated overnight at 4°C. Appropriate controls, including those for effects introduced by the hapten alone, were prepared. It has previously been determined that anticomplementary effects were seen at a final concentration of the hapten above 0.25 µM/ml. The remaining assay was performed as described previously. A 50% inhibition of C-fixation was achieved with a 25-fold molar excess with better than 90% inhibition by progressive increments.

The finding that C-fixation by the isolated antigen-antibody complexes was specifically inhibited by the presence of monovalent hapten correlated well with earlier ultracentrifugation studies demonstrating that the heterogeneous polymeric forms of the complexes as well as the dimers revert to the IgG monomer form in the presence of monovalent hapten.

The specificity of the quaternary structural requirements for C-fixation was also demonstrated by the sensitivity to even gentle heating at 37°C shown by C-fixing complexes: although the C-fixing efficiency of a particular column fraction would remain stable for at least 1 wk if the samples were kept at 4°C, exposure to 37°C for an hour resulted in a 15% loss of activity, and a 90% loss occurred after 24 hr of incubation (the period generally recommended for pepsin and papain digestions of IgG).

Study of Mechanism of C-Fixation by Soluble Complexes: $C\bar{1}$ Fixation.—$C\bar{1}$ fixation assays were performed on individual samples to determine if the fractions evidencing fixation of whole complement did so on the basis of initial

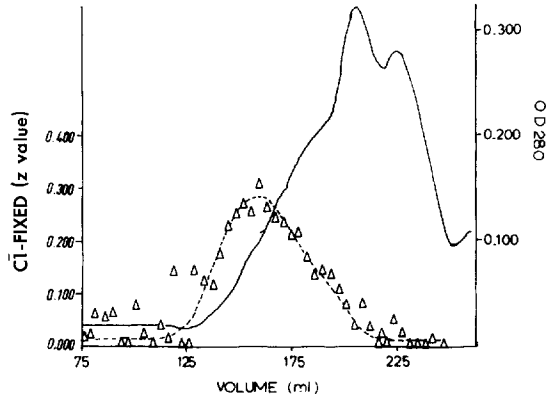

FIG. 8. $C\bar{1}$-fixation profile of fractionated hapten-antibody complexes: Sepharose 4B gel in TCB-S. Every third tube assayed. Amount of $C\bar{1}$ fixed (△) by individual fraction is expressed as the difference in z-value between the sample and the $C\bar{1}$ control (centrifugation method; 15-min incubation at 30°C with EAC4). The solid line represents the protein concentrations of the fractions.

fixation of the first component with subsequent activation of the complement cascade. For these experiments hapten-antibody mixtures were fractionated in TCB-S rather than TCB to enhance $C\bar{1}$ fixation and reduce underestimation of fixation due to site-to-site transfer by $C\bar{1}$.

The $C\bar{1}$-fixation profile is compared with the elution profile in Fig. 8; when this data is compared with Fig. 6, it is apparent that there is a direct correlation between the molecular species showing $C\bar{1}$ fixation and those capable of fixing whole complement. These conclusions based on relative elution position were confirmed by electronmicroscopy. It is likely that a similar pattern would be found for C1 fixation (21, 22) although the efficiency of activation of C1 by IgG complexes might affect the absolute values (10).

The kinetic studies supporting the choice of assay system chosen for the column analysis are shown in Fig. 9. Conventional t_{max} experiments were con-

ducted with $C\bar{1}$-fixing complexes, using the direct stepwise addition of reagents in the method of Ishizaka et al. (22) and the modified system ultimately adopted which employed a centrifugation step to separate the indicator cells from the reaction system before reaction with C2. Initial reaction of $C\bar{1}$ with hapten-antibody complexes at 0°C caused no appreciable difference (23) in

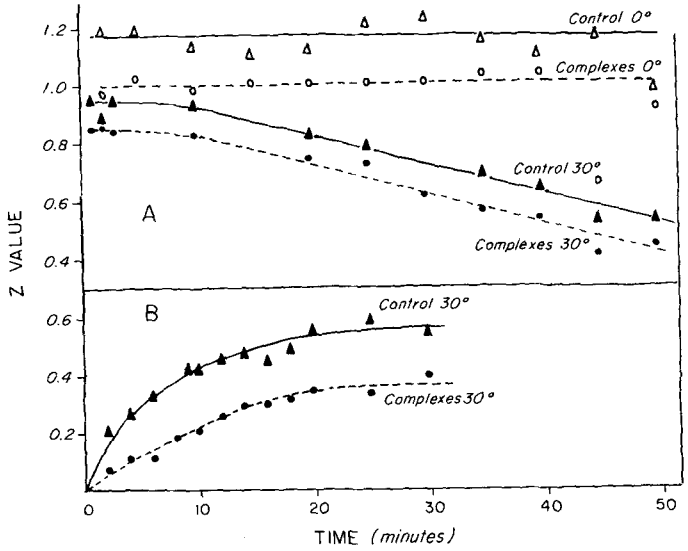

FIG. 9. Kinetics of $C\bar{1}$-fixation by C-fixing polymers. A. *Direct stepwise addition of reagents:* abscissa indicates duration of preliminary incubation between $C\bar{1}$ and polymers or buffer; results expressed as z values. [$z = -1n\ (1 - y)$, where y = per cent hemolysis]. Initial incubation of controls (triangles) and polymer samples (circles) with $C\bar{1}$ were studied at 0°C (open symbols) and 30°C (solid symbols), followed by 15-min 30°C incubation with EAC4, 10-min 30°C incubation with excess C2, and 90-min 37°C incubation with C-EDTA. B. *Centrifugation method:* abscissa indicates duration of reaction between EAC4 and mixture of $C\bar{1}$ with polymers or buffer; results expressed as z-values. At $t = 0$, $C\bar{1}$ and EAC4 were added to reagent flask containing either active polymers or buffer, contents mixed, and 1.5-ml samples removed at indicated intervals, cells sedimented, resuspended in 1.0-ml TCB-S, treated 10 min at 30°C with excess C2 and then 90 min at 37°C with C-EDTA.

$C\bar{1}$ fixation over an initial incubation at 30°C (Fig. 9A). In fact, no preliminary incubation between complexes and $C\bar{1}$ was necessary, as the complexes appeared to compete favorably with EAC4 for $C\bar{1}$ (Fig. 9B). The t_{max} for the reaction of 12–14 min was only definable with the centrifugation method, as the direct system employed a 15 min incubation step with EAC4 after the preliminary incubation of complexes with $C\bar{1}$.

When a dose-response curve was constructed using the two methods, shown in Fig. 10, it was apparent that dimers had no significant ability to fix $C\bar{1}$,

while the large complexes did so in direct proportion to concentration. The scatter of points obtained from the current centrifugation method assays in this figure and Fig. 8 make the data unsatisfactory for quantitative extrapolations such as calculating moles of $C\bar{1}$ fixed per polymer of complex. Despite its technical imperfections, it was preferable to the direct addition system which appeared in 4°C overnight-experiments to cause an underestimation of number of $C\bar{1}$ molecules fixed, presumably due to generation of SAC42 sites by fluid-phase complex-bound $C\bar{1}$.

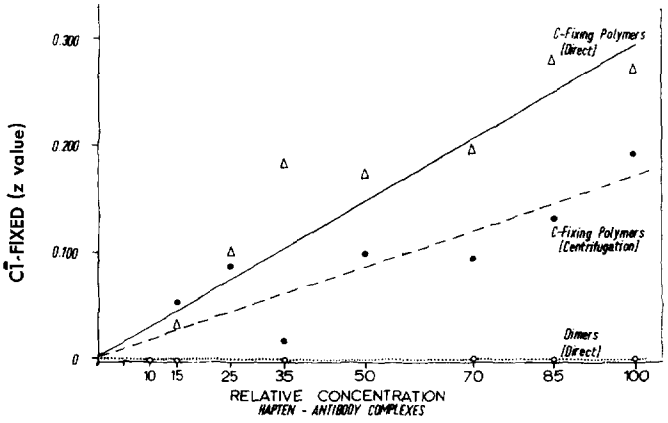

FIG. 10. Dose-response analysis of $C\bar{1}$ fixation by varying amounts of dimers and C-fixing (and $C\bar{1}$-fixing) polymers. Amount of $C\bar{1}$ fixed expressed as difference in z value between sample and $C\bar{1}$ control; in relative protein concentration 100 = 15.6 μg protein of complex obtained by pooling samples from highly active region of TCB-S fractionation for C-fixing complexes and from main peak for dimer source. $C\bar{1}$-fixation analysis done by both direct and centrifugation systems.

DISCUSSION

From the data presented, it appears that dimers and probably trimers of rabbit IgG antibody linked by bivalent hapten do not possess the capacity to fix whole guinea pig complement while higher polymers do. In view of present knowledge of rabbit IgG antibody populations, these results cannot reasonably be attributed to subclass differences in biological activity (24). It is likely that a difference in the ability to bind the first component of complement is the basis of this phenomenon, as the same pattern was found for $C\bar{1}$ fixation. Since the binding of the first component presumably is critically dependent on the physical structure of the IgG polymers, these findings help to define the conditions required for this event.

The minimal size of the predominant polymers in the fractions binding whole complement and $C\bar{1}$ most efficiently appeared to be either the tetramer, the

pentamer, or a slightly higher polymer. Only few trimers were found in these fractions and decreased efficiency was obtained with trimer-rich fractions, so it seems unlikely that the trimer has significant complement-fixing activity. It was not possible to define an optimal or maximally efficient size because of the heterogeneity observed in the higher active fractions.

The presence of dimers and trimers in fractions containing chiefly larger polymers can be explained by the tendency noted among the isolated larger polymers to dissociate and form smaller rings under a variety of normally mild conditions such as concentration by ultrafiltration, heating at 37°C, and even standing for prolonged periods at room temperature, in each case accompanied by a reduction in C-fixing activity of the sample. This difficulty makes it inefficient to attempt improved segregation of different-sized polymers by refractionation until a system is devised with a more stable hapten-antibody bond.

Using a different system, Cohen (25) concluded that a dimer of rabbit IgG was the minimal unit in a soluble antigen-antibody complex necessary to initiate whole complement fixation. From our experiments it seems clear that formation of tetramers or larger rings is a necessary condition for binding of C1 by hapten-antibody complexes. Since reversion to the monomeric state by simple addition of monovalent hapten to the polymers abolished complement fixation, the requisite structural features for C1 binding must be due to noncovalent bonds.

Of the various possible configurational changes which may confer biological activity on the IgG polymers, an analysis of the dose-response experiments for whole complement fixation and $C\bar{1}$ fixation eliminates any significant contribution from aggregated polymers. In contrast to the linear proportionality observed in Fig. 7 between C-fixation and complex concentration over a 20-fold range of protein concentrations, one would expect increasing efficiency of C-fixation with increased concentration if cooperative effects between polymers were a major factor in C1 binding. This conclusion is supported by the linear response for $C\bar{1}$-fixation by the large polymers (Fig. 10) and the inactivity of dimers over the same range of protein concentrations.

At present it is not possible to distinguish between contributions to the production of an active polymer by alterations in tertiary structure of the individual IgG molecule subunits and those arising from quaternary structural relationships between these molecules. It is possible that assumption of an appropriate quaternary structure is alone sufficient to provide the necessary conditions for C1 fixation in a manner analogous to the requirement for more than one IgG molecule in formation of a $C\bar{1}$-fixing site on erythrocytes (10, 26).

Since the accumulated evidence assigns the primary role in complement binding to the Fc portion of the molecule (1), it is possible that the ability to bind C1 is determined solely by the number and relative positions of the Fc

sections within the various polymers. No changes in tertiary structure need occur if the relative density and orientation of the Fc components of the various polymers is the critical feature controlling C1 binding. However, in view of the inability of single molecules of IgM, containing five Fc fragments, to fix and activate complement except when bound to antigen, it seems probable that at least in the case of IgM some conformational change is required in addition to a high density of C1 binding-sites.

The only consistent structural difference between the active and inactive polymers that can be detected by electron microscopy is in the angle between the Fab arms of each IgG molecule. In the inactive polymers the angle is less than 60°, while in the active polymers it lies between 90° and 180°. This is consistent with the suggestion (27) that opening up of the IgG molecule is accompanied by conformational changes in the Fc fragment which are required for fixation of complement. The extent of such conformational changes and possible methods for detecting them have been discussed in a recent review (20).

SUMMARY

Hapten-antibody complexes prepared at equivalence with the bivalent hapten *bis*-DNP-octamethylene-diamine and purified rabbit anti-DNP antibody were fractionated by Sepharose gel-filtration and the fractions examined by electron microscopy. Individual fractions were tested for whole-complement fixation and C1 fixation. Dimer forms did not show this type of biological activity, while fractions containing tetramers and larger polymers exhibited both C and C1 fixation, which could be inhibited by prior exposure of the complexes to the univalent hapten epsilon-DNP-caproic acid. The dose-response result indicated that the C-fixation observed was not due to interpolymeric cooperative effects.

It was concluded that in the generation of biological activity by soluble antigen-antibody complexes made with complement-fixing antibody, quaternary structural changes following specific combination with antigen may be as important as any tertiary structural alterations that occur in the individual immunoglobulin molecule.

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