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The Molecular Basis of Self-Association of Antibodies to IgG (Rheumatoid Factors) in Rheumatoid Arthritis

(IgG-rheumatoid factor/ultracentrifugal analysis)

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ABSTRACT **The serum and synovial fluid of many patients with rheumatoid arthritis contain immune complexes composed of immunoglobulin G (IgG). In this study such complexes from one patient are shown to be formed by self-association of IgG-antibodies to IgG (IgG-rheumatoid factors), so that each molecule serves as an antibody as well as an antigen. All F(ab')$_2$ and Fab' fragments derived from these complexes have antibody binding sites for normal IgG. Due to a high association constant in the formation of a cyclic complex by these antibodies, normal IgG is excluded as an antigen. These studies serve as a model for further elucidation of presence of similar immune complexes in the serum and synovial fluid of patients with rheumatoid arthritis.**

Kunkel *et al.* (1) first described in serum of some patients with rheumatoid arthritis abundant immune complexes sedimenting at a rate between the 6.6S and 19S components of normal serum. Further studies have shown that the antibodies in these intermediate complexes are IgG-rheumatoid factors (antibodies to IgG) (2). Furthermore, similar and faster-sedimenting immune complexes, composed of IgG, have been identified in the synovial fluid of patients with rheumatoid arthritis (3, 4). In addition, materials eluted from rheumatoid synovial tissues by acid buffers included complexes that contained IgG-rheumatoid factors (5). Thus, mounting evidence indicates that the immune complexes containing IgG-rheumatoid factors participate in the immunologically induced synovitis of rheumatoid arthritis.

The molecular composition of these intermediate complexes in serum or synovial fluid of patients with rheumatoid arthritis has eluded precise characterization. Therefore, experiments were undertaken to isolate the intermediate complexes from plasma of one patient with rheumatoid arthritis and to characterize their molecular composition.

MATERIALS AND METHODS

Plasma was obtained from a patient (Seattle USPHS Hospital no. 35-12-23) with rheumatoid arthritis during plasmapheresis therapy for hyperviscosity syndrome. The sterile plasma was stored at 4°. Once sterility was broken, sodium azide was added (to 0.01%) to prevent bacterial growth.

Isolation, Immunologic Characterization, and Pepsin Digestion of Intermediate Complexes. The initial presence of intermediate complexes was determined by analytical ultracentrifugation of plasma. The complexes were separated from other plasma components by gel filtration on a Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N.J.) column (20 cm^2 × 83 cm), equilibrated and run at 4° with 0.1 M Tris·HCl, 0.15 M NaCl, pH 7.6 buffer. The purified complexes were concentrated by Diaflow XM-100A membranes (Amicon Corp., Lexington, Mass.); their concentration was determined by absorbance at 280 nm using an absorbance for human IgG $A^{1\%}_{1cm} = 13.7$.

Pepsin digestion of the complexes was performed by the method of Nisonoff (6). The monomeric F(ab')$_2$ fragments were isolated by gel filtration on a column of Sephadex G-200 (Pharmacia Fine Chemicals), equilibrated with 0.2 M sodium borate, 0.15 M NaCl, pH 8.0.

The isolated complexes or the F(ab')$_2$ fragments derived from these complexes were trace-labeled with ^{125}I at 1–2 mol of iodine per mol of protein (7). Radioactivity was measured by an automatic well-type gamma counter.

The immunological purity of complexes was ascertained by double diffusion in 2% agarose in borate buffer, using monospecific antisera to γ, α, μ, κ, and λ chains of immunoglobulins (8) and an antiserum to human serum. A search for idiotypic antigenic determinants (9) was made by hyperimmunizing six rabbits with the purified complexes in complete Freund's adjuvant. Antisera were obtained 3 and 10 weeks after the last immunization. The presence of precipitating idiotypic antibodies was examined by double diffusion in agarose.

Several methods were employed to examine antibody activity in complexes or their fragments. For these systems pooled normal human IgG was obtained from Cohn Fraction II (Schwarz/Mann, Orangeburg, N.Y.) by ion-exchange chromatography on DEAE. The material eluted with 0.01 M phosphate buffer, pH 8.0, was used for obtaining monomeric IgG by gel filtration on Sephadex G-200. Heat-aggregated human IgG was prepared from Cohn Fraction II, by the method of Christian; only fractions SS_1 and SS_2 were employed (10). Quantitative assays for binding of complexes or their F(ab')$_2$ fragments to normal 6.6S IgG or aggregated IgG were performed. Radiolabeled complexes or their fragments were added to IgG or aggregated IgG, incubated at 23° for 1 hr and at 4° for 1–2 hr. The interaction of these reactants was examined by distribution of radioactivity after zone ultracentrifugation. Previously described procedures were employed for zone ultracentrifugation (11). Either borate (0.2 M sodium borate, 0.15 M NaCl, pH 8.0) or acetate (0.05 M sodium acetate, 0.15 M NaCl, pH 3.5) buffers were used to build linear sucrose gradients.

Analytical Ultracentrifugation. Analytical ultracentrifugation studies were performed with a Beckman-Spinco model E analytical ultracentrifuge equipped with both the schlieren and

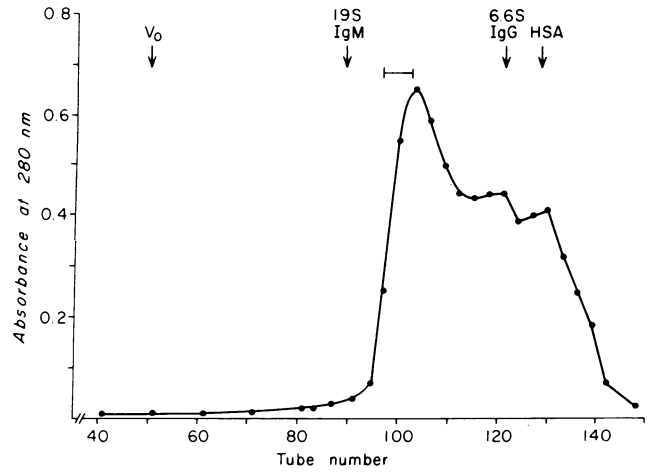

FIG. 1. Gel filtration pattern of plasma. 4 ml of plasma was applied to a Sepharose 6B column running at 34.5 ml/hr and 3 tubes per hr. A large peak of intermediate complexes is present between the elution positions of 19S IgM and 6.6S IgG. The area marked with the *horizontal bar* was processed for further studies. *HSA* indicates human serum albumin.

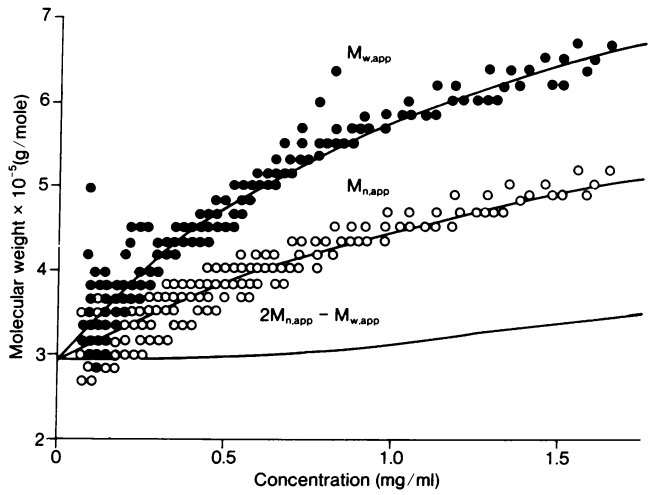

FIG. 2. Apparent weight-average ($M_{w,app}$) and number-average ($M_{n,app}$) molecular weights as a function of concentration from high-speed sedimentation equilibrium experiments. Isolated complexes were run at 8000 or 6400 rpm for approximately 30 hr at initial loading concentrations between 0.3 and 1.2 mg/ml. Data from two preparations and five centerpiece channels are shown on the graph. The lines are predicted values from smallest molecular weights, $M_1 = 292 \times 10^3$ g/mol, equilibrium constant, $K = 2.5 \times 10^5$ liters/mol and virial coefficient $B = 1.1 \times 10^{-7}$ liter mol/g^2. The line for $2M_{n,app} - M_{w,app}$ was calculated from equation 17 of Teller (15) when terms higher than C^2 equaled zero.

interference optical systems. The procedures of Behnke *et al.* (12) were used for sedimentation velocity and equilibrium experiments. Plates were read on an automatic microdensitometer linked to a PDP-12 computer (13). For analysis of self-associating stoichiometries, the procedures and computer programs discussed by van Holde *et al.* (14) and Teller (15) were used. For the analysis of complex formation between dissimilar molecules, the procedure and computer program of Behnke *et al.* (12) were utilized.

Unless otherwise specified, the buffer for all experiments was 0.05 M phosphate, 0.15 M NaCl, pH 7.3. A partial specific volume of 0.738 ml/g at 20° (16) was used for all IgG molecules and their fragments.

RESULTS

The initial analytical ultracentrifugation experiments on plasma of this patient disclosed that 50% of protein sedimented between the 6.6S and 19S components of normal plasma, analogous to the observations described by Kunkel *et al.* (1) in their Fig. 1. Very little material sedimented in the 19S component in this plasma.

Immunologic Characterization of Isolated Complexes. Separations of complexes from other plasma proteins were performed with a Sepharose 6B column and pools were made as indicated in Fig. 1. The pooled material was concentrated, but whenever concentrations over 1.0–1.5 mg/ml were attempted in neutral Tris, borate, or phosphate buffers, precipitates formed and the soluble protein remained at 1.0–1.5 mg/ml. Higher concentrations could only be achieved in a plasma ultrafiltrate solvent (filtrate of PM-30 Diaflow membrane). The purified complexes contained predominantly IgG; less than 5% of total protein was IgA. No other serum proteins, including IgM, were detected by double diffusion in agarose. With specific antisera κ and λ chain antigenic determinants were detected in the intact complexes as well as in the $F(ab')_2$ fragments derived from the complexes. On immunoelectrophoresis the $F(ab')_2$ fragments showed a broad distribution. These observations indicated that the antibodies in complexes were not structurally homogeneous and did not have restricted heterogeneity. Furthermore, no evidence was found for idiotypic antigenic determinants on the complexes.

Ultracentrifugation Studies on Isolated Complexes. Moving boundary sedimentation velocity experiments at 20°–21° on the isolated complexes showed a single schlieren peak which was slightly asymmetric. The centrifugal portion of the boundary was sharper than the trailing edge. The concentration dependence of the sedimentation coefficient indicated dissociation upon dilution of the protein.

The molecular weight distributions observed in high-speed sedimentation equilibrium experiments are shown in Fig. 2. The data show reasonable superposition of the molecular weight averages as a function of concentration, which indicates self-association of a single thermodynamic component (17). This technique is not sufficiently sensitive, however, to prove that all molecules are identical and in chemical equilibrium. Since it is demonstrated below that this system is indeed a self-associating one, we proceeded to analyze it as such. To obtain the molecular weight of the smallest molecules that participated in the chemical equilibrium, equation 17 of Teller (15) was fitted by least squares. The molecular weight obtained was $(292 \pm 2.5) \times 10^3$ g/mol. Thus, the smallest molecular weight component that could be determined from the data of Fig. 2 consisted of two IgG molecules. This value of the molecular weight and the technique of van Holde and Rosetti (18) were employed for determination of the equilibrium constant and the virial coefficient from both the $M_{n,app}$ and $M_{w,app}$ data (Fig. 2). The average equilibrium constant from $M_{n,app}$ and $M_{w,app}$ data for isodesmic aggregation of IgG dimers is 2.5×10^5 liters/mol and the virial coefficient is 1.1×10^{-7} liter mol/g^2. Since both the number-average and weight-average molecular weights yielded the same equilibrium constant (within 2.5%) and the same virial coefficient (within 20%), this model of association seems reasonable.

TABLE 1. *Equilibrium parameters for discrete self-association of IgG complex dimers*[a]

Postulated stoichiometry[b]	Equilibrium constants ($\times 10^{-5}$), liters/mole[c]				
	K_2	K_3	K_4	K_5	K_6
A. 0.05 M phosphate 0.15 M NaCl, pH 7.3 buffer					
2–4–6	0.73	2.17	—	—	—
2–4–6–8	1.28	1.69	0.19	—	—
2–4–8	4.20	—	1.48	—	—
B. Plasma ultrafiltrate as solvent					
2–4–6	6.35	22.9	—	—	—
2–4–8	24.76	—	47.95	—	—
2–4–8–12	23.87	—	28.79	—	17.10

[a] The method of choosing the best stoichiometry has been discussed by Behnke *et al.* (12). No simple choice can be made between dimer–tetramer–hexamer–octamer and dimer–tetramer–octamer systems in part *A* of the table. In part *B*, a dimer–tetramer–octamer–dodecamer stoichiometry is the best choice (14).

[b] Numbers represent degree of polymerization of monomeric IgG, e.g., 2–4–6 represents a dimer–tetramer–hexamer stoichiometry.

[c] Subscripts for equilibrium constants refer to the reactions of dimer; e.g., K_2 is written for the association of two dimers to form a tetramer.

The discrete calculation of van Holde *et al.* (14) was also applied to the weight-average molecular weight data. Table 1*A* shows the stoichiometries, which yielded positive equilibrium constants when tested to hexamers of the units weighing 292×10^3 g/mol. While the stoichiometry of complex formation in sodium chloride–phosphate buffer was somewhat uncertain, since multiple chemical models fit the data, it is important to note that the dimer of IgG was the aggregating unit.

Since complex formation might be altered by the ionic mileu, a sedimentation equilibrium experiment was performed with plasma ultrafiltrate as buffer. The pH of this ultrafiltrate was 7.47 and the density was 1.0030 ± 0.0005 g/ml. In this solvent the self-association was much more extensive. The molecular weight distributions superimposed well when graphed as a function of concentration. An isodesmic association model could not be fitted reasonably to these data. Table 1*B* presents the discrete stoichiometries that could be fit to the data using the molecular weight of 292×10^3 g/mol as the smallest species. The self-association of dimers was much stronger and quite specific in this solvent leading to tetramer, octamer, and dodecamer formation.

The isolated and radiolabeled complexes were also examined by density gradient ultracentrifugation. At neutral pH no 6.6S material was seen, but at pH 3.5, 85–90% of the labeled protein sedimented at 6.6S. The remainder of material sedimented as a shoulder of up to 11S and was not further characterized (see part of Fig. 5).

Ultracentrifugation Studies on F(ab')$_2$ Fragments of Isolated Complexes. In order to further prove that the complexes were formed due to self-association, experiments were undertaken to show that all IgG molecules in the complexes were antibodies to IgG. F(ab')$_2$ fragments were prepared; they sedimented as a single peak in zone centrifugation and no evidence for aggregation was found by velocity and equilibrium analytical ultracentrifugation.

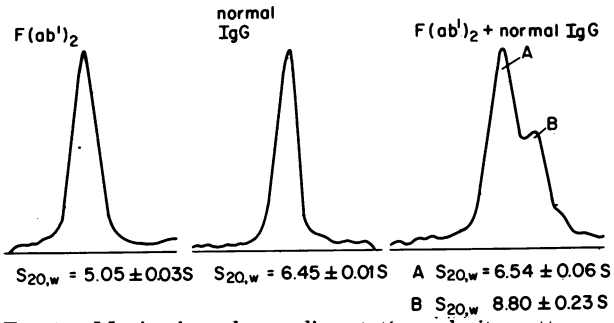

FIG. 3. Moving boundary sedimentation velocity patterns of F(ab')$_2$, normal IgG, and a mixture of the two. The patterns are taken from the distance derivative of the Rayleigh interference patterns. The initial concentrations (from radial dilution) are 2.01 ± 0.02, 3.91 ± 0.02, and 3.26 ± 0.01 mg/ml, respectively. Patterns are from 72, 40, and 48 min at 60,000 rpm. The initial mole fraction of IgG in the third pattern was 0.73.

Thereafter, the F(ab')$_2$ fragments were mixed with monomeric normal human IgG for sedimentation velocity and equilibrium experiments. When the normal IgG mole fraction was 0.73, no free F(ab')$_2$ was found in the mixture, as determined by interference patterns during sedimentation velocity experiments (see Fig. 3). In addition, when normal IgG was added to radiolabeled F(ab')$_2$ fragments, up to 90% of the label sedimented faster than the 5.3S peak of free fragments on zone centrifugation on density gradients. Therefore, nearly all molecules of F(ab')$_2$ fragments participated in the chemical equilibrium with normal IgG.

Sedimentation equilibrium of the mixture of F(ab')$_2$ with normal human IgG indicated that 1:1 and 1:2 complexes were formed between these reactants and allowed the calculation of equilibrium constants for these reactions. The presence of 1:1 and 1:2 complexes was supported by the analysis presented in Fig. 4. Only two stoichiometries of complex formation were consistent with the data: (*a*) both 1:1 and 1:2 complexes of F(ab')$_2$ and IgG, and (*b*) a single 2:1 complex of F(ab')$_2$ and IgG. Two reasons indicated that alternative (*a*) was the correct stoichiometry. First, 1:1 and 1:2 F(ab')$_2$–IgG complexes are most reasonable due to the bivalency of F(ab')$_2$ and lack of self-association of these fragments. Second, the sedimentation coefficient of the fast peak observed in sedimentation velocity experiments increased with increasing mole fraction of IgG from 8.15 ± 0.10S at mole fraction IgG = 0.41 to 8.80 ± 0.23S at mole fraction IgG = 0.73. The equilibrium constants observed for this stoichiometry were $(1.50 \pm 0.38) \times 10^5$ liters/mol for 1:1 complex and $(1.45 \pm 0.58) \times 10^5$ liters/mol for formation of the 1:2 complex from the 1:1 complex.

As a final check on the nature of the complex formation, the F(ab')$_2$ fragments were reduced and alkylated. This material did not self-associate as determined by velocity and equilibrium analytical ultracentrifugation. In sedimentation velocity experiments in excess normal human IgG, a slight trailing peak was observed (less than 10%). Sedimentation equilibrium experiments indicated the formation of only 1:1 complex of Fab' with IgG. The possible formation of a 2:1 complex of Fab' with IgG was examined but not found in these mixtures.

Thus, the experiments with the Fab' and F(ab')$_2$ fragments prove that all of these fragments derived from the isolated

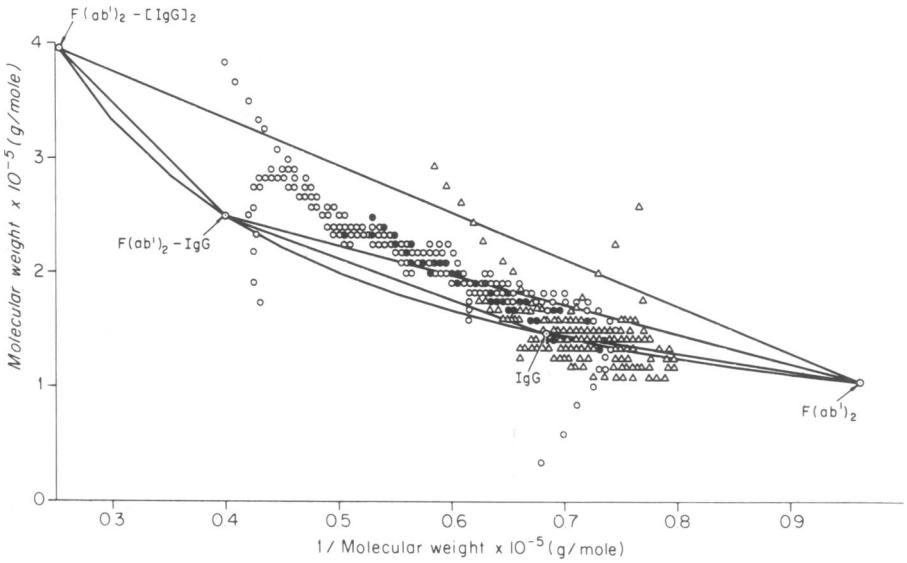

FIG. 4. "Allowed space" graph of the molecular weight averages determined by high speed equilibrium for a mixture of $F(ab')_2$ and normal IgG. (O) M_z vs $1/M_w$, (●) M_w vs $1/M_n$, (△) $2M_w - M_z$ vs $2/M_n - 1/M_w$. In this analysis (12, 17) the molecular weight averages are plotted on the ordinate and the next lower average on abscissa. In the absence of experimental errors and thermodynamic nonideality all points should lie above and to the right of the curved line that represents the locus of homogeneous materials. If no complexes were formed, all data points should lie along the line between $F(ab')_2$ and IgG. If only 1:1 complexes were formed between the reactants, then the points should lie in the triangle on the lower right. For both 1:1 and 1:2 complex formation between $F(ab')_2$ and normal IgG, the points should lie in the quadrilateral as observed for most data points.

complexes had binding sites for IgG and, therefore, all IgG molecules in the complexes were antibodies.

Antibody Activity in Isolated Complexes. In the self-associating complexes two antibody binding sites should remain free until a cyclic structure is formed. To test this experimentally, the isolated and radiolabeled complexes (0.025 mg) were added to variable amounts of aggregated human IgG, and adjusted to a final volume of 0.40 ml. After incubation these mixtures were separated by sucrose density gradient centrifugation. When 55-fold excess of aggregated IgG by weight was added, 90% of the radiolabeled complexes reacted with the aggregated IgG and sedimented faster than the complexes alone.

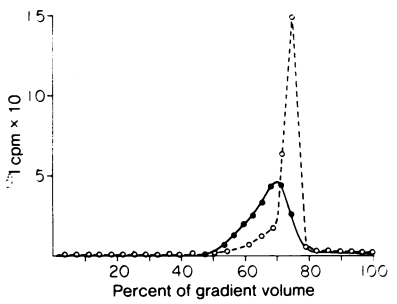

FIG. 5. Sucrose density gradient patterns of acid-dissociated complexes and complexes neutralized in the presence of excess normal IgG. Sucrose gradients were 10–30%; centrifugation was performed at 37,000 rpm for 16 hr. The radiolabeled complexes at pH 3.5 show a 6.6S peak with a faster-sedimenting shoulder (O- - -O). Complexes (0.11 mg) were mixed with 1.8 mg of normal human IgG at pH 3.5, neutralized slowly, and applied to a pH 8.0 gradient (●——●). The complexes are reformed and no 6.6S radiolabeled material is released. The numbers on the ordinate have been obtained by multiplying the data by the indicated factor.

Since all the IgG in isolated complexes was IgG-rheumatoid factor due to self-association, the acid-dissociated complexes should reform upon neutralization even in the presence of normal IgG. To test this, mixtures were made at pH 3.5 of radiolabled complexes and up to 16-fold excess normal IgG by weight. The mixtures were dialyzed to pH 8.0 and then analyzed by sucrose density gradient ultracentrifugation. The complexes reformed the original sedimentation pattern and no 6.6S radiolabeled material was released, indicating that normal IgG did not replace IgG-rheumatoid factor in dimer formation (Fig. 5).

DISCUSSION

The presented results demonstrate that in the rheumatoid immune complexes, composed of IgG molecules, each IgG molecule serves both as an antibody and an antigen, resulting in a self-associating system. The association constant of the antigen-antibody reaction at each site was calculated to be $(1.50 \pm 0.38) \times 10^5$ liters/mol. Since the dimer of IgG was the smallest molecular weight species that was found in dilute complexes, the association constant for dimer formation could not be measured by the employed methods. However, since all molecules were shown to be antibodies, a cyclic dimer is

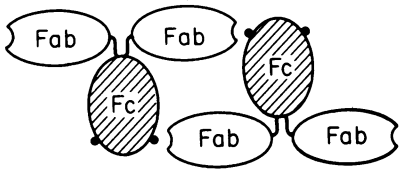

FIG. 6. Proposed model for the self-association of two molecules of antibody to IgG. The T-shaped molecules are drawn according to best available hydrodynamic data (11). A cyclic structure is formed with an estimated association constant of 10^{10} liters/mol.

proposed, as depicted in Fig. 6. Such a cyclic structure would have an approximate association constant of 10^{10} liters/mol (19). Therefore, the cyclic structure would form in preference to binding normal IgG with an association constant of 10^5 liters/mol per binding site. The dissociation of the complexes to monomeric antibody molecules at pH 3.5 clearly ruled out covalent interactions in dimer formation. Self-association was demonstrated by the fact that all $F(ab')_2$ and Fab' fragments had antibody activity. This was further substantiated by the failure to incorporate normal IgG even when the complexes were dissociated and pooled normal IgG was added prior to neutralization and reformation of complexes. The further aggregation of dimers, particularly when dissolved in plasma ultrafiltrate, showed tetramers, octamers, and dodecamers, with absence of hexamers and decamers. These observations may indicate that the higher complexes have a specific geometry. The failure to find trimers and further isodesmic polymers of IgG among the isolated complexes argues that the complexes are not due to "monogamous bivalency" of antibodies as described by Klinman and Karush (20).

The presence of small amounts (<5%) of IgA in the complex preparations does not negate the above conclusions, for it could not have served as an antigen or antibody in the reactions described due to the small mole fraction of this contaminant.

The studies described herein on the intermediate complexes of one patient with rheumatoid arthritis serve as a model for further investigations on serum and synovial fluid from patients with rheumatoid arthritis. The self-association of IgG rheumatoid factors on thermodynamic reasons may well be a general phenomenon. The results of several investigators indicate that this may be the case, even though the basis of this phenomenon has not been explained previously. Grey *et al.* (21) noted that all $F(ab')_2$ fragments of some cryoglobulins with monoclonal IgG-rheumatoid factors bound to normal IgG. The work of Winchester *et al.* (22) suggested that IgG-rheumatoid factors may be the primary constituent in synovial fluid complexes from patients with rheumatoid arthritis, since these materials failed to adsorb to solidified normal IgG, even after acid dissociation. Munthe and Natvig (23) showed that IgG complexes may already exist within plasma cells in the synovial tissue of patients with rheumatoid arthritis.

If the self-association of IgG-rheumatoid factors is a general phenomenon in rheumatoid arthritis, immune complexes of IgG can form in the synovial fluid without the presence of another antigenic molecule. This in turn may be important in maintaining the immunologically mediated synovitis of rheumatoid arthritis, once the synthesis of IgG-rheumatoid factor is initiated.

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