Aggregation of IgG Globulin *in Vivo*. II. Physicochemical Properties of the Isolated Protein*

Shaul Kochwa, Eugene Smith, Marilyn Brownell, and Louis R. Wasserman

ABSTRACT: Study of *in vivo* aggregated IgG globulin, isolated from a case of multiple myeloma with hyperviscosity syndrome, permitted characterization of an associating–dissociating IgG system without demonstrable antibody activity. The main component has a molecular weight of 808,000 ± 33,000, is homogeneous on electrophoresis, and can be dissociated into 7S IgG molecules by lowering the pH below 4.0. Progressive dissociation occurs either on dilution or by increasing NaCl concentration to 40 mg/ml. This protein does not precipitate at low temperature although its reduced viscosity in the cold is highly temperature dependent.

Digestion by either pepsin or papain produces fragments that retain some associating–dissociating properties. On the basis of ultracentrifugal analysis of the products of enzymatic degradation, and assuming from its molecular weight that the aggregated molecule is a pentamer, a tentative structure of this protein is proposed.

Paraprotein is a common name for qualitatively and/or quantitatively abnormal immunoglobulins or related serum protein components (Heremans, 1959, 1961). Two major groups are recognized, one associated with multiple myeloma and the other with Waldenström-type macroglobulinemia, but considerable heterogeneity exists within each group. The myeloma proteins are IgG or IgA globulins[1] and possess, in general, a sedimentation constant of about 7 S; they are heterogeneous in regard to carbohydrate content and antigenic structure. The IgM Waldenström type macroglobulins have an S value of 18–19, are composed of 7 S subunits bound by S–S bridges, and represent a specific entity cross reacting with other immunoglobulins on the basis of "light" chains (Olins and Edelman, 1962).

Aggregates encountered in IgA myeloma are based upon S–S bridges, have S values of 9–15, and are broken down to 7 S units by sulfhydryl reagents such as mercaptoethanol and cysteine (Deutsch and Morton, 1958). Precipitation of globulins in the cold represents another type of aggregation of paraproteins (Jahnke *et al.*, 1958) the supporting mechanism of which is not yet fully understood. At 37°, in general, cryoglobulins exist in a monomeric state, and aggregation, precipitation, or gel formation occurs only when the temperature is lowered. Some cryoglobulins may exist in an aggregated form at 37°; these are responsible for pathological symptoms *in vivo* and inhibit rheumatoid factor (RF) agglutination *in vitro* (Smith *et al.*, 1965). Aggregation and cryoprecipitation may be prevented by low pH (Pedersen, 1950) but is unaffected by increase in ionic strength (Smith *et al.*, 1965). Aggregation resembling antigen–antibody complexes has been encountered in rheumatoid arthritis (Franklin *et al.*, 1957a) and in other disorders. These complexes dissociate at low pH, yielding 7 S and 19 S components from initial 22 S complexes (Franklin *et al.*, 1957b) and 7 S subunits from 12–15 S complexes (Kunkel *et al.*, 1961). The latter, thought to be composed of 7 S RF and 7 S IgG globulin molecules, vary in size relative to 7 S IgG globulin concentration.

In this report the properties of a hitherto undescribed type of aggregation will be described. The *in vivo* aggregated protein isolated from the serum of a patient with multiple myeloma was found to be composed of 7 S IgG globulin subunits, was dissociated by low pH or high salt concentration, and did not precipitate on cooling, but its viscosity was temperature and concentration dependent and produced, in the patient, clinical symptoms of Waldenström macroglobulinemia (Smith *et al.*, 1965). The physicochemical characterization to be presented contributes to the understanding of noncovalent association of protein molecules.

Materials and Methods

Serum. Serum was separated from clotted blood or from citrated plasma obtained by plasmapheresis (Smith *et al.*, 1965) after clotting with 4 ml of 10% $CaCl_2$ solution per 250 ml of plasma. Serum was stored frozen at −20° and, if obtained from plasma, was dialyzed

* From the Department of Hematology, The Mount Sinai Hospital, New York, New York 10029. *Received April 5, 1965; revised October 20, 1965.* Preliminary report of this work was presented at the 47th Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1963. This work was supported in part by a research grant (AM-04434) from the National Institutes of Health and by the Albert A. List, Frederick Machlin, and Anna Ruth Lowenberg Research Funds.

[1] The nomenclature of the immunoglobulins is in accordance with recommendations of the Committee of the World Health Organization ((1964), *Bull. World Health Organ. 30*, 447). Other abbreviation used: RF, rheumatoid factor.

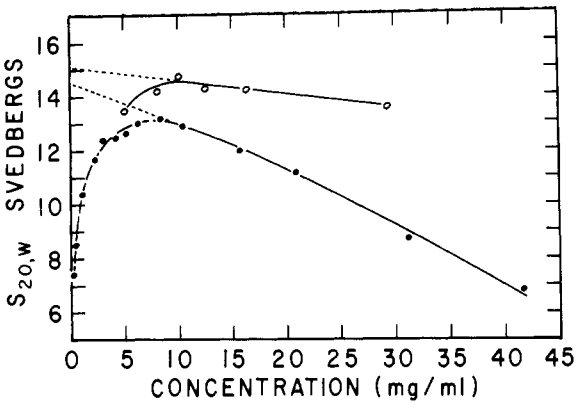

FIGURE 1: Dependence of $s_{20,w}$ value on concentration of the paraprotein in 0.9% NaCl buffered with phosphate to pH 7.9 (●——●) and in unbuffered 0.9% NaCl (pH 6.6) (○——○). Ultracentrifugal analyses at protein concentrations of 0.1–1.0 mg/ml were performed in a standard 12-mm cell with an ultraviolet absorption optical system. At concentrations of 1.0–5.0, 5.0–15, and >15 mg/ml, schlieren optics with 30, 12, and 3-mm cells, respectively, were used.

against three changes of 100 volumes of 0.9% NaCl at 4° before use.

Purification of the Aggregated IgG Globulin. The proteins precipitated from serum at a concentration of 0.96 M Na_2SO_4 were reprecipitated from a 9% solution with 0.62 M Na_2SO_4, yielding a product containing 85% of the paraprotein. After several reprecipitations a final fraction, precipitated from a 1% solution with Na_2SO_4 concentration between 0.50 and 0.57 M, contained 92% of 14.9 S paraprotein. Details of this purification and antigenic analysis of the paraprotein will be published elsewhere (S. Kochwa *et al.*, in preparation).

Euglobulin. Euglobulin fraction was prepared from serum by dilution in ten volumes of distilled water. The precipitate containing the paraprotein was dissolved in 0.9% NaCl and usually reprecipitated in distilled water. Lipoproteins, when present, were removed after centrifugation at 4° and 37,000*g* in a Sorvall RC2 centrifuge.

Human IgG Globulin. Human IgG globulin (Squibb fraction II, Lot No. 1812) was aggregated at 56° for 30 min. The 30 S component was separated with Na_2SO_4 as described by Christian (1958).

Protein Concentration. Absorption at 280 mμ, biuret reaction, or micro-Kjeldahl analyses were used interchangeably for determination of protein concentration.

Ultracentrifugation. A Spinco Model E ultracentrifuge equipped with phase plate schlieren diaphragm, ultraviolet absorption optical system, and RTIC was used. The temperature of the rotor was controlled at 18–20°. Measurement of the ultracentrifuge schlieren pattern was performed with a Nikon two-dimensional comparator having an accuracy of ±0.001 mm. Ultraviolet absorption patterns were traced with an Analytrol. The calculations of the S and relative concentration values were made by conventional methods (Schachman, 1959). The molecular weight estimations were made by a modification (Klainer and Kegeles, 1956) of the Archibald procedure (Archibald, 1947).

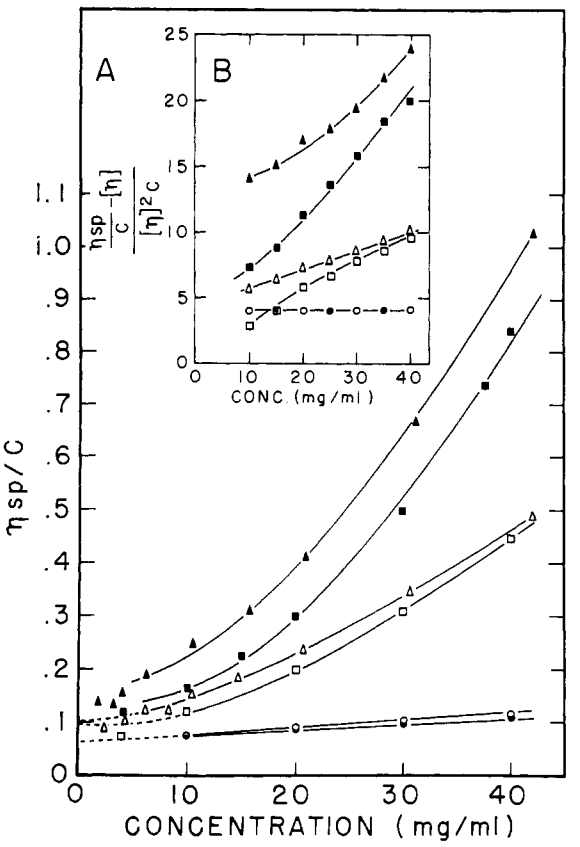

FIGURE 2: Dependence of the viscosity of the purified paraprotein on temperature and concentration. (A) Reduced viscosity plotted against protein concentration. (B) Plot of calculated *K* (see text) against protein concentration. Purified paraprotein in 0.9% NaCl buffered with phosphate to pH 7.9 at 23° (▲——▲) and at 36° (△——△). Purified paraprotein in 0.9% NaCl unbuffered (pH 6.6) at 23° (■——■) and at 36° (□——□). Squibb fraction II in 0.9% NaCl buffered with phosphate to pH 7.9 at 23° (●——●) and at 36° (○——○).

Electrophoresis. Spinco Model H electrophoresis apparatus was used for determination of free electrophoretic mobility. Mobilities were calculated from the δ and ε boundaries at pH 3.5 and from the distance from the starting boundary at pH 8.6.

Acrylamide electrophoresis was performed as previously described (Kochwa *et al.*, 1964). The electrophoresis system for the acid range was constructed according to the theory of disk electrophoresis (Ornstein, 1964; Davis, 1964) so that proteins separated at pH 3.0–3.2.

Potentiometric Titration. Radiometer equipment, including TTT1 titrator with PHA 630T scale ex-

TABLE I: Comparison of the Physicochemical Constants of the Aggregated IgG Globulin with Normal IgG Globulin.

	Paraprotein	IgG Globulin
	15.1	6.56–7.2[a]
$s^0_{20,w}$ pH 6.6	13.2	6.6
$s^{0.5\%}_{20,w}$ pH 3.5	6.1	6.3
in 5% NaCl	6.3	6.4
Mol wt	808,000 ± 33,000	150–177,000[a]
Electrophoretic mobility, $cm^2/v/sec$		
pH 8.6, Γ/2 0.1	$+0.6 \times 10^{-5}$	-1.0×10^{-5}[b]
pH 3.5, Γ/2 0.1	$+6.6 \times 10^{-5}$	$+6.1 \times 10^{-5}$
$[\eta]$	0.095	0.060[c]
Hexose, %	1.1	1.22[a]
$E^{1\%}_{cm}$ 280 mμ	15.6	14.9

[a] Kabat and Mayer (1961). [b] Dole and Braun (1944). [c] Edsall (1953).

pander, SBR2c Titrograph, and SBUIa syringe buret, was used for potentiometric titration. The titration and the graphic presentation was, in general, as described by Winzor and Scheraga (1964), except that stepwise titration in 0.1 pH unit was used throughout. Lack of response after the instrument was twice started to show no requirement of additional titrant was accepted as end point in each 0.1 pH unit step. The overall sensitivity of the instrument was better than 0.005 pH. Protein solutions containing between 9 and 12 mg/ml were exhaustively dialyzed at 4° against 0.1 and 0.7 M KCl, respectively, prior to titration. Eight milliliters of a solution containing 70–90 mg of either paraprotein or γ-globulin in 0.1 or 0.7 M KCl was titrated in a vessel thermostated at 25 ± 0.02°. The final titration values of the protein were obtained after subtraction of values for similar titration of the solvent alone.

Viscosity. Viscosity measurements were made in an Ostwald-type viscosimeter with an outflow time for solvent of about 60 sec at 36° and 73 sec at 23°. Water bath temperatures were ±0.1°.

Enzymatic Degradation and Reaggregation of the Fragments. Pepsin and pepsin–cysteine degradations were performed at 2–3% concentration of protein according to the method used by Nisonoff *et al.* (1960) for digestion of rabbit γ-globulin. Cleavage by papain was according to the method of Hsiao and Putnam (1961).

Results

Electrophoretic Mobility and Molecular Weight Determination. A comparison of the physicochemical properties of the aggregated IgG globulin with the known constants of normal human IgG globulin is given in Table I.

On electrophoresis at pH 8.6 an asymmetric boundary indicated strong concentration dependence while at pH 3.5 sharpening of the ascending and spreading of the descending boundary probably resulted from lower conductivity of the protein than of the buffer. This boundary was always narrower than the boundary of normal γ-globulin (fraction II) observed under similar conditions, an indication of electrophoretic homogeneity confirmed by electrophoresis on acrylamide where only single bands were observed at pH 8.3 for aggregates (Kochwa *et al.*, 1964) and below pH 3.5 for dissociated molecules.

The asymmetric shape of the electrophoretic boundary and, on ultracentrifugation, some evidence of piling up at the bottom of the cell indicate polydispersity of these high molecular weight aggregates. The results of the ultracentrifugation studies were in agreement with the characteristics of such systems because, during purification of this protein, the relative concentration of components with different sedimentation coefficients was dependent on the total protein concentration.

When sedimentation constants were determined at different concentrations of this aggregated IgG globulin abnormal properties were found (Figure 1). Below a concentration of 8 mg/ml the sedimentation constant was found to decrease, indicating dissociation of aggregates. On extrapolation to infinite dilution the calculated $s^0_{20,w}$ value for the aggregated form, analyzed in 0.9% NaCl buffered with phosphate to pH 7.9, was 14.6. Similar experiments performed in unbuffered 0.9% NaCl at pH 6.6 yielded slightly higher S values ($s^0_{2,0w}$ = 15.1 S), especially at high protein concentration.

The average molecular weight of complexes was determined on a solution of 9.6 mg/ml of purified protein in 0.9% NaCl. Sedimentation velocity analysis revealed two components with sedimentation coefficients of 14.9 (94%) and 19.8 S (6%). The determination was performed under the following conditions: 6150 rpm, temperature 20°, photographs taken every 10 min at a phase plate angle of 80°. The mean molecular weight (average of five photographs) was found to be 808,000 ± 33,000 assuming the partial specific volume of γ-globulin to be 0.739 (Hughes, 1954). These values were calculated from the meniscus. Because of the presence of

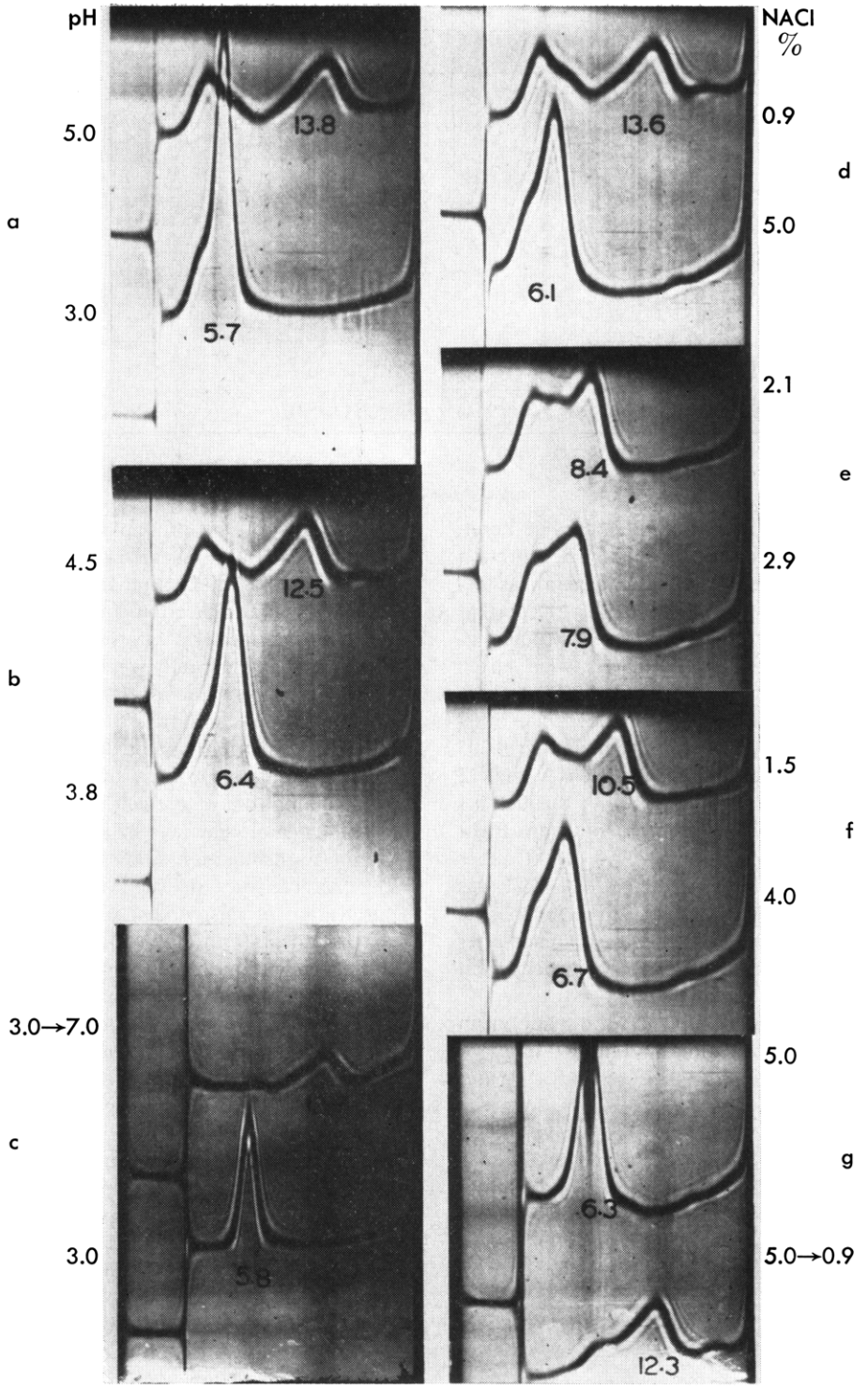

FIGURE 3: Dissociation and reaggregation of the paraprotein. Effect of pH (a and b) and NaCl concentration (d, e, and f) on the sedimentation pattern of the euglobulin fraction. For the reaggregation experiments from low pH (c) and high NaCl (g) paraprotein had been isolated by Na_2SO_4 precipitation. Euglobulin fraction in 0.9% NaCl (50 mg/ml total protein and >30 mg/ml paraprotein) was diluted with four volumes of either 0.1 M glycine buffer of corresponding pH (a and b) or NaCl of desired concentration (d, e, f). For reaggregation studies (c and g) the dissociated paraprotein was dialysed overnight at 4° against 100 volumes of 0.9% NaCl buffered with phosphate to pH 7.0. The photographs were taken after 32 min at 59,780 rpm. Paraprotein peaks are identified by the $s_{20,w}$ values.

the heavier component the values calculated at the bottom of the cell were higher. Comparable values were found by the analysis of the unimolecular layers (Muramatsu and Sobotka, 1963) of this protein (Demeny *et al.*, 1966).

Viscosity. The viscosity measurement revealed both concentration and temperature dependence (Figure 2A). Whole serum and purified aggregated IgG globulin showed similar behavior consistent with the ultracentrifugation data. Reproducible measurements could not be obtained below a concentration of 8 mg/ml and the calculated values, especially at 23°, were considerably lower than expected from the continuation of the reduced viscosity–concentration curve. The effects of the solvent and pH were also comparable with the findings on ultracentrifugal analysis. The viscosity measurements in phosphate-buffered 0.9% NaCl solution at pH 7.9 were constantly higher than those obtained in unbuffered 0.9% NaCl solution at pH 6.6. A certain degree of concentration-dependent association at lower temperature was observed when the K value was calculated for two different temperatures.

Assuming the formula for elongated protein molecules to be

$$\eta_{sp} = [\eta](1 + [\eta]Kc) = \frac{\frac{\eta_{sp}}{c} - [\eta]}{[\eta]^2 c}$$

where the η_{sp} was the observed relative viscosity minus 1, and the intrinsic viscosity $[\eta]$ was determined as 0.095 (Figure 2A), the plot of calculated K values against protein concentration (c) disclosed (Figure 2B) a more pronounced effect of concentration on the K values at 23° than at 36°.

Dissociation and Reaggregation. The sedimentation coefficient and the relative viscosity of the aggregated IgG globulin were not affected by 0.06 M cysteine known to disrupt the S–S bonds between the subunits of IgM macroglobulins and IgA globulin aggregates.

However, lowering the pH below 4.0 (0.1 M glycine–HCl buffer) or increasing the ionic strength of the medium dissociated these aggregates. The lowering of pH caused an abrupt dissociation below pH 4.0 and the transition from aggregated to monomeric form was within a very narrow pH range (Figure 3a, 3b). The dissociation by increased ionic strength was stepwise with full dissociation occurring at 4% NaCl (Figure 3d, 3e, 3f). The relative concentrations of other serum components remained unchanged. The main difference seen was the change in sedimentation coefficient of the aggregates. It is evident that the dissociated complex is ultracentrifugally indistinguishable from 7 S IgG globulin normally present in serum. Normal human serum analyzed in the ultracentrifuge under similar conditions did not show these changes although, after prolonged dialysis against 0.1 M glycine buffer of pH 3.0, gel formation was observed in some samples.

Dissociation of aggregated IgG globulin by either acid or salt was reversible by dialysis against 0.9% NaCl buffered with phosphate to pH 7.0, but the reformed complexes always had slightly lower S values than the original material. Deaggregation and reaggregation did not differ in tests of patient serum, euglobulin, or Na_2SO_4-precipitated paraprotein. Figures 3c and 3g depict the dissociation and reassociation of the 0.62 M Na_2SO_4 precipitated fraction.

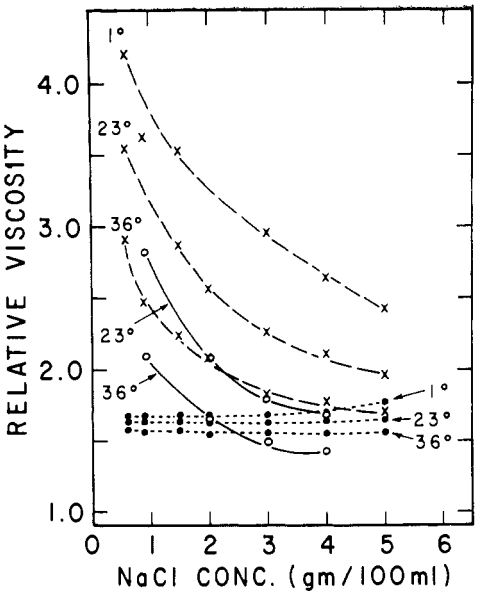

FIGURE 4: Effect of increasing NaCl concentration and temperature on the relative viscosity of patient's serum and isolated paraprotein. Patient's serum, total protein 90 mg/ml (×– – ·×); isolated paraprotein 30 mg/ml (O——O); normal human serum, total protein 60 mg/ml (●– · –●).

The relative viscosity of the patient's whole serum and purified aggregated IgG globulin was similarly affected by increased NaCl concentration (Figure 4). Although the relative temperature effect on the viscosity at different NaCl concentration was unchanged, the relative viscosity of the solution was strongly reduced with increasing concentration of NaCl. Concentration and temperature had negligible effects in tests of normal human serum under identical conditions (Table II).

Potentiometric Titration of Protein in Aggregated and Dissociated Form. Typical titration curves are shown in Figure 5A. Transient turbidity was observed between pH 7.5 and 9.0 in 0.1 M KCl, and opalescence was less pronounced in 0.7 M than in 0.1 M KCl. Of special interest is that, in the region between pH 3.1 and 3.5 (Figure 5B), the protein in 0.1 M KCl took up more protons per molecule of monomer than the same protein in 0.7 M KCl. A comparable difference of 4.0 protons/molecule was found on back titration in this region. The reaggregation occurring in the region above pH 4.5 could be observed by an increase in opalescence on back titration and is possibly indicated by the flattening of the back titration curve in this region. No excessive uptake of protons was observed in the pH

TABLE II: Effect of NaCl Concentration and pH on the Viscosity of Normal Human Serum, Serum Containing *in Vivo* Aggregated IgG Globulin, and Cryoglobulinemic Serum.

	Concn (%)	Temp (°C)	Rel Visc in NaCl (0.9%)	Rel Visc in NaCl (5%)	Rel Visc in Glycine (pH 3.0)	(Rel visc 0.9%)/(Rel visc 5.0%)	(Rel visc 0.9%)/(Rel visc pH 3.0)
		1	3.45	2.34	1.76	1.47	1.96
Patient's	6	23	2.95	1.96	1.71	1.51	1.73
serum 45%		36	2.47	1.56	1.79	1.41	1.58
aggregated		1	2.15	1.72		1.25	
IgG globulin	4	23	2.02	1.55		1.30	
		36	1.77	1.46		1.23	
		1	1.66	1.66	*a*	1.00	
Normal	6	23	1.69	1.57		1.07	
human		36	1.60	1.53		1.04	
serum		1	1.44	1.46		0.99	
	4	23	1.42	1.39		1.02	
		36	1.39	1.38		1.01	
Serum with	6	23	8.6	8.4	4.05	1.02	2.12
cryoglobulin		36	3.2	3.7	3.55	0.86	0.90

[a] Several samples have shown gel formation after dialysis (Smith *et al.*, 1965).

TABLE III: Enzymatic Cleavage of Normal and Aggregated IgG Globulin.

				0.62 M Na_2SO_4 Precipitated Fraction of			
		IgG Globulin[a]		56° 30′ Heat-Aggregated IgG Globulin		*in vivo* Aggregated IgG Globulin	
Treatment	pH	($s_{20,w}$)	(%)	($s_{20,w}$)	(%)	($s_{20,w}$)	(%)
Native	7.9	11.9	6	30.3	76	14.8	>95
		9.6	19	Intermed.	11		
		6.4	75	6.2	13		
	4.0	10.8	4	*b*		5.9	>99
		8.8	16				
		6.0	79				
Pepsin	4.0	4.6	100			4.4	100
	7.5	4.6	100			11.4	7
						7.7	77
						7.0	16
Pepsin and	4.0	3.3	100			3.2	100
cysteine	7.5	3.2	100			4.2	100
Papain cysteine	6.0	3.3	100	3.4	100	4.3	100
activated	3.0			3.3	100	3.2	100

[a] Squibb fraction II Lot No. 1812. [b] Over 80% of the material irreversibly precipitated.

3.1–3.5 range when 7 S IgG globulin in 0.1 M KCl was substituted and titration was performed under identical conditions.

Enzymatic Degradation of the Purified Protein. At pH 4.0, where dissociation of the molecule takes place and the enzyme could act on the monomeric form, the paraprotein was split by pepsin and pepsin with cysteine into fragments having sedimentation coefficients similar

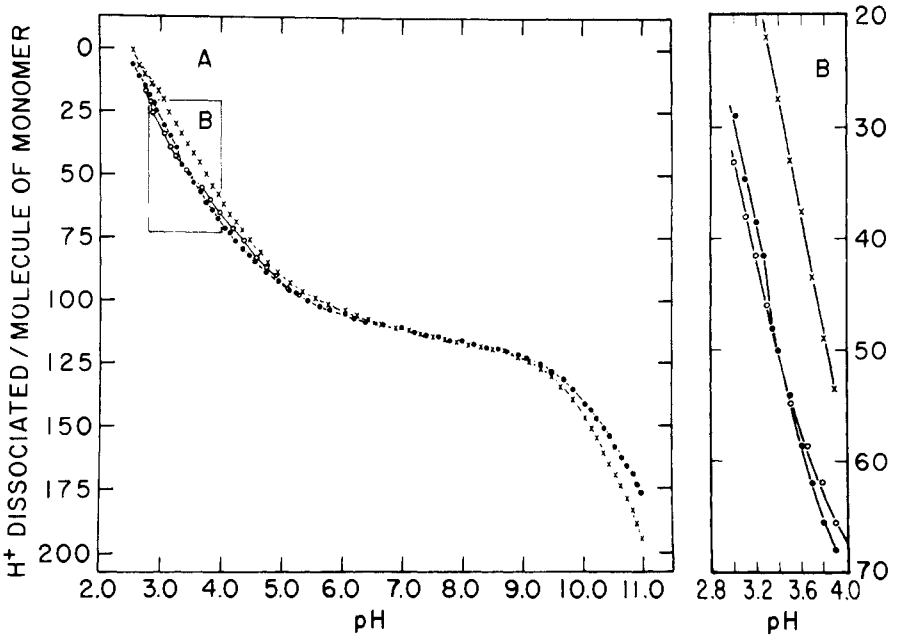

FIGURE 5: Potentiometric titration of isolated paraprotein.[2] (A) Titration curve between pH 2.5 and 11.0. (B) Titration curve between 3.0 and 4.0. Titration curve of the paraprotein in 0.1 M KCl (●——●) and in 0.7 M KCl (X——X); back titration between pH 2.5 and 6.0 in 0.1 M KCl (O——O).

to those produced from normal IgG globulin (Table III). However, when the pH of the digested protein solution was raised to 7.5, aggregation independent of S–S bonds occurred; the $s_{20,w}$ value of the main component rose from 4.4 to 7.7 S and some heterogeneity of reformed aggregates was observed. The final product of pepsin–cysteine treatment (irrespective of whether the paraprotein had been digested by pepsin in the presence of cysteine or peptic fragments had been subsequently treated with cysteine) was found to have, at pH 4.0, an $s_{20,w}$ value of 3.2 S which rose to 4.2 after change of pH to 7.5.

Papain digestion performed on intact aggregates at pH 6.0 produced fragments of $s_{20,w}$ = 4.3 S, from which, in lowering the pH below 4.0, fragments of $s_{20,w}$ = 3.3 S were obtained similar to those from normal IgG globulin.

Such aggregation of fragments derived from proteolysis was not found after digestion of heat-aggregated (56°, 30 min) IgG globulin that had been purified in the same way as the *in vivo* aggregated paraprotein. Fragments from heat-aggregated IgG globulin were indistinguishable from those found after digestion of normal 7 S IgG globulin with cysteine-activated papain. Data could not be obtained for pepsin, or pepsin with cysteine, because of irreversible precipitation of heat-aggregated IgG globulin that occurred in acetate buffer of pH 4.0.

Discussion

The properties of an *in vivo* aggregated IgG globulin that have been described are consistent with reported data pertaining to the structure of other proteins. Dissociation at low pH has been observed for several proteins and enzymes (Kunkel *et al.*, 1961; Winzor and Scheraga, 1964) and has been attributed to disruption of hydrogen bonds. A vertical shift of titration curves in the region of dissociation has been described (Riddiford and Edsall, 1963; Winzor and Scheraga, 1964). On the basis of the difference in electrophoretic mobility between the paraprotein and normal IgG globulin at pH 8.6, as compared with pH 3.5, it may be assumed that a certain number of negative charges are neutralized upon aggregation. Similarly, the effect of high salt concentration seems to have a counterpart in the described effect of 1 M NaBr at pH 5.3 on the association of thrombin-treated fibrinogen (Donnelley *et al.*, 1955). The dissociation at low protein concentration, apparent on ultracentrifugation, is similar to that described for a cryoglobulin (Pedersen, 1950) and for some Bence-Jones proteins (Bernier and Putnam, 1963) and is in agreement with viscosity studies.

The acid-dissociable IgG globulin aggregate of this report differs from others reported by Kunkel *et al.* (1961). On addition of normal IgG globulin (fraction II) the described shift in the molecular weight of the aggregates toward lower values, due to partial dissociation of antigen–antibody complexes, has not been ob-

[2] These titration curves were obtained with the aggregated (in 0.1 M KCl) and the dissociated molecules (in 0.7 M KCl). In addition a dissociation of the pentamer to monomer takes place in 0.1 M KCl below pH 4.0. To avoid unnecessary complication in presentation of data the assumed molecular weight of the monomer (160,000) was used for the calculation of titrable groups.

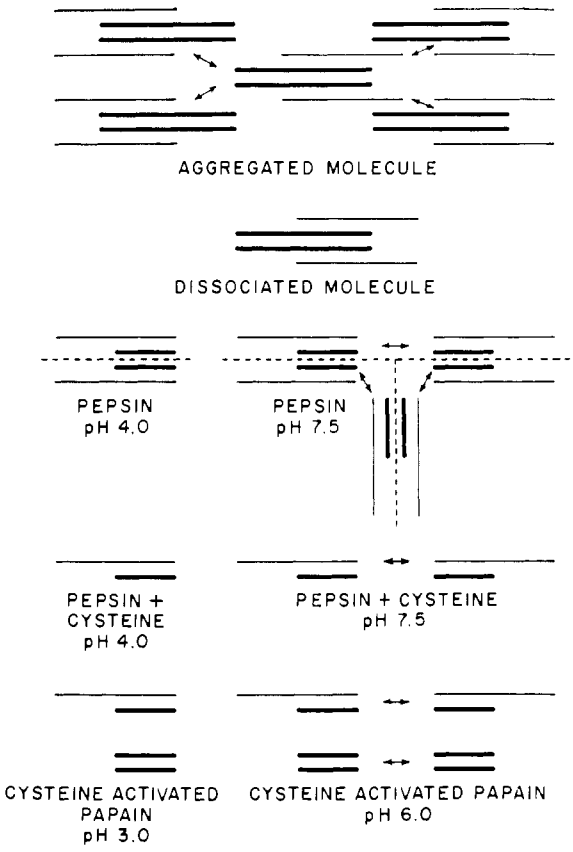

FIGURE 6: Hypothetical structure of the *in vivo* aggregated γ-globulin and its enzymatic degradation products. Light chain (———); heavy chain (———); hypothetical bond (←——→); dissociation of S–S bonds by cysteine (– – – –).

served (S. Kochwa *et al.*, in preparation). The acid-dissociable macroglobulins described by Rees and Resner (1959) could represent a different type of aggregated IgG globulin, but unfortunately only ultracentrifugation data were reported.

It is of interest to note that the S values of the different fractions recovered in Na_2SO_4 preparations were considerably lower than those reported by Christian (1958) in the separation of heat-aggregated IgG globulins at similar salt concentrations. His suggestion that further aggregation of heated IgG globulin occurs during isolation procedures is supported by differences now observed in relative concentrations of successive precipitated fractions. However, the phenomenon would have to be much more pronounced in the artificially produced aggregates than in the *in vivo* complex of IgG globulin. The homogeneity of the electrophoretic peak at pH 3.5 is in accord with the assumption that the heterogeneity on ultracentrifugal analysis is due to different degrees of polymerization and not due to contamination. On immunoelectrophoretic analysis (S. Kochwa *et al.*, in preparation) only antigenic determinants of IgG globulin were found.

The question must be considered as to whether this *in vivo* aggregation is based upon a mechanism related to that governing the behavior of cryoglobulins. Differences were not observed when $s_{20,w}$ values for the *in vivo* aggregated protein were calculated from runs at 37° and 18°, whereas pronounced differences were found in similar studies of cryoglobulins (Smith *et al.*, 1965). Similarly indices of the relative viscosities of undissociated and dissociated proteins (Table II) remained relatively unchanged for the *in vivo* aggregated paraprotein whereas those for cryoglobulin were relatively temperature dependent even when dissociated by pH 3.0. In spite of this evidence, however, the effect of concentration upon the *in vivo* aggregates was temperature dependent, as seen from the calculation of K (Figure 2B). If cryoglobulins are defined as proteins that precipitate in the cold this aggregate is not a cryoglobulin. On the other hand, it could be included if the definition were high-temperature dependence of viscosity with or without precipitation in the cold. Therefore, the behavior of this protein may be similar to the first stages in the aggregation of cryoglobulins.

The *in vivo* aggregated globulin molecule has a molecular weight of over 800,000 and a specific arrangement of its subunits. Although only one homogeneous component was found on free electrophoresis the high isoelectric point indicates a positively charged molecule. The lack of incorporation of normal IgG globulin (S. Kochwa *et al.*, in preparation) and the complexes obtained after enzymatic splitting indicate that the electrostatic charges holding the complex together are located on definable parts of the molecule. The electrostatic nature of this bond was confirmed by potentiometric titration, and the shift in the titration curve at pH 3.2 resembled the transition from native to unfolded molecules (Tanford, 1962). The number of groups responsible for the bonding were calculated to be 4–4.5 per molecule of monomer (mol wt 160,000). The relationship of fragments obtained under different conditions and by different enzymes is of special interest and permits the construction of a hypothetical model of the aggregated molecule (Figure 6). It is clear from the ultracentrifugal analysis of the enzymatic degradation products that the part of the IgG heavy chain that is destroyed by pepsin digestion (Edelman and Gally, 1964) must bear some of the groups that are responsible for aggregation (Table III). From the calculated molecular weight the main aggregate can be assumed to be a pentamer which dissociates into monomers below pH 4.0. Since a trimer of 5 S components is probably the major product after reaggregation of pepsin-digested fragment (s = 7.7 S), two of the sites of aggregation must have been destroyed when part of the H chain was digested. Furthermore, three dimers (s = 4.2 S) were produced from this trimer by reduction of S–S bonds with cysteine. At pH 4.0 these dimers dissociated into monomers having the same sedimentation coefficient (s = 3.2 S) as those obtained from digestion of normal IgG globulin (Table III) (Nisonoff *et al.*, 1960). This association–dissociation reaction, unaffected by iodoacetamide, must depend on electro-

static forces. The effect of cysteine-activated papain is also consistent with this model because the dimers so produced were susceptible to dissociation by low pH (Table III). Heat-aggregated IgG globulin, while possessing similar salting out properties (Christian, 1960), provided digestion fragments indistinguishable from those obtained from unaggregated IgG globulin (Table III).

In tests with rheumatoid arthritis serum, the *in vivo* aggregated paraprotein was found to resemble heat-aggregated IgG globulin by inhibiting the RF agglutinating activity of Rh sensitized cells and by supporting characteristic specific precipitin curves with RF (S. Kochwa *et al.*, in preparation). However, the products of pepsin digestion lost their activity in this system while papain products remained active (S. Kochwa *et al.*, in preparation). These properties are in contrast to those of heat-aggregated IgG globulin and may assist in determining structures responsible for the RF–heat-aggregated IgG globulin interaction.

Acknowledgment

The authors are indebted to Dr. Mary L. Peterman, Sloan Kettering Institute, New York, N. Y., for performance and interpretation of the electrophoretic mobility determinations and to Dr. Harold A. Scheraga, Cornell University, Ithaca, N. Y., for helpful discussion during preparation of this manuscript.

References

Archibald, W. J. (1947), *J. Phys. Colloid Chem. 51*, 1204.

Bernier, G. M., and Putnam, F. W. (1963), *Nature 200*, 223.

Christian, C. L. (1958), *J. Exptl. Med. 108*, 139.

Christian, C. L. (1960), *J. Immunol. 84*, 112.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Demeny, M., Kochwa, S., and Sobotka, H. (1966), *J. Colloid Sci.* (in press).

Deutsch, H. F., and Morton, J. I. (1958), *J. Biol. Chem. 231*, 1107.

Dole, V. P., and Braun, E. (1944), *J. Clin. Invest. 23*, 708.

Donnelly, T. H., Laskowski, M., Jr., Notley, N., and Scheraga, H. A. (1955), *Arch. Biochem. Biophys. 56*, 369.

Edelman, G. M., and Gally, J. A. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 846.

Edsall, J. T. (1953), *Proteins 1*, 692.

Franklin, E. C., Holman, H. R., Müller-Eberhard, H. J., and Kunkel, H. G. (1957a), *J. Exptl. Med. 105*, 425.

Franklin, E. C., Kunkel, H. G., Müller-Eberhard, H. J., and Holman, H. R. (1957b), *Ann. Rheum. Dis. 16*, 315.

Heremans, J. (1959), *Clin. Chim. Acta 4*, 639.

Heremans, J. (1961), Les Globulines Sériques du Systeme Gamma, leur Nature et leur Pathologie, Paris, Masson, p. 149.

Hsiao, S. H., and Putnam, F. W. (1961), *J. Biol. Chem. 236*, 122.

Hughes, W. L. (1954), *Proteins 2*, 720.

Jahnke, K., Sholtan, W., and Heinzler, F. (1958), *Helv. Med. Acta 25*, 2.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, 2nd ed., Springfield, Ill., Charles C Thomas, p. 765.

Klainer, S. M., and Kegeles, G. (1956), *Arch. Biochem. Biophys. 63*, 247.

Kochwa, S., Smith, E., Davis, B. J., and Wasserman, L. R. (1964), *Ann. N. Y. Acad. Sci. 121*, 445.

Kunkel, H. G., Müller-Eberhard, H. J., Fudenberg, H. H., and Tomasi, T. B. (1961), *J. Clin. Invest. 40*, 117.

Muramatsu, M., and Sobotka, H. (1963), *J. Colloid Sci. 18*, 625.

Nisonoff, A., Wissler, F. C., Lipman, L. N., and Woernley, D. L. (1960), *Arch. Biochem. Biophys. 89*, 230.

Olins, D. E., and Edelman, G. M. (1962), *J. Exptl. Med. 116*, 635.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.

Pedersen, K. O. (1950), *Cold Spring Harbor Symp. Quant. Biol. 14*, 140.

Rees, E. D., and Resner, R. (1959), *Clin. Chim. Acta 4*, 272.

Riddiford, L. M., and Edsall, J. T. (1963), Abstracts, 143rd National Meeting of the American Chemical Society, Cincinnati, Ohio, April 1963, p. 23A.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, p. 63.

Smith, E., Kochwa, S., and Wasserman, L. R. (1965), *Am. J. Med. 39*, 35.

Tanford, C. (1962), *Advan. Protein Chem. 17*, 108.

Winzor, D. J., and Scheraga, H. A. (1964), *Arch. Biochem. Biophys. 104*, 202.