Aggregation of γG3 Proteins: Relevance to the Hyperviscosity Syndrome

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ABSTRACT Studies on the sera and isolated proteins from 14 patients with γG3 multiple myeloma revealed a concentration- and temperature-dependent aggregation which was not encountered in 26 sera from patients with multiple myeloma involving other γG subgroups. When the γG3 myeloma sera were diluted and characterized by analytical ultracentrifugation, complex formation was minimal. However, when these sera were examined undiluted, marked complex formation was observed. Studies on the isolated proteins, their enzymatic fragments, as well as their heavy and light polypeptide chains localized the aggregating sites to the Fd fragment of the heavy chains. The findings were also documented by acrylamide-gel electrophoresis and capillary tube viscometry.

The hyperviscosity syndrome was observed in six patients: three with γG3 myeloma and three with γG1 myeloma. In the latter group extreme protein concentrations appeared essential for the development of the clinical symptoms. The γG3 cases, however, because of the aggregation phenomenon, showed the syndrome at relatively low protein concentrations.

INTRODUCTION

Previous studies have documented the existence of at least four subgroups of γG globulin based on antigenic differences in their heavy polypeptide chains (1, 2). While the γG3 (Vi,γ_{2c}) subgroup accounts for only 8% of the normal γG globulin and a similar percentage of γG myeloma proteins (3), several lines of evidence suggest that this subgroup plays a significant role in human immunology. It is known that γG3 proteins are catabolized at a faster rate than any other γG globulins (4, 5) and the quantitative level of γG3 in serum is influenced by the specific genetic makeup of the individual (3). Patients with hypogammaglobulinemia have been shown to have a disproportionate increase in γG3 globulin (6). Cryoglobulins of the γG type with rheumatoid factor activity are predominately γG3 proteins (7) and preliminary evidence suggests that γG3 proteins are important in the binding of complement.[1]

An interesting physical property of γG3 proteins of both normal sera and myeloma sera has been their partial adsorption near the well of origin in immunoelectrophoresis (3). Even isolated γG3 proteins that have been purified frequently show a characteristic biphasic pattern in the agar system. In the present study this phenomena has been further investigated and a concentration and temperature-dependent aggregation of these proteins has been delineated. This aggregation apparently plays a major role in the hyperviscosity syndrome which occasionally accompanies multiple myeloma (8, 9) and may be the explanation for the rapid catabolism and special complement binding of γG3 proteins.

Aggregates with S values of 9–15S are frequently encountered in γA myeloma and are due to S-S bridges (10). Presumably these stable high molecular weight aggregates are responsible for the occasional occurrence of the hyperviscosity syndrome in γA myeloma (11). Smith, Kochwa, and Wasserman (12) were the first to demonstrate aggregate formation in hyperviscosity syndrome in γG myeloma. These aggregates were isolated and found to be acid dissociable indicating that the intermolecular bonds were noncovalent. Since their report, however, other workers have failed to document aggregate formation in the hyperviscosity syndrome in γG myeloma despite analytical ultracentrifugation (13–15).

METHODS

Isolation, purification, and characterization of myeloma proteins. Myeloma proteins were isolated by zone electrophoresis on polyvinyl copolymer (Pevikon). Further purification was accomplished by gel filtration on Sephadex G-200 equilibrated with 0.1 M tris(hydroxymethyl)aminomethane (Tris) and 0.5 M sodium chloride. The isolated proteins were concentrated by vacuum dialysis or by dialysis against polyethylene glycol. For determination of molecular weight by low speed analytical ultracentrifugation an addi-

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[1] Müller-Eberhard. 1968. *Advan. Immunol.* **8**: 1.

tional purification step was utilized. After Sephadex G-200 chromatography each protein was concentrated by vacuum dialysis in membranes which will exclude material of less than 70,000 molecular weight. Each preparation was then centrifuged at 35,000 rpm in a Spinco model L preparative ultracentrifuge for 2 hr at 4°C in a swinging bucket rotor. The top one-third was aspirated and used for molecular weight measurements by the field relaxation technique. γG subgroup analysis and Gm typing were done as described previously (1, 16).

Enzymatic cleavage of γ-globulin. Fab and Fc fragments of myeloma proteins were prepared by papain digestion (17) using a protein: enzyme ratio of 50:1. γG3 proteins were subjected to enzymatic digestion for 30 min at 37°C in the presence of 0.01 M cysteine and 0.002 M EDTA at pH 7.4. γG1 proteins were digested for 16 hr. The reaction was stopped by dialysis against cold saline, and Fab and Fc were separated by zone electrophoresis on Pevikon. They were then concentrated by vacuum dialysis and further purified by gel filtration by Sephadex G-100 chromatography. Enzymatic fragments were tested for purity by immunoelectrophoresis and Ouchterlony analysis with appropriate antisera.

Pepsin digestion was accomplished in pH 4.0 acetate buffer with a protein: enzyme ratio of 50:1. Digestion proceeded at 37°C for 24 hr and was terminated by dialysis at 4°C against pH 7.2 phosphate-buffered saline (PBS). The 5S pepsin $(Fab')_2$ fragment was then isolated by Sephadex G-200 chromatography and purity was ascertained by ultracentrifugation, immunoelectrophoresis, and acrylamide-gel electrophoresis.

Preparation of H and L chains. Isolated myeloma proteins at 10 mg/ml were dialyzed into 0.5 M Tris buffer pH 8.6. The buffer was then made 0.1 M in β-mercaptoethanol and dialysis was continued overnight at 4°C. The buffer was then made 0.15 M in iodoacetamide and dialysis was continued for 24 hr at 4°C. Chain separation was accomplished by dialyzing against 1 M propionic acid at 4°C for 72 hr. Separation of H and L chains was performed by gel filtration on a Sephadex G-200 column in 1 M propionic acid and 0.001 M iodoacetamide. Chains were concentrated by dialysis against polyethylene glycol in 0.5 M propionic acid. For some experiments utilizing acrylamide-gel electrophoresis whole myeloma proteins were reduced and alkylated in small volumes and placed directly on the gels. Chain separation in these instances was accomplished by the gels themselves. For acid-urea gels, urea was added to the reduced and alkylated proteins as it was to all samples before placing them on the gels.

Radioiodination. 50 mg of isolated myeloma proteins G. J. and E. T. were separately dissolved in 10 cc of 0.05 M phosphate buffer pH 7.0. A modification of the chloramine T method of McConahey and Dixon (18) was used to label G. J. with ^{131}I and E. T. with ^{125}I. 5 μg chloramine T per mg protein and 5 μg sodium metabisulfite per mg protein was used. Over 97% of the radioactivity was precipitated with 10% trichloroacetic acid. Radioactivity was measured on a dual channel scintillation counter (Nuclear-Chicago Automatic Gamma Counter, model 4233). After radioiodination the labeled proteins were purified on a Sephadex G-200 column in order to remove higher molecular weight aggregates.

Recombination of myeloma proteins. Recombination and reoxidation of the labeled myeloma proteins was accomplished by a modification of the method of Olins and Edelman (19). Each of the radiolabeled and column-purified proteins was reduced with β-mercaptoethanol and dialyzed against 1 M propionic acid for 72 hr. Chain separation was accomplished on a Sephadex G-200 column made 1 M in propionic acid and 0.01 M mercaptoethanol. Hybrid molecules were formed using H chains from a γG3 myeloma protein labeled with ^{131}I (G. J.) and the light chains from a γG1 myeloma protein labeled with ^{125}I (E. T.). Recombination was usually done on a milligram per milligram basis of H chain per L chain. Similar hybrids were made with a γG1 heavy chain and the light chains derived from a γG3 protein. After prolonged dialysis against acid and then neutral buffers containing 0.001 mercaptoethanol, dialysis was continued for 72 hr against phosphate-buffered saline pH 7.2 with no mercaptoethanol. The recombined molecules were then concentrated slowly by dialysis against 3% polyethylene glycol (PEG) in 1 M propionic acid. They were then purified on Sephadex G-200 columns made 1 M in propionic acid. The γG peak usually accounted for over 80% of the radioactivity. This was pooled and concentrated by dialysis against 3% PEG and then utilized in the acrylamide-gel electrophoresis experiments. In most experiments recombined molecules contained from 1.17 to 1.25 moles of H chain per L chain.

Analytical ultracentrifugation. A Spinco model E analytical ultracentrifuge equipped with schlieren optics was used for all studies. Unless otherwise noted, studies were performed in 12 mm double-sector cells at 20°C and 52,640 rpm. Plate measurements were made according to procedures described by Trautman (20). In correcting the sedimentation coefficients to the $s_{20,w}$ value the partial specific volume was assumed to be 0.73. Studies were done on undiluted sera, sera diluted 1:1 with PBS and with purified myeloma proteins and their fragments in PBS. Sedimentation constants at infinite dilution were derived from plots of $s_{20,w}$ vs. concentration.

Molecular weights were determined by the method of Kegeles and Sia (21) using the field relaxation procedure. Fluorochemical Fc 43 (Beckman/Spinco Division, Palo Alto, Calif.) was used in a capillary synthetic boundary cell. Molecular weights were determined in pH 7.2 PBS as well as 0.1 M acetate buffer pH 4.0. In each study the protein concentration was 8.0 ±0.2 mg/cc. Computation of the molecular weight at the cell bottom was made from the following equation:

$$M_\omega = \frac{RT \left.\frac{dc}{dr}\right|_b}{(1 - \bar{V}\rho) r_b (A_1 + A_2)\omega^2}$$

where M_ω is the weight average molecular weight at the cell bottom, $\left.\frac{dc}{dr}\right|_b$ is the extrapolated deflection above the estimated baseline of the schlieren diagram at the lower meniscus, r_b is the absolute position in the cell and A_1 and A_2 the areas below the free peak and the peak near the cell bottom. These areas were measured by the trapazoid method.

Acrylamide-gel electrophoresis. Acid–acrylamide-gel electrophoresis was performed in an E-C Apparatus Corp. vertical gel electrophoresis cell. The basic procedure was the Canalco modification of the system of Williams and Reisfield (22). The acid gel buffer was KOH-citric acid pH 2.9 while the tank buffer was glycine-citrate pH 4.0. For urea-acid gels both the gel and the tank buffers contained 8 M urea. 7.5% acrylamide gels were constructed from a stock solution (5% bisacrylamide, 95% acrylamide) which contained ascorbic acid, ferrous sulfate, and 0.001 M iodo-

acetamide. Gels were polymerized by the addition of 5–10 μl of 30% cold hydrogen peroxide (23). Acid gels were run for 4 hr at 300 v while acid-urea gels were run for 8–12 hr. The slabs were stained with amido black 0.25% in methanol: water glacial acetic acid 5:5:1. Destaining was accomplished in an electrophoretic destainer (E-C Apparatus Corp.) in a similar solution without amido black.

Tests for anti-γ-globulin activity. The Rh agglutination test was done according to Waller and Vaughan (24). Anti-CD serum Ripley (kindly supplied by Dr. Marion Waller, Richmond, Va.) was used for these studies. The latex fixation test was obtained from Hyland Laboratories, Los Angeles, Calif. (RA-Test). Both the Rh agglutination test and the latex fixation test were performed at 4° and 22°C.

In separate experiments Fab and $(Fab')_2$ from each protein were mixed with normal γ-globulin as well as isolated myeloma proteins from each γG heavy-chain subclass and examined in the analytical ultracentrifuge for interactions. The area under the curve of Fab or $(Fab')_2$ was compared to the same material in a wedge cell without additional γ-globulin in order to assess for complex formation.

Test for cryoglobulins. Attempts were made to demonstrate significant amounts of cryoglobulins in each of the sera. Despite prolonged standing in the cold, dilution, and pH changes from 5 to 8.5 no cryoproteins were evident. In a final attempt each serum was spun at 35,000 rpm in an SW 39 Rotor of a Spinco model L ultracentrifuge for 18 hr at 4°C. No cryoprecipitate was evident in the bottom of any tube.

Viscosity studies. All solutions introduced into the viscometers were filtered repeatedly through a Millipore filter pad. The density of the solutions was measured with a 5 ml capillary vent pyknometer. Capillary viscosity measurements were done in a constant temperature bath utilizing an Ostwald Viscometer. Phosphate-buffered saline was the reference solvent in all studies. Measurements were performed at 4°, 20°, 22°, and 37°C and each determination was the average of three separate studies.

The specific viscosity was derived from the expression: $\eta_{sp} = \eta_{rel} - 1$. The intrinsic viscosity was determined by a plot of η_{sp}/c vs. C where C represents the protein concentration in grams per cubic centimeter. Studies were performed over a protein concentration range of 0.001–0.067 g/cc.

A modified version of the G. D. M. viscometer was used to determine the viscosity at low shear rates (25). The principle of this viscometer, which consists of coaxial cylinders separated by a small anulus in which the sample is located has been described (26, 27). Utilizing a water bath connected to a channel inside the rotor and a thermistor connected to a thermo regulator at the exit from the channel, sample temperature could be maintained at ±0.1°C. Studies were done at 4°, 20°, and 37°C. Measurements were performed at five shear rates (52, 5.2, 0.52, 0.104, and 0.052 sec^{-1}).

RESULTS

Analytical ultracentrifugation of serum and isolated myeloma proteins. When γG3 myeloma sera were diluted 1:1 with phosphate-buffered saline and studied in the analytical ultracentrifuge they did not appear conspicuously different from the sera from patients with multiple myeloma involving other subgroups (Fig. 1, top). However, when studied undiluted, marked differences were obvious (Fig. 1, bottom). Seven γG3 myeloma sera were studied and each showed a shelf of complexes arising from the 7S peak and moving to the bottom of the cell. That this was not an artifact of examining undiluted sera was shown by the fact that in the undiluted sera of several patients with multiple myeloma involving other subgroups no such complexes were noted (Fig. 1, E. T.). In addition, under these conditions marked convective disturbances were noted only

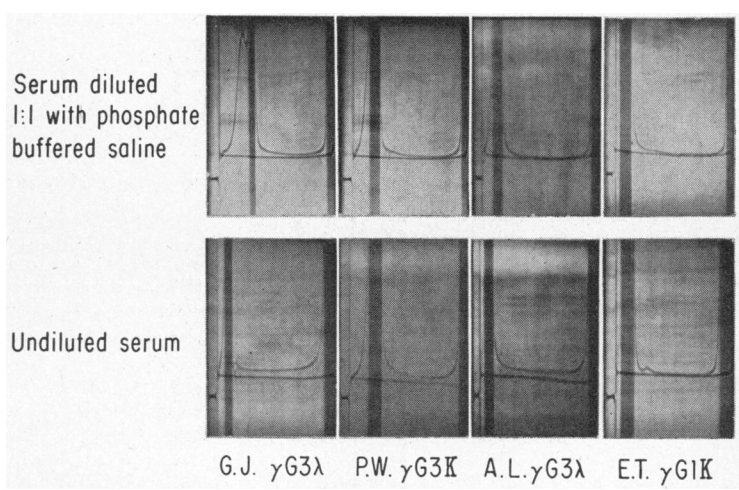

FIGURE 1 Analytical ultracentrifuge analysis of three sera from patients with γG3 multiple myeloma (G. J., P. W., and A. L.) and serum E. T. γG1. Each photograph was taken 64 min after reaching speed of 52,640 rpm. The shelf of aggregating material is only seen in the undiluted sera of the γG3 cases.

in the undiluted sera of the patients with γG3 myeloma. The amount of complexes seen was correlated with the amount of myeloma protein in the patient's serum and γG3 myeloma sera with less than 15 mg/ml of myeloma protein did not readily demonstrate complex formation on analytical ultracentrifugation.

In order to determine the sedimentation constant for these γG3 proteins three of the isolated myeloma proteins were sedimented at various concentrations and on S vs. C plot was constructed (Fig. 2). Protein G. J. had an extrapolating value of 6.72 at infinite dilution. Protein A. L. had a value of 6.81 and protein P. W. was 6.78. At higher concentrations each isolated myeloma protein was seen to aggregate and the S value reported in based on the analysis of the main peak alone (Fig. 2). The radius of gyration of each profile was calculated with the aid of a computer program. In each case, with increasing protein concentration there was accelerated skewing of the schlieren profile towards the base of the cell. Thus, the increase in the radius of gyration suggests that the heavier components seen at higher concentrations are the product of the higher concentration and not secondary to better visualization of these components. Each of the isolated proteins was dialyzed against acetate and phosphate buffers ranging from pH 4.0 to 8.0. Each solution was then adjusted to 20 mg/ml and studied in the analytical ultracentrifuge. Complex formation was maximal at pH 6.8. This is illustrated in a later section for γG3 fragments (Fig. 6).

Low-speed determinations of molecular weights were performed in an attempt to rule out an effect of the extreme pressures in the cell during high-speed centrifugation contributing to the complexing phenomenon. For these studies special care was taken that the isolated proteins did not contain stable aggregates which might have arisen from the isolation procedure (see Methods). As illustrated in Fig. 3 the weight average molecular weight at the bottom of the cell was considerably higher in the isolated protein from patient G. J. with γG3 myeloma than the protein isolated from E. T. who had γG1 multiple myeloma. In each case the molecular weight determined in pH 4.0 acetate buffer compared favorably with the accepted values for γG globulin in the literature.

Acrylamide-gel electrophoresis. Acrylamide-gel electrophoresis proved to be a sensitive technique for demonstrating aggregation phenomena, apparently because of the concentrating effect that occurs before entrance into the gel. The concentration-dependent aggregation was seen even at low pH. Thus while at pH 4.0 no aggregation could be demonstrated in the analytical ultracentrifuge, aggregation was clearly seen in acrylamide gels at pH's as low as 2.9. Fig. 4 represents an acid–acrylamide-gel electrophoresis of seven sera from

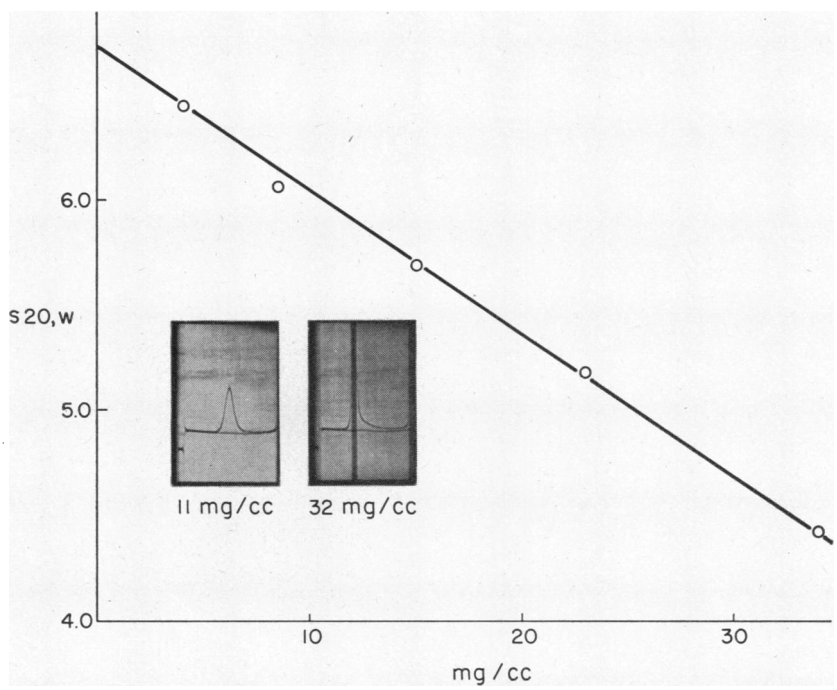

FIGURE 2 The concentration dependence of sedimentation of G. J. isolated γG3 protein. The extrapolated value of $s_{20,w}$ (in Svedberg units) at infinite dilution was 6.72. The insert shows aggregation at higher concentration.

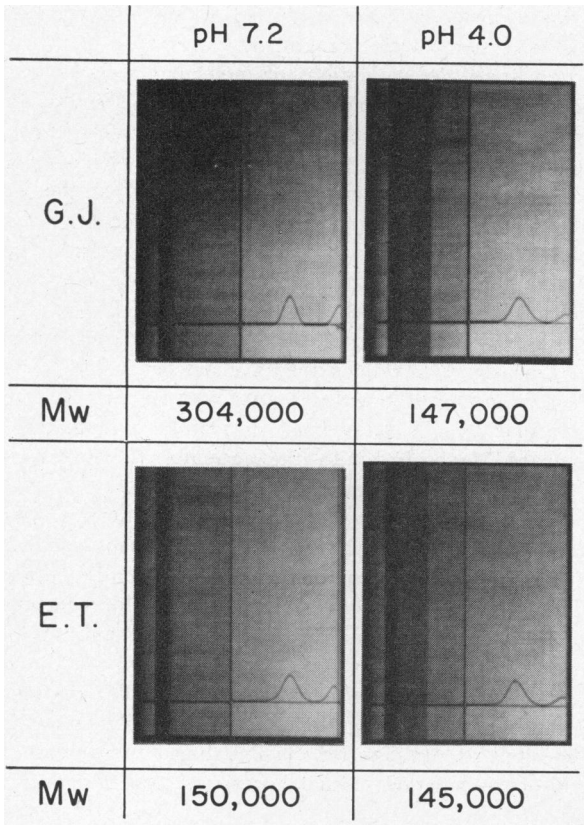

FIGURE 3 Apparent molecular weights of isolated myeloma proteins by the Field Relaxation Technique. In each experiment the sample was accelerated to 20,000 rpm for 4 min and decelerated at approximately 1000 rpm min^{-1} to 4000 rpm. Photographs were taken after 20 min at the lower speed.

patients with γG3 myeloma and one serum from a patient with γG1 myeloma, all with large protein peaks. It was clear that the γ-globulin in serum from the patients with γG3 myeloma did not significantly enter the gel while the γG from serum E. T. (γG1 myeloma) did enter the gel. Fig. 5 represents the same experiment except the gel contained 8 M urea. Here the γ-globulin from all patients entered the gel and appeared virtually identical. Sera from four other patients with γG3 myeloma were studied and behaved in an identical way with those shown in Figs. 4 and 5. Gm (b) or Gm (g) and kappa or lambda proteins behaved similiarly. 25 sera from patients with multiple myeloma of the γG1, 2, and 4 subgroups were similarly studied and in each instance the γG paraprotein peak entered both acid–acrylamide-gel systems in a normal fashion.

In order to determine whether γG3 proteins in normal serum also displayed a concentration-dependent aggregation, normal serum was run on acid–acrylamide-gel electrophoresis. Without staining, the origin was cut out as well as the position where the γG normally migrates. Each portion of the gel was then forced through an 18-gauge needle and agar plate analysis was performed with γG subgroup–specific antisera. Only γG3 proteins were detected at the origin and no γG3 was found in the main γG band.

Molecular localization of aggregation site. In order to determine which portion of the γG3 molecule was responsible for this unusual property of aggregation, enzymatic fragments as well as H and L chains were studied. Fab and $(Fab')_2$ from these proteins failed to enter the acid–acrylamide gels while similar fragments from control γG1 proteins entered normally. Studies in acid-urea gels did not distinguish the two groups, again indicating the noncovalent nature of the forces of aggregation. Fc fragments from γG3 proteins entered the acid gels in a normal position. Analytical ultracentrifugation of A. L. $(Fab')_2$ is depicted in Fig. 6. Like several γG3 whole proteins, aggregation was maximal around pH 6.8. Fc fragments from γG3 proteins did not aggregate in the analytical ultracentrifuge.

Reduction and alkylation of the isolated proteins and controls revealed that on acid-urea gels both H and L chains entered the gels. On acid gels after reduction and

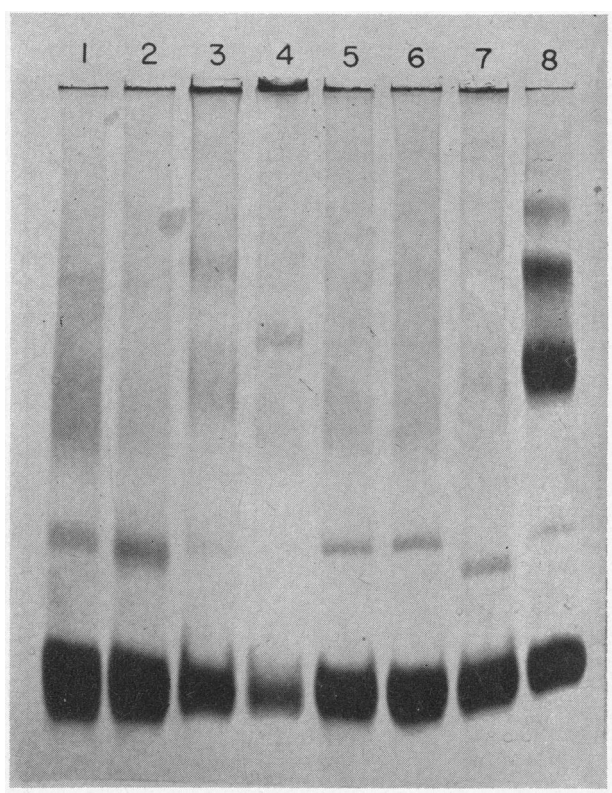

FIGURE 4 Acid–acrylamide-gel electrophoresis of seven γG3 myeloma sera (1–7) and a γG1 myeloma serum (slot 8). None of the γG3 proteins are visualized because of failure to enter the gel.

alkylation the L chains of all γG3 proteins entered normally but the H chains aggregated at the origin. All control H chain preparations from proteins of γG1, 2, and 4 subgroups entered the acid gels under these conditions.

While these results strongly implicated the Fd portion of the H chain as the aggregating unit, studies were done with recombined and reoxidized molecules for confirmation. Hybrid molecules were formed between G. J. H. chains labeled with ^{131}I and E. T. light-chains labeled with ^{125}I. Also, G. J. light-chains labeled with ^{131}I were recombined with E. T. H chain labeled with ^{125}I. As seen in Fig. 7 (left) when the H chain in the hybrid molecule arose from the γG3 protein, the entire recombined molecule would not enter the acid gel. However, when the H chain arose from a γG1 protein, the entire hybrid entered the gel normally. On acid-urea gels the two hybrids could not be distinguished from each other as both entered normally. Finally on re-reduction only the H chains from the γG3 protein accumulated at the origin. Thus the light chains seem to play no role in the aggregation phenomena whereas the H chain and specifically the Fd portion seems to be implicated.

Tests for anti-γ-globulin activity. Whole sera as well as the isolated paraproteins were tested for rheumatoid factor activity by the Rh agglutination test and the latex fixation test. Serum A. L. had a titer of 1000 in the Rh agglutination test but the isolated γG3 protein was devoid of rheumatoid factor activity. Pevikon block electrophoresis separated the rheumatoid factor in serum A. L. from the myeloma protein. All other sera in this study as well as the isolated proteins from most were

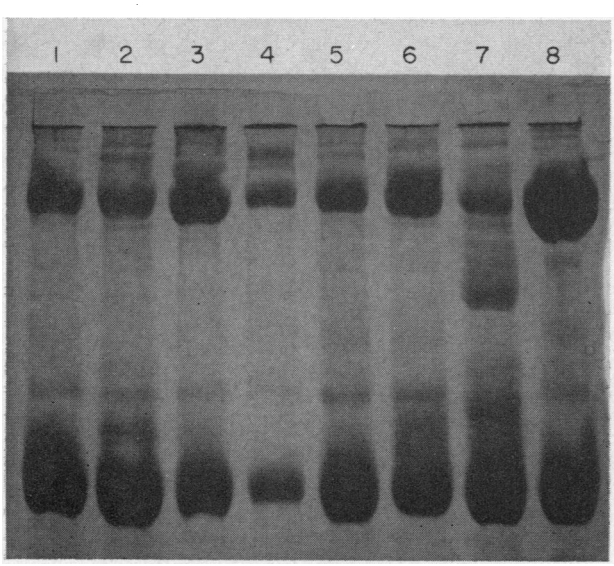

FIGURE 5 Same experiment as Fig. 4 except the gel was made 8 M in urea. The myeloma components are now apparent for the γG3 proteins.

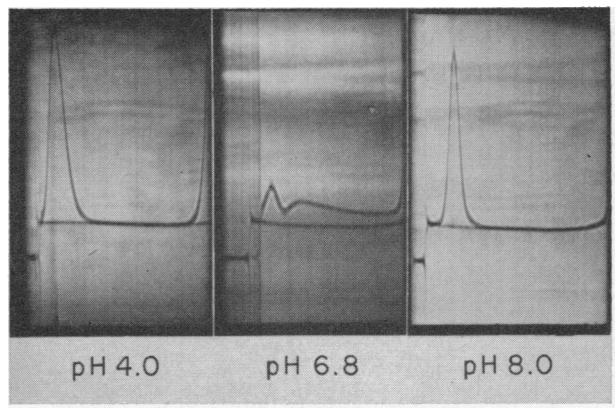

FIGURE 6 Effect of pH on aggregation of the pepsin $(Fab')_2$ fragment of the γG3 protein A. L.

devoid of rheumatoid factor activity both at 4°C and 20°C. In extensive experiments with the analytical ultracentrifuge neither pepsin $(Fab')_2$ nor papain Fab fragments prepared from three of the γG3 proteins could be demonstrated to combine with either autologous Fc or Fc prepared from pooled γ-globulin.

Viscosity studies. During the early stages of these studies two of the patients with γG3 myeloma proteins developed the hyperviscosity syndrome. This initiated viscosity studies of their proteins, other γG3 myeloma proteins, and myeloma proteins of other subgroups. The initial serum relative viscosity in the two cases was 14.6 and 10.0 at 22°C. The concentration-dependent aggregation of these proteins was presented earlier in the analytical ultracentrifuge studies (Fig. 1, G. J. and A. L. as well as P. W. without hyperviscosity syndrome).

The pronounced effect of temperature on the relative viscosity of γG3 proteins is shown in Table I. All sera with γG3 proteins demonstrated an increased relative viscosity at lower temperatures. This is evident whether the hyperviscosity syndrome is present or not. No patient with γG1 myeloma or with macroglobulinemia demonstrated a significant increase in relative viscosity at lower temperatures. In γG3 myeloma hyperviscosity becomes significant at much lower concentrations of myeloma protein. Thus with only 6.2 g/100 ml of γG3 protein patient G. J. had a relative viscosity at 22°C of 14.6. Patient M. K. with γG1 myeloma with 19.0 g of γG1 protein had a serum relative viscosity of only 6.0 at 22°C. This distinction between the effect of protein concentration on relative viscosity in γG3 vs. γG1 myeloma is shown again in Fig. 8. The markedly different slopes indicate that per unit of concentration a γG3 protein results in a far more viscous serum than a γG1 protein.

Each serum studied in Table I was diluted with equal volumes of PBS and the relative viscosity determined. Table II depicts some of the results. The relative viscosity of NHS and the sera of patients with multiple

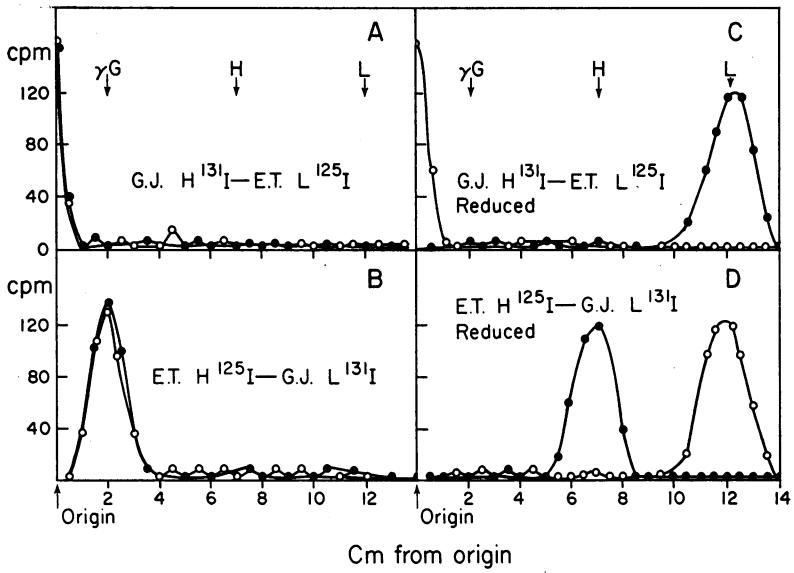

FIGURE 7 Acid–acrylamide-gel electrophoresis of radioiodinated recombined and reoxidized molecules. 7 A shows that when the γ-chain of the hybrid molecule comes from the γG3 protein, the molecule does not enter the gel. 7 B indicates that when the γ-chain of the hybrid molecule comes from the γG1 protein, the molecule enters the gel normally. 7 C and 7 D show that on re-reduction only the γ-chain from the γG1 protein enters the gels. For further details see text.

myeloma of the γG1, two or four subgroups without hyperviscosity syndrome, was reduced less than 10% by dilution. The sera of patients with Waldenström's macroglobulinemia and hyperviscosity syndrome was reduced less than 20% and those from patients with γG1 myeloma and hyperviscosity syndrome was reduced approximately 50%. In contrast, the sera from patients with γG3 myeloma and hyperviscosity syndrome was reduced 75% or greater.

Intrinsic viscosity was determined on some of the isolated proteins and was found to be 4.1 cc/g for G. J. and 4.6 cc/g for A. L. Thus at infinite dilution these proteins have intrinsic viscosity values which do not differ significantly from pooled gamma globulin.

Plasma viscosity determinations at low shear rates (Table III) showed no shear dependence. Thus the viscosity in centipoises did not change over a 1000-fold change in shear rate for any plasma. The clear temperature dependence is again noted for the γG3 proteins but not for the γG1 control. These studies were performed on plasma obtained via plasmapheresis and were thus diluted. This accounts for the lower relative viscosity values.

DISCUSSION

The initial observations of concentration-dependent aggregation of γG3 proteins were made on analytical ultracentrifugation of whole serum. The large shelf of complexes arising from the 7S peak and in a continuum to the cell bottom suggested an unstable associating-dissociating system. The convective disturbances seen in Fig. 1 probably represent density inversions due to slow interconversion of monomers into polymers and vice versa. The sawtooth spikes are indicative of convective disturbances which lead to extremely sharp concentration gradients (28). The effect of pressure on aggregation phenomena are well described (29). At maximum angular rotation, pressures of over 250 atmospheres occur in ultracentrifuge cells (30) so the experiments at low speeds were designed to minimize the effect of pressure on the aggregation phenomena. The particular method of purifying the isolated myeloma proteins was designed to reduce all factors which might lead to aggregation thereby allowing the effect of concentration at the cell bottom to be the major determinant in the molecular weight measurement. The value of 305,000 for G. J represents a weight average molecular weight not of the original sample, but instead, is the apparent molecular weight of the material present at the lower meniscus at a given instant (21). Thus the demonstration of an apparent molecular weight of over 300,000 provides strong evidence that the aggregation phenomena is concentration and not pressure dependent.

Aggregation of γG3 proteins was also documented by their failure to penetrate acrylamide gels in which proteins with molecular weights less than a million will

TABLE I

Comparative Results of Protein and Viscosity Studies on the Serum of Patients with γG3, γG1, and Macroglobulin Peaks with and without the Hyperviscosity Syndrome

Patient	Type	Hyperviscosity syndrome	Total protein	Peak	Serum relative viscosity		
					4°C	22°C	37°C
			g/100 ml	*g/100 ml*			
Normal	—	−	7.1	—	1.5	1.4	1.4
G. J.	γG3 lambda	+	11.2	6.2	37.0	14.6	5.8
A. L.	γG3 lambda	+	11.0	5.8	16.4	10.0	4.0
W. P.	γG3 kappa	+	10.5	4.2	10.2	6.4	3.1
P. W.	γG3 kappa	−	10.0	3.6	7.7	4.2	2.2
C. V.	γG3 lambda	−	9.6	2.8	3.3	2.0	1.4
L. A.	γG3 kappa	−	7.4	2.0	2.0	1.7	1.4
M. K.	γG1 kappa	+	25.0	18.0	5.2	5.1	5.0
D. P.	γG1 kappa	+	24.1	17.2	4.9	4.9	4.8
C. F.	γG1 kappa	+	22.2	15.0	4.7	4.5	4.4
N. B.	γG1 kappa	−	19.0	12.0	3.6	3.5	3.5
R. B.	γG1 kappa	−	14.4	9.5	2.8	2.8	2.7
A. G.	γG1 lambda	−	12.6	7.5	2.2	2.1	2.1
E. T.	γG1 kappa	−	12.0	6.2	1.7	1.6	1.6
S. N.	γG1 kappa	−	11.9	6.2	1.6	1.5	1.5
A. G.	γM lambda	+	12.6	6.2	6.8	6.5	6.4
B. D.	γM kappa	+	12.0	7.0	8.2	8.2	8.1
S. W.	γM kappa	+	11.4	5.8	5.8	5.6	5.5

readily enter. The so-called "stacking" effect of the gel at the point of entry apparently produces a high concentration of the γG3 proteins to effect their aggregation.

Studies on enzymatic fragments and reduction products localized the aggregating portion of the molecule to Fab and H chain. This strongly suggests that the γG3 Fd fragment is responsible for this phenomenon. There

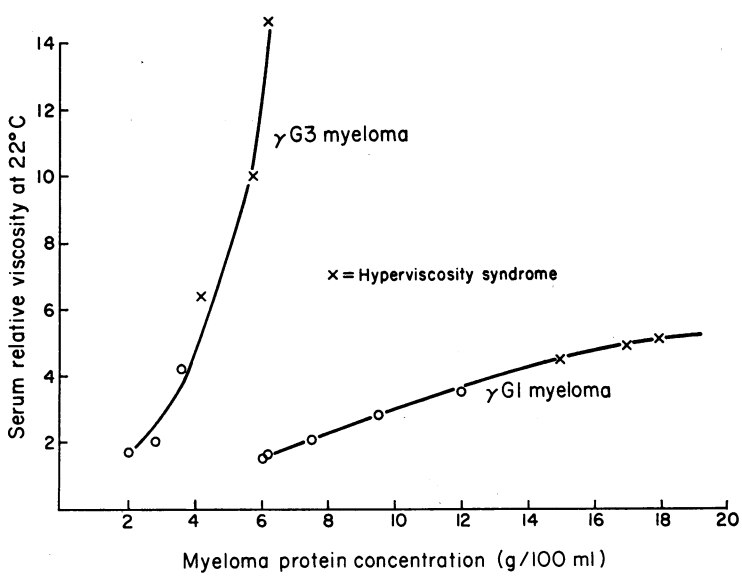

FIGURE 8 A graphic representation of some of the data in Table I. γG1 myelomas fall on a line with a much lower slope than γG3 proteins. The points represent the individual patients.

TABLE II

Dilution Test for Concentration-Dependent Aggregation

Patient	Relative viscosity at 20°C of whole serum	Relative viscosity at 20°C of serum diluted with PBS	Reduction
NHS*	1.51	1.40	7%
Multiple myeloma without hyperviscosity syndrome‡	2.06	1.90	8%
Waldenström's macroglobulinemia with hyperviscosity syndrome§	6.60	5.40	18%
M. K.	6.01	3.12	52%
C. F.	5.07	2.61	51%
G. J.	13.00	2.81	88%
A. L.	8.04	2.02	75%
W. P.	7.52	1.88	75%

* Average of five normals.

‡ Sera from 30 patients, γG1, γG2, and γG4 multiple myeloma.

§ Sera from three patients.

is a considerable body of data which suggests that subclass-specific antigens reside on both the Fc and the Fd portion of the H chain (16, 31). This is especially true in the γG3 subgroup where the Fd antigens are particularly potent. It is likely that these same areas of the molecule render it more susceptible to aggregating potential. It may be that a string of strongly hydrophobic residues mutually attract and lead to aggregation. Frangione and Milstein (32) report that the γG3 subgroup is unique among the γG globulins having five inter-heavy-chain disulphide bonds. One and perhaps two of these is in the Fd region. The role the additional half-cysteines play in this aggregation phenomena is not clear at the present time.

Recent studies on the catabolism of human myeloma proteins (4, 5) show that those of the γG3 subclass are catabolized much faster than any other subgroup. Spiegelberg and Fishkin (4) also report that this difference is not accounted for by more recent studies on the Fc fragment. It is reasonable to assume on the basis of the present study that the different catabolic rate may be related to in vivo aggregation and subsequent destruction, which would be dependent on the Fd fragment.

The hyperviscosity syndrome is a constellation of signs and symptoms including tinnitus, mucous membrane bleeding, stupor, stroke, convulsions, blurred vision, dilated retinal veins, gastrointestinal hemorrhage, and congestive heart failure (33). Clinically the most common disease associated with this syndrome is Waldenström's macroglobulinemia (9). In multiple myeloma, however, the hyperviscosity syndrome is rare.

Bjørneboe and Jensen (13) described a patient with hyperviscosity syndrome and γG multiple myeloma in which measurement of colloid osmotic pressure was normal and the plasma volume was only slightly increased. They postulated aggregation of the paraprо-

TABLE III

Plasma Viscosity at Low Shear Rates

Temperature	Shear rate	Viscosity in centipoises			Relative viscosity			
		G. J.	A. L.	E. T.	Water	G. J.	A. L.	E. T.
°C	sec^{-1}							
37	52	2.47	2.98	1.82	0.6947	3.56	4.29	2.62
	5.2	2.47	2.98	1.82				
	0.52	2.47	2.98	1.82				
	0.104	2.47	2.98	1.82				
	0.052	2.47	2.98	1.92				
20	52	5.13	5.04	2.61	1.0050	5.10	5.01	2.60
	5.2	5.13	5.04	2.61				
	0.52	5.13	5.04	2.61				
	0.104	5.13	5.04	2.61				
	0.052	5.13	5.04	2.61				
4	52	13.4	11.2	3.92	1.5674	8.55	7.15	2.51
	5.2	13.4	11.2	3.92				
	0.52	13.4	11.2	3.92				
	0.104	13.4	11.2	3.92				
	0.052	13.4	11.2	3.92				

tein as a mechanism to explain these findings but were unable to demonstrate aggregation despite analytical ultracentrifugation studies. However, since they diluted the serum about 10-fold before centrifugation, concentration-dependent aggregation would not have been detected. Kopp, Beirne, and Burns (14) described a similar patient with γG myeloma and hyperviscosity syndrome with a serum relative viscosity of 6.0 and again analytical ultracentrifugation did not demonstrate aggregate formation. However, the centrifuge photograph shown suggests that the sera had been diluted 5–10 times and concentration-dependent aggregates as described herein would not have been observed. They measured relative viscosity at 37°C and found it to be 4.8 while at 24°C it was 7.0. This increase in relative viscosity from 37°C to 24°C suggests that they were probably confronted with the same phenomenon reported here, i.e., concentration- and temperature-dependent aggregation. MacKenzie and Fudenberg (15) reported on two patients with hyperviscosity syndrome secondary to γG myeloma in which no aggregates could be documented by analytical ultracentrifugation. The intrinsic viscosity of the isolated proteins was determined and it was normal in one patient while in the other it was reported to be twice normal. They concluded that hyperviscosity syndrome in γG myeloma was due to the primary structure of the individual proteins, not their potential for aggregation. In view of the data presented in this report it is likely that in some of the above patients concentration- and temperature-dependent aggregation of γG3 proteins lead to the hyperviscosity syndrome.

Two potentially useful simple tests for unstable aggregating γG myelomas producing hyperviscosity syndrome are the serum relative viscosity determination at two different temperatures and the serum relative viscosity on whole serum and serum diluted 1:1. The temperature-viscosity index (TVI) was introduced by Waldenström (34). TVI = (100 × rel. visc. 13°C)/(rel. visc. 37°). Values greater than 120 were considered suggestive of macroglobulinemia. Somer (35) in his extensive study of viscosity in myeloma and macroglobulinemia reexamined this test. He found that of 16 cases of γG myeloma only three had TVI 120 and in only two was the serum relative viscosity at 20°C 5.0. Only 5 of 15 sera from patients with Waldenström's macroglobulinemia had TVI values above 120. He concluded that the TVI was of somewhat limited usefulness clinically except in cases of cryoglobulinemia. It should be emphasized that in the present study cryoglobulins were not present in any of these sera. The temperature viscosity index can only be extrapolated from the present study as measurements were not done at 13°C. However, it is clear that none of the three patients with Waldenström's macroglobulinemia who served as controls had a TVI > 120 (Table I). In addition, patients with multiple myeloma without hyperviscosity syndrome had a TVI within the normal range. However, the marked effect of temperature on viscosity is evident in the two patients with hyperviscosity syndrome with γG3 multiple myeloma, for A. L., 276 and for G. J., 460. These values are higher than any value obtained by Somer (35) in his study of sera from 15 patients with macroglobulinemia, 7 with γA myeloma and 16 with γG myeloma. These studies would therefore suggest that viscosity determination at two different temperatures can be useful to distinguish this form of hyperviscosity syndrome from that caused by Waldenström's macroglobulinemia when analytical ultracentrifugation is not readily available.

Another clinically useful test utilizing the Ostwald viscometer is the dilution test. Steel (36) introduced the "interpolated 7% viscosity" based on a fixed protein concentration. He noted that by diluting the serum with elevated viscosity to a protein content of 7 g/100 ml the relative viscosity of myeloma sera is reduced to the normal range but in cases of macroglobulinemia the "7% viscosity" remains abnormal. The value of the test as reported here, however, is that protein concentration need not be measured as shown in Table II. A simple 1:1 dilution of the serum reduces the serum relative viscosity of these concentration-dependent aggregating myelomas to close to the normal range, while the relative viscosity of the sera of patients with Waldenström's macroglobulinemia is much less affected.

Capillary tube viscometers in general measure viscosity at relatively high shear rates. Measurements of flow velocity in post capillary venules suggest that the shear rate in this portion of the microcirculation is low (37). It was therefore desirable to study the effect of low shear rates on viscosity in this unique group of patients. The results (Table III) indicate that there is no shear dependence and suggest Newtonian behavior of the plasma. However, it has long been known that whole blood is non-Newtonian (38, 39) and that there is a tendency for the non-Newtonian behavior to increase as the viscosity level rises (27). Thus it appears that for maximum information whole blood instead of the diluted citrated plasma will have to be studied at low shear rates and such studies are now underway.

The γG heavy-chain subgroups of myeloma proteins associated with the hyperviscosity syndrome shows a disproportionate number belonging to the γG3 subgroup. Three in the present report are γG3 proteins; however, the two patients of MacKenzie and Fudenberg (15) were of the γG1 subgroup but a third patient provided to us by these authors was a γG3 protein. Studies of the sera of patients with hyperviscosity syn-

drome with γG1 myeloma indicate that: (*a*) the myeloma protein concentration in each case was 15 g or over (see Table I); (*b*) no aggregates could be documented by analytical ultracentrifugation; (*c*) all entered the acid-acrylamide gel; and (*d*) none demonstrated a significant increase in relative viscosity at lower temperatures. Although these studies are somewhat preliminary, it would appear that the phenomenon of hyperviscosity syndrome in γG1 myeloma seems more fundamentally related to extreme levels of myeloma protein in the circulation.

The exact proportion of hyperviscosity syndrome cases that are due to γG3 proteins remains uncertain because of the limited number of cases available. A total of nine cases of γG myeloma with hyperviscosity syndrome have passed through the authors' laboratory including those described above; five of the nine proved to involve γG3 proteins. Since only 7–10% of myeloma proteins belong to the γG3 subgroup, this high proportion appears impressive. It seems probable that the concentration- and temperature-dependent aggregation described for these proteins played a significant role in the usual incidence. The evidence obtained in the present study indicates that the hyperviscosity syndrome develops when γG3 proteins reach a concentration of approximately 4 g/100 ml. Other γG globulins require considerably higher concentrations before hyperviscosity develops.

The finding of multiple myeloma with a γG3 protein should alert the clinician to the possibility of hyperviscosity playing a role in the symptomatology at some point in the course of the disease particularly if the protein peak is above 4 g/100 ml. In addition, whenever γG3 myeloma is diagnosed, reduction of the level of myeloma protein should be a definitive goal of therapy.

The preponderance of the γG3 subgroup in this disorder recalls the group of γG cryoglobulins with anti-γ-globulin activity recently reported by Grey, Kohler, Terry, and Franklin (7). Of the five proteins in their series with anti-γG activity, four were γG3 and one was γG1. However, the proteins recorded in this present study were not cryoglobulins and lacked anti-γ-globulin activity by several criteria. Thus the mechanism of aggregation of the two groups of proteins appears to be fundamentally different.

It appears, therefore, that there are at least three mechanisms whereby the hyperviscosity syndrome may develop in γG myeloma. (*a*) The proteins may form relatively stable aggregates of 13–15S as in the cases of Smith et al. (12, 40); (*b*) extreme levels of myeloma proteins as our cases of γG1 myeloma with hyperviscosity syndrome; and (*c*) unstable complex formation of γG3 proteins.

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