STUDIES ON STRUCTURAL UNITS OF THE γ-GLOBULINS

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In studying the molecular structure of the γ-globulins, one is confronted at the outset with the problem of whether these proteins consist of one or of several polypeptide chains. The solution of this problem has significance in determining the chemical basis of antibody specificity, and in formulating a detailed theory of antibody production. In addition, it bears upon the relation between normal γ-globulins and those of disease.

A previous report (1) showed that normal human 7S γ-globulin and a pathological macroglobulin dissociated to components of lower molecular weight when treated with reagents that cleave disulfide bonds. The present study is concerned with an extension of this approach to a variety of normal and abnormal γ-globulins. Partial separation of the dissociation products of γ-globulin has been achieved by means of column chromatography and starch gel electrophoresis. The results suggest that γ-globulin molecules are composed of several discrete subunits or polypeptide chains linked by disulfide bonds.

Materials and Methods

γ-Globulins and Other Proteins.—Lyophilized human fraction II γ-globulin was obtained from Lederle Laboratories. This material was free of 19S γ-globulin, although it contained small amounts (2 to 3 per cent) of aggregated material with sedimentation coefficients between 7 and 19S. Fresh human γ-globulin was isolated from the serum of four individuals by zone electrophoresis on starch (2); rabbit γ-globulin was prepared in the same way from the pooled serum of 3 rabbits. Macroglobulin was isolated by precipitation following water dilution of serum from a patient with macroglobulinemia of Waldenström and was further purified by starch zone electrophoresis. Myeloma globulins isolated by starch zone electrophoresis were obtained from Dr. H. Fudenberg and Dr. H. G. Kunkel. Highly purified native and reduced carboxymethylated ribonuclease samples were provided by Dr. A. M. Crestfield and Dr. W. H. Stein. Protein concentrations were determined by a modification of the Folin-Ciocalteu method (3).

Salts, Buffers, and Reagents.—All chemicals used were of reagent grade or better. Urea was further purified by treatment with amberlite MB-1 ion exchange resin as proposed by Benesch *et al.* (4). Guanidine·HCl was recrystallized from hot methanol by the method of Kolthoff *et al.* (5). β-Mercaptoethylamine·HCl[1] was obtained from Evans Chemetics, Inc., New York, and recrystallized from ethanol. β-mercaptoethanol (Matheson, Coleman and Bell, East Rutherford, New Jersey) was redistilled and stored at 4°C. Iodoacetamide (K & K Labora-

[1] MEA, β-mercaptoethylamine·HCl.

tories) was recrystallized from water. Tris(hydroxymethyl)aminomethane buffer[2] was made from "sigma 121" primary acidometric standard reagent.

Chemical Treatment of γ-Globulins.—A number of procedures were used to reduce γ-globulin. 1. For most of the ultracentrifugal studies, lyophilized γ-globulin was mixed with a small amount of reagent solution (usually 0.1 M MEA and 6 M urea) to make a paste. This was then made up to volume in 5 ml. volumetric flasks to protein concentrations of 3 to 5 mg./ml. After standing for varying periods of time at room temperature, certain samples were dialyzed against large volumes of 0.02 M iodoacetamide in 6 M urea at 4°C. for 12 to 16 hours. In some cases the solutions were buffered, and all samples prepared for ultracentrifugal analysis were made 0.1 to 0.2 M in KCl or NaCl to minimize charge effects.

2. When larger amounts of γ-globulin (up to 200 mg.) were reduced, the sample was dissolved in 5 ml. of 0.1 M mercaptoethanol in 6 M urea, and placed in a 23/32 Visking casing. This was surrounded by 100 ml. of 0.1 M mercaptoethanol in 6 M urea and stirred for 12 hours at room temperature. A 20-fold molar excess of iodoacetamide was added to the inner solution and after 10 to 20 minutes at room temperature the solution was dialyzed at 4°C. against large volumes of 6 M urea or water. In the latter case, the products were lyophilized.

3. The reduction procedure employed to prepare samples for column chromatography and starch gel electrophoresis was as follows: 100 mg. of γ-globulin was added to 5 ml. of a solution that was 6 M in urea and 0.1 M in mercaptoethanol brought to pH 8.0 by addition of small amounts of 1 M tris solution. After 4 to 5 hours at room temperature, 2 gm. of iodoacetamide in 10 ml. of 6 M urea were added and the pH was kept at 8.0 by addition of 1 M tris solution. After 10 to 20 minutes at room temperature, the samples were dialyzed at 4°C. against large volumes of water and then lyophilized. In some experiments, 0.15 M NaCl was substituted for 6 M urea in this procedure.

4. When only small amounts of protein were available, the following procedure was used to prepare samples for starch gel electrophoresis: 1 per cent solutions of protein were prepared in 0.1 M borate buffer pH 8.5, 0.1 M borate buffer in 8 M urea, and 0.1 M borate buffer in 8 M urea + 0.02 M mercaptoethanol, and were allowed to stand at room temperature for times varying from 15 minutes to 2 hours. Iodoacetamide was added to a final concentration of 0.05 M to some of the solutions that contained the reducing agent. The solutions were subjected simultaneously to starch gel electrophoresis as described below. Tris buffer 0.1 M, pH 8.5, and barbital buffer 0.1 M, pH 8.5, were also used in this procedure with equivalent results.

In addition to the above procedures, reaction of 700 mg. of human fraction II γ-globulin with sulfite (6) in urea was carried out using the method of Pechère *et al.* (7), without modification. Performic acid was used to oxidize γ-globulin at room temperature. To 100 mg. of fraction II human γ-globulin in 9.0 ml. of 88 per cent formic acid, 1.0 ml. of 30 per cent H_2O_2 was added. After 30 minutes at room temperature the reaction was stopped by adding 200 ml. of H_2O as described by Hirs (8), and the sample was lyophilized.

Chemical Analyses.—Sulfhydryl groups were measured using the amperometric titration method of Benesch *et al.* (4). Titrations were generally done in 8 M urea to make "masked" SH groups more reactive. The electrode response was sometimes sluggish even in the presence of urea. In such cases, an estimated slight excess of $AgNO_3$ was added to the protein sample in urea and it was placed in the dark for 30 minutes. The titration was then completed in the usual fashion. From 0.3 to 1.0 micromole of protein was used for each titration. All titrations were standardized by measuring the SH content of a weighed standard of reduced glutathione. To obtain an approximate measure of the number of disulfide bonds split after reduction of certain samples by MEA, the procedure of Markus and Karush (9) was used to remove the reducing agent.

[2] Tris, tris(hydroxymethyl)aminomethane buffer.

Amino acid analyses were done using ion exchange chromatography as described by Moore *et al.* (10) and Spackman *et al.* (11). Qualitative N-terminal amino acid analyses were performed following the procedures of Levy (12) and Fraenkel-Conrat *et al.* (13) except that dinitrophenylation was performed in solutions containing 6 M urea as well as in aqueous solutions. Carbohydrate determinations were done by the methods employed by Müller-Eberhard and Kunkel (14).

Ultracentrifugation.—A Spinco model E ultracentrifuge equipped with phase plate schlieren optics was used. Most runs were done using double sector 12 mm. cells; for double runs, one cell was fitted with a wedge quartz window. Plate measurements were made according to procedures described by Trautman (15). Molecular weights were determined in strong urea solutions by the method of Trautman and Crampton (16). This method yields a reduced apparent molecular weight, M_{app} $(1-\bar{V}\rho)$ as the slope of a least squares line;[3] the molecular weight average obtained initially emphasizes the heavier components of a polydisperse solution (17). Weight average molecular weights of a few samples were determined by equilibrium ultracentrifugation in short columns.

Density measurements were made at 20°C. using calibrated 3.0 ml. pycnometers. To correct the sedimentation coefficients for viscosity of urea, values from the International Critical Tables were used, interpolating when necessary. All ultracentrifugal patterns are reproduced here with the direction of the field proceeding from left to right.

Chromatography.—Chromatography of reduced γ-globulin was done on 25 × 0.9 cm. columns of carboxymethyl cellulose (Schleicher and Schuell, 0.71 equivalents per gm.) in 6 M urea solutions at 4°C. Gradient elution was accomplished by running 1.0 M sodium acetate buffer in 6 M urea (apparent pH 6.0 at 25°C.) into a mixing chamber of 500 ml. volume initially containing 0.01 M sodium acetate in 6 M urea (apparent pH 6.0 at 25°C). Buffers were prepared in the manner described by Cole (18). The amount of sample applied to the column varied from 50 to 100 mg. in the chromatographic experiments.

Starch Gel Electrophoresis.—Vertical starch gel electrophoresis according to Smithies (19) was performed in an essentially identical apparatus except that platinum electrodes were substituted for the Ag/AgCl electrodes. Gels were prepared from commercially available starch (starch-hydrolysed, Connaught Medical Research Laboratories, Toronto, Canada) in formate buffer (0.05 M formic acid and 0.01 M NaOH), pH 2.9–3.0, prepared in 8 M urea (20, 21). The electrode vessels contained formate buffer of higher concentration (0.2 M formic acid and 0.08 M NaOH). A voltage gradient of 6V/cm. was applied and electrophoresis was conducted at room temperature for 16 to 18 hours. After completion of the experiment, the electrophoretic tray was placed in the refrigerator for 15 to 30 minutes to facilitate the subsequent slicing of the gel. Both halves of the sliced gel were stained for 3 to 5 minutes with amido black 10B and were decolorized (22) using 10 per cent acetic acid. The gels were rendered rigid for both photography and storage by placing them in a methanol:water:acetic acid (50:50:10) bath after the decolorizing procedure.

Two-dimensional zone electrophoresis (23, 24) in gels prepared in 8 M urea was employed to exclude the possibility that the bands in one dimension represented metastable polymers or aggregates. After separation in a vertical gel, a 3 mm. wide slice encompassing all the bands was transferred to the origin of an identically prepared gel run horizontally. The efficacy of starch gel electrophoresis in urea was also tested using highly purified ribonuclease and reduced carboxymethylated ribonuclease. Both samples migrated as single bands in the gel. Further corroboration of the efficacy of this method has been obtained by Dixon and Wardlaw (25) who used it to separate the chains of insulin.

[3] M_{app} = the apparent molecular weight of the solute.
$\bar{V}$ = the partial specific volume of the solute.
ρ = the density of the solvent.

RESULTS

The Ultracentrifugal Behavior of Reduced γ-Globulin

Reduced alkylated γ-globulin was found to be very insoluble in aqueous solvents except at extremes of the pH scale. Ultracentrifugal patterns of the material that did dissolve usually showed extensive aggregation. Solutions of ionic strength greater than 0.2 showed Tyndall effects, and in some cases visible precipitates appeared as the ionic strength was raised. A search for effective solvents revealed that the reduced alkylated γ-globulin was soluble

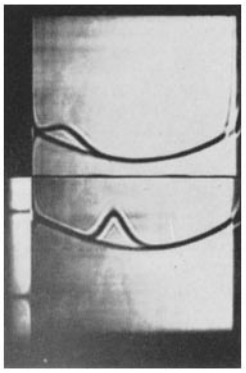


FIG. 1. Simultaneous sedimentation velocity experiments to compare reduced and alkylated human fraction II γ-globulin (upper frame) with untreated fraction II γ-globulin (lower frame). Solvent: 6 M urea, 0.2 KCl. Speed: 52,640 R.P.M. Time of photograph: 192 minutes. Phase plate angle: 60°.

in strong urea solutions (6 M or greater), in guanidine·HCl solutions (3 M or greater), in 0.5 to 1 per cent sodium dodecyl sulfate solutions, and in 50 per cent acetic acid. Urea solutions appeared to promote disaggregation effectively and for this reason they were chosen for most of the experiments.

A direct comparison in the ultracentrifuge of untreated fraction II human γ-globulin, and of γ-globulin reduced in the presence of 6 M urea and treated with iodoacetamide, is shown in Fig. 1. This photograph represents a double sedimentation velocity experiment with the menisci of the two cells in identical positions. The uncorrected sedimentation coefficient of the untreated material was 2.7S, and that of the reduced material was 1.1S. After correction for the density and viscosity of the urea, the values were 5.2S and 2.1S respectively. To obtain a more accurate estimate of the ultracentrifugal behavior of reduced alkylated γ-globulin in urea solutions, a sample was run at 5 different concentrations from 15.8 mg./ml. to 1.8 mg./ml. The sedimentation coefficient at infinite dilution corrected for the presence of the urea was 2.3S.

An analysis of the apparent molecular weights of the starting material and

of variously treated human γ-globulin was performed. The values obtained are given in Table I. Corresponding to each value of M_{app} $(1 - \bar{V}\rho)$ is an approximate value of M_{app} calculated on the assumption that $\bar{V} = 0.74$ (26) and using measured values for the solvent densities rather than the calculated values used in a previous report (1). The somewhat high value of 189,000 for the starting material appeared to be correlated with the presence of a small amount of aggregated material in the sample. It was difficult to determine whether the drop in M_{app} for this sample measured in 6 M urea was solely due to dispersion of aggregates. A striking difference was found for γ-globulin reduced in urea, however. In this case, the apparent molecular weight dropped to about ⅓ of the control value for untreated γ-globulin in urea.

TABLE I

*Effect of Various Reagents on M_{app} $(1-\bar{V}\rho)$ Values of Human γ-Globulin**

Solvent	M_{app} $(1 - \bar{V}\rho)$ ± standard deviation of slope	M_{app}‡
0.2 M KCl	$(4.8 \pm 0.1) \times 10^4$	189,000
6 M urea + 0.2 M KCl	$(3.0 \pm 0.3) \times 10^4$	167,000
0.1 M MEA§ + 0.2 M KCl	$(3.5 \pm 0.1) \times 10^4$	189,000
0.1 M MEA + 6 M urea + 0.2 M KCl	$(0.93 \pm 0.07) \times 10^4$	48,000
Reduced in 8 M urea + 0.1 M MEA, next dialyzed against 6 M urea + 0.02 M iodoacetamide, then 6 M urea + 0.2 M KCl	$(0.92 \pm 0.05) \times 10^4$	51,000

* Fraction II of Cohn, Lederle lot C-543. This sample contained a small amount of heavy material sedimenting faster than the main 7S component.

‡ Calculated assuming that $\bar{V} = 0.74$ and using measured values for ρ.

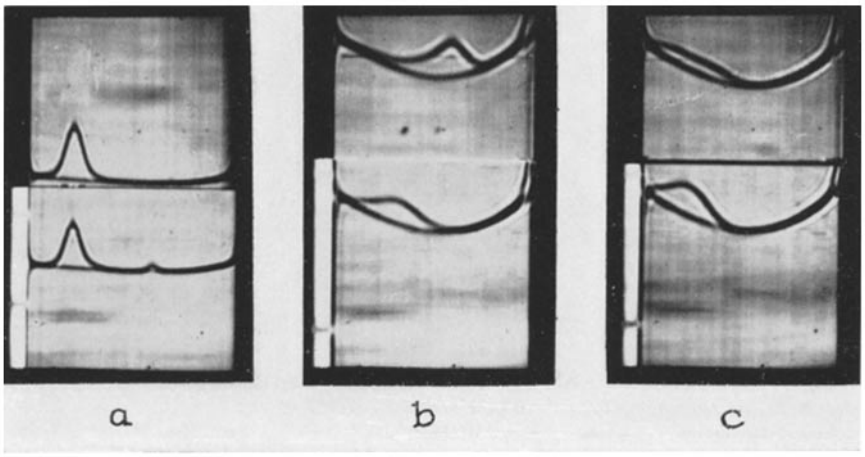
§ MEA, β-mercaptoethylamine·HCl.

No buffer was used in these experiments in order to minimize the complexity of the solvent system. Experiments done on γ-globulin samples in buffered solutions gave results comparable to those shown in Table I. Fraction II human γ-globulin reduced and analyzed in a solvent composed of tris 0.05 M, pH 7.3, 0.2 M NaCl, 6 M urea, and 0.1 M MEA gave a value of $(1.0 \pm 0.03) \times 10^4$ for M_{app} $(1 - \bar{V}\rho)$. The reaction of human fraction II γ-globulin with sulfite in the presence of urea and Cu^{++} ions (6) led to a product that was partially aggregated even in 6 M urea. The smallest component of this polydisperse mixture had a value of 0.81×10^4 for M_{app} $(1 - \bar{V}\rho)$, determined in a solvent composed of 6 M urea, 0.2 M KCl, and tris 0.05 M at pH 8.0.

Because of the general similarity of rabbit and human γ-globulins, their behavior after reduction with sulfhydryl compounds was compared (Fig. 2). The upper frames in Figs. 2 *a*, 2 *b*, and 2 *c* represent rabbit γ-globulin at various stages of treatment, and the lower frames show the human protein at the same

stages. Before reduction, the two proteins were compared at concentrations of 3 mg./ml. in 0.15 M NaCl (Fig. 2 *a*). The small peak in advance of the main 7S component is the normal 19S component which was present in higher concentration in the human γ-globulin sample.

Both samples were reduced under identical conditions in a solvent composed of 6 M urea, 0.1 M MEA, and 0.2 M KCl. After 1 hour at room temperature,



FIGS. 2*a*, 2*b*, and 2*c*. Comparison of electrophoretically isolated human and rabbit γ-globulins before and after reduction.

(*a*) Unreduced γ-globulins. Lower frame: human γ-globulin. Upper frame: rabbit γ-globulin. Solvent: 0.15 M NaCl. Speed 52,640 R.P.M. Time of photograph: 40 minutes. Phase plate angle: 60°.

(*b*) γ-globulins examined after reduction for 1 hr. Lower frame: human γ-globulin. Upper frame: rabbit γ-globulin. Solvent: 6 M urea, 0.1 M MEA, 0.2 M KCl. Speed: 52,640 R.P.M. Time of photograph: 300 minutes. Phase plate angle: 45°.

(*c*) γ-globulins examined after reduction for 24 hours. Lower frame: human γ-globulin. Upper frame: rabbit γ-globulin. Solvent: 6 M urea, 0.1 M MEA, 0.2 M KCl. Speed: 52,640 R.P.M. Time of photograph: 264 minutes. Phase plate angle: 45°.

the samples were again simultaneously analyzed in the ultracentrifuge in the same solvent (Fig. 2 *b*). The reduced human γ-globulin shows a pattern similar to that shown in Fig. 1. In contrast, the peak of rabbit γ-globulin has sedimented further than that of human γ-globulin and it is skewed, showing a tail representing slower sedimenting components. After 24 hours at room temperature, the rabbit γ-globulin solution became slightly turbid. The turbidity was cleared by centrifugation and the supernate was compared with the human γ-globulin solution (Fig. 2 *c*). Little or no change appeared in the pattern of reduced human γ-globulin. The reduced rabbit γ-globulin appeared to consist

of polydisperse material with a sedimentation velocity more or less comparable to that of reduced human γ-globulin.

These results are consistent with dissociation of rabbit γ-globulin proceeding at a slower rate than that of human γ-globulin. In Table II are given the M_{app} values for untreated rabbit γ-globulin and for the reduced protein after 2 and 48 hours. After 48 hours the apparent molecular weight fell to about ¼ of the value obtained at 2 hours.

TABLE II

*The Effect of Duration of Reduction on the Apparent Molecular Weight of Rabbit γ-Globulin**

Solvent and treatment	M_{app} $(1 - \bar{V}\rho)$	M_{app}‡
0.2 M KCl	4.1×10^4	155,000
6 M urea + 0.2 M KCl + 0.1 M MEA§ 2 hrs., room temperature	3.4×10^4	170,000
6 M urea + 0.2 M KCl + 0.1 M MEA 48 hrs., room temperature	$(0.84 \pm 0.02) \times 10^4$	42,000

* Rabbit γ-pseudoglobulin (electrophoretically purified).

‡ M_{app} calculated using value of 0.73 for $\bar{V}$, and measured values for ρ.

§ MEA, β-mercaptoethylamine·HCl.

Separation of the Reduction Products of γ-Globulin

Reduced carboxamidomethylated fraction II human γ-globulin was chromatographed on carboxymethyl cellulose ion exchanger in buffers prepared with 6 M urea. The chromatographic pattern obtained is shown in Fig. 3. The first peak appeared within one column volume and thus the material in this peak did not bind to the ion exchanger. Further resolution of the material in the second peak was not obtained by changing the gradient or the pH. Material from both peaks behaved consistently upon rechromatography, indicating that true separation had taken place. The average yield of material from both peaks was approximately 60 per cent. The weight average molecular weight in urea of the products isolated from the first peak was approximately 17,000. Ultracentrifugal analysis in urea of the material from the second peak yielded a weight average molecular weight of 103,000, and indicated the presence of aggregates.

In an attempt to achieve greater resolution, starch gel electrophoresis in urea at acid pH values was employed. The results obtained for reduced carboxamidomethylated human fraction II γ-globulin are shown in Fig. 4. Unreduced γ-globulin and unreduced γ-globulin treated with iodoacetamide showed a diffuse slow band with a suggestion of an additional minor band. After reduction and carboxamidomethylation, the pattern was entirely altered. At least 4 bands appeared, and a characteristic feature was the presence of 2 main

bands of slightly different mobility. Material from areas *A*, *B*, *C*, and *D* of the chromatogram shown in Fig. 3 was compared on the same gel. Pooled material from the first peak (marked *A*) appeared to be identifiable with the fastest band of reduced alkylated γ-globulin in the starch gel. The approximate yield of this peak on chromatography was 10 per cent. Succeeding cuts from the second peak of the chromatogram (*B*, *C*, and *D*) showed less and less of this fast band, but all other components were present. None of the bands appeared to be metastable as tested by 2-dimensional electrophoresis.

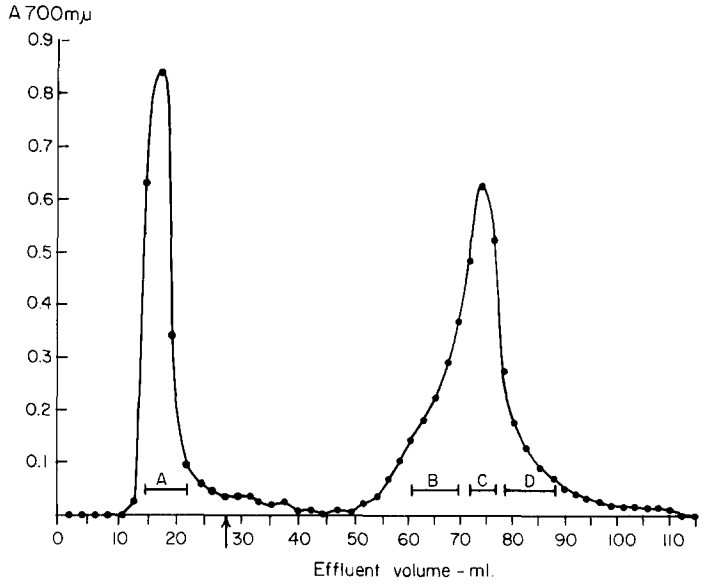


FIG. 3. Chromatogram on carboxymethyl cellulose of human fraction II γ-globulin reduced and alkylated by procedure 3. Column load: 100 mg. of treated γ-globulin. $A_{700m\mu}$ optical density of Folin reaction at 700 mμ. *A*, *B*, *C*, and *D* indicate fractions pooled for further analysis. Arrow on abscissa indicates start of gradient.

Partial reduction of γ-globulin with mercaptoethanol in the absence of urea followed by carboxamidomethylation resulted in a product that was soluble in aqueous solutions. Although the molecular weight of this material in aqueous solution was similar to that of untreated γ-globulin, the weight average molecular weight in 6 M urea was approximately 135,000, suggesting partial dissociation in this solvent. After chromatography on carboxymethyl cellulose in 6 M urea, a pattern similar to that of more completely reduced γ-globulin emerged. On the starch gel in urea, however, the pattern was somewhat different than that obtained for the more completely reduced γ-globulin. There were fewer components (Fig. 5) and the mobilities of the bands appeared to be greater.

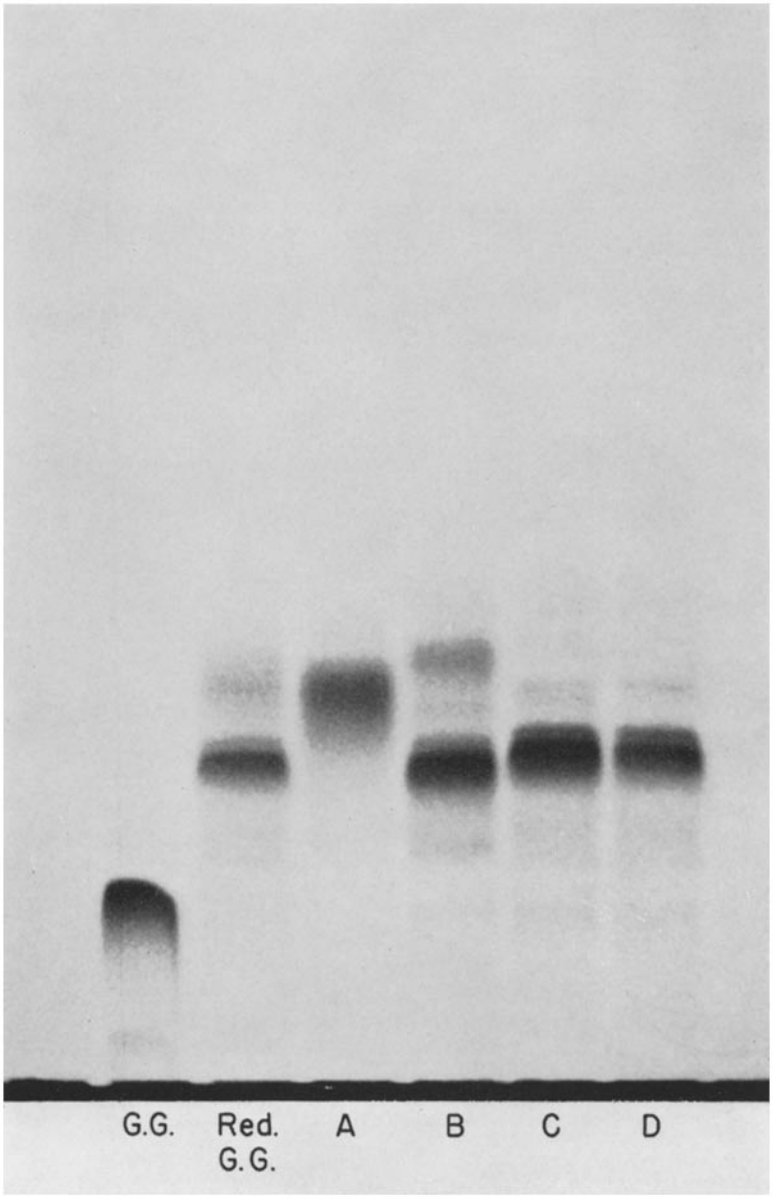


FIG. 4. Comparison of untreated human fraction II γ-globulin with reduced alkylated γ-globulin before and after chromatography. G.G. = untreated γ-globulin. Red. G.G. = γ-globulin treated following procedure 3. *A*, *B*, *C*, *D*,: successive fractions from chromatogram in Fig. 3.

Again, material from the first peak of the chromatogram was identifiable with the fastest component, and material from the second peak showed less of this component.

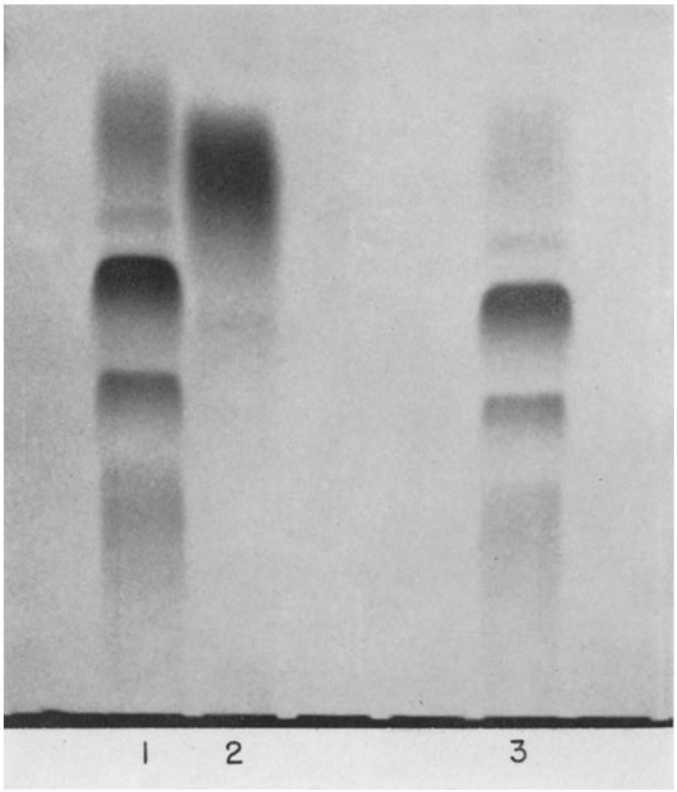


FIG. 5. Starch gel electrophoresis in urea-formate buffer of human fraction II γ globulin reduced in the absence of urea (procedure 3).

1. Pattern of material prior to chromatography.
2. Pattern of material from first peak obtained by chromatography on carboxymethyl cellulose.
3. Pattern of material from second peak of same chromatogram.

Reduced alkylated rabbit γ-globulin was compared on the starch gel with similarly treated human γ-globulin. Although chromatography of reduced rabbit γ-globulin produced two peaks, as was the case with human γ-globulin, notable differences were found on starch gel electrophoresis (see Fig. 6 *b*). The main difference appeared in the fast moving band which was more prominent in human γ-globulin treated by procedure 4. In addition, a distinct band migrating more slowly than the 2 main bands was seen in the treated human

γ-globulin. This band appeared to have no counterpart in the similarly treated rabbit γ-globulin. It is possible that these differences resulted from differences in rates of dissociation of these proteins.

In view of the relative homogeneity of the myeloma globulins, a comparison was made of their behavior after reduction, using starch gel electrophoresis in urea.[4] Two myeloma proteins that were reduced and alkylated by method 4 are compared with similarly treated fraction II human γ-globulin in Fig. 6 *a*. A striking difference in the number and position of the bands is apparent, although before reduction the broad diffuse patterns of these proteins were quite similar. Both reduced myeloma proteins had at least one band of mobility similar to one of the bands of reduced normal γ-globulin, yet each reduced myeloma protein had one or more bands differing from those of reduced normal γ-globulin. It is notable that the patterns obtained for the myeloma proteins differed from each other. This apparent uniqueness in the starch gel patterns of reduced alkylated myeloma proteins is clearly seen in Fig. 6 *b*. Notwithstanding the differences in their over-all patterns, the reduced myeloma proteins appeared to possess certain bands with mobilities in common.

Characterization of Reduction Products of γ-Globulin

Titration of Sulfhydryl Groups in Native and Reduced γ-Globulin.—Two methods were used to demonstrate that reduction of γ-globulin by thiol compounds was taking place. Free sulfhydryl groups were titrated amperometrically at a rotating platinum electrode after removal of reducing agent (MEA) on a Dowex 50 ion exchange resin under anaerobic conditions. In some samples, the extent of reduction was determined by estimating the conversion of cystinyl and cysteinyl groups to S-carboxymethylcysteinyl groups using ion exchange chromatography.

Before titrating the SH groups appearing after reduction, an estimate was made of the titratable SH groups of unreduced human γ-globulin (Table III). Between 0.4 and 0.9 gm. atoms of silver were bound per mole of 7S γ-globulin (assumed molecular weight = 160,000). Neither 7S γ-globulin nor pathological macroglobulin showed titratable SH groups in aqueous solutions. A measurement of the macroglobulin sample made at a different time than that shown in Table III yielded a value of 1.9 gm. atoms of silver bound by a mole of protein assuming a molecular weight of 1 million. This value, and the value shown in Table III, are of the same order of magnitude as that obtained by Caputo and Appella (27) for a different macroglobulin sample.

Estimation of the number of disulfide bonds cleaved by reduction in urea of 7S γ-globulin for short periods of time yielded values ranging from 7 to 9

[4] Preliminary ultracentrifugal studies indicated that the myeloma globulins were dissociated to components of lower molecular weight after reduction and alkylation.

disulfide bonds per mole (Table III). That not all bonds were cleaved in these instances was shown by the determination of the S-carboxymethylcysteine content of γ-globulin reduced by the bulk dialysis procedure (procedure 2). This appeared to be the most efficient of all procedures employed, yielding a total of 12 bonds cleaved per mole of protein.

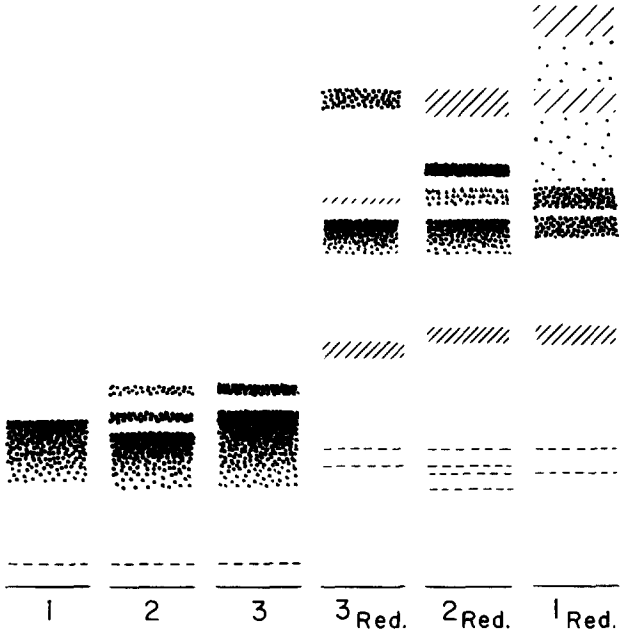


FIG. 6 *a*. Diagram of starch gel, comparing human fraction II γ-globulin and two myeloma globulins before and after reduction by procedure 4.

1. Human fraction II γ-globulin.
2. Myeloma globulin.
3. Myeloma globulin.

$3_{Red.}$ Reduced and alkylated myeloma globulin 3.

$2_{Red.}$ Reduced and alkylated myeloma globulin 2.

$1_{Red.}$ Reduced and alkylated human fraction II γ-globulin.

Macroglobulins are known to dissociate to 7S fragments after treatment with reducing agents in the absence of urea (28). A concurrent drop in the molecular weight from approximately 1 million to about 160,000 has been established. Because the products of this treatment are quite soluble in aqueous solutions, the approximate number of SH groups released could be determined with less difficulty than in the case of 7S γ-globulins. After reduction of a pathological macroglobulin with MEA alone, the reagent was removed anaerobically on Dowex 50 ion exchange resin. One aliquot of the protein in the effluent was

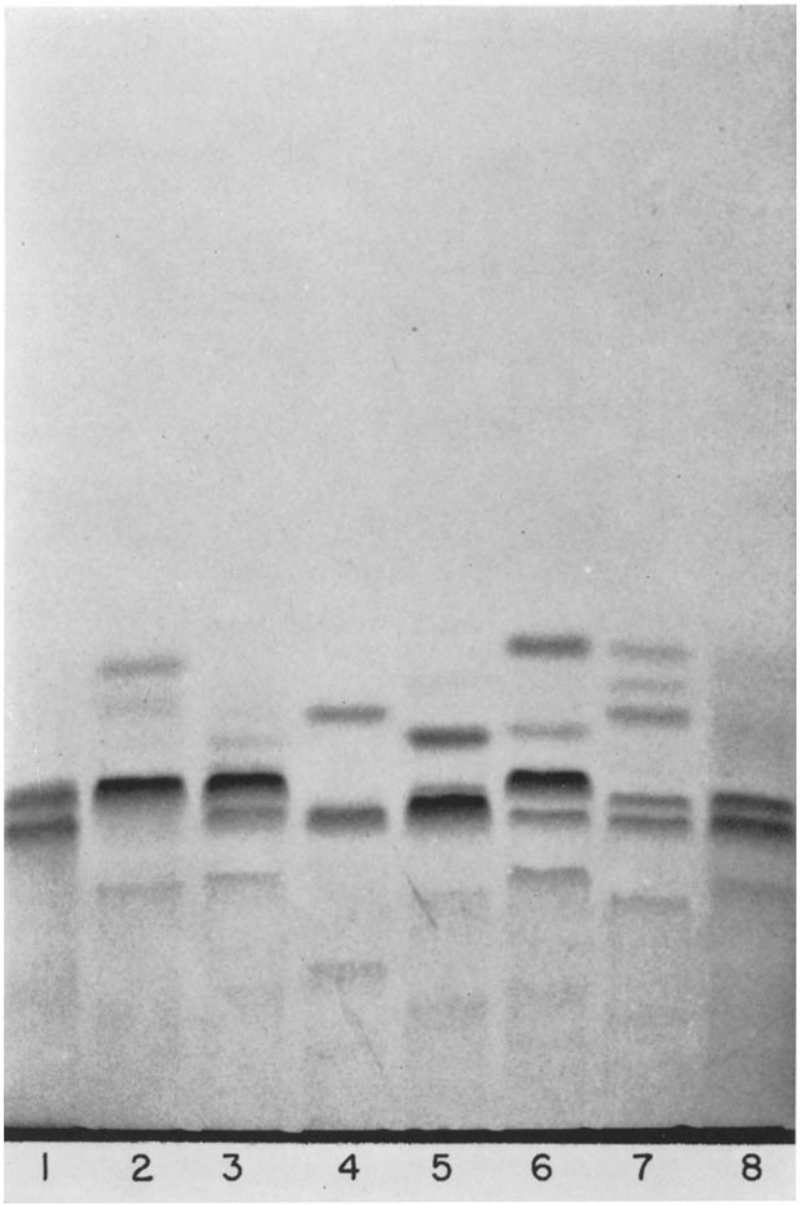


FIG. 6 *b*. Comparison of normal γ-globulins and human myeloma globulins after reduction and alkylation by procedure 4. (Starch gel electrophoresis in urea-formate buffer)

1. Rabbit γ-globulin.
2–7. Individual myeloma globulins.
8. Human fraction II γ-globulin.

titrated immediately; another was treated with iodoacetamide and then dialyzed against 0.15 M NaCl for ultracentrifugal analysis.

In Fig. 7 are shown patterns of untreated macroglobulin and of the iodoacetamide treated product from the effluent of the Dowex 50 column. The untreated material possessed a characteristic macroglobulin pattern: one major 19S peak and two minor peaks with higher sedimentation velocities (Fig. 7 *a*). Dissociation to 7S units following reduction was over 90 per cent complete

TABLE III

Titratable Sulfhydryl Groups of Unreduced and Reduced Human γ-Globulins

Sample	Procedure*	Moles SH / Mole protein‡
Fraction II γ-pseudoglobulin	Excess titration procedure in 8 M urea	0.9§
Fraction II γ-globulin	Titrated immediately in 8 M urea	0.5
Fraction II γ-globulin	Reduced in 8 M urea + 0.1 M MEA 1.5 hrs. Titrated immediately in 8 M urea	14.6
Electrophoretically isolated γ-globulin	Excess titration procedure in 8 M urea	0.4
Electrophoretically isolated γ-globulin	Reduced in 6 M urea + 0.1 M MEA 30 minutes under N_2. Titrated immediately in 8 M urea	18.5
Pathological macroglobulin	Excess titration procedure in 8 M urea	1.2
Pathological macroglobulin	Reduced in tris 0.1 M pH 7.4 + 0.1 M MEA 1 hr. under N_2.	29.2
Pathological macroglobulin	Reduced in 8 M urea + 0.1 M MEA 1 hr. under N_2	86.1

* All titrations and reductions done at 19–20°C.

‡ Molecular weight of all samples except macroglobulin taken to be 160,000. Macroglobulin assumed to have a molecular weight of 1 million.

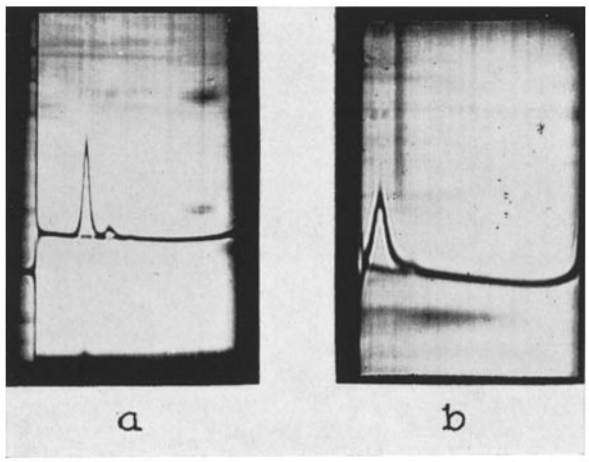
§ Average of 3 separate determinations.

(Fig. 7 *b*). Concurrent with this dissociation there appeared 29.2 SH groups per mole of protein (Table III). In several other experiments dissociation was less complete or reaggregation was greater than in the case illustrated. All these products showed an SH titer proportional to the amount of 7S dissociation product and lower than 29.2.

An attempt was made to dissociate macroglobulin to even smaller units by reduction in the presence of urea. When this was done, a dissociation product with an apparent molecular weight of 41,000 was obtained. Concurrently 86 SH groups appeared per mole of pathological macroglobulin (Table III).

Amino Acid Analysis of the Reduction Products of Human γ-Globulin.—Amino acid analyses were done on fraction II γ-globulin and on the reduction products obtained by the bulk dialysis procedure (procedure 2). Any significant

losses of small peptides could be detected by this procedure. To accomplish this, the outer solution of 6 M urea and 0.1 M mercaptoethanol was passed over a 6 × 1 cm. column of IRC 50 ion exchange resin in the acid form. After washing the column exhaustively with water, 50 per cent acetic acid was passed over the column and the eluate was lyophilized. About 5 mg. of recoverable material was obtained by this procedure when 100 mg. of γ-globulin was reduced within the dialysis sac. Less than 5 per cent of this material was accounted for as amino acids, and the distribution of these amino acids was



FIGS. 7 *a* and 7 *b*. Ultracentrifugal patterns of a pathological macroglobulin before and after treatment with thiol compound.

(*a*) Untreated macroglobulin. Solvent: 0.15 M NaCl. Speed: 52,640 R.P.M. Time of photograph: 16 minutes. Phase plate angle: 60°.

(*b*) Macroglobulin after treatment with 0.1 M MEA, passage over Dowex 50 column, and alkylation with iodoacetamide. Solvent: 0.15 M NaCl. Speed: 52,640 R.P.M. Time of photograph: 16 minutes. Phase plate angle: 45°.

identical with that found for the reduced protein inside the dialysis sac. Thus, no appreciable amount of dialyzable small peptides were discerned by this method.

No striking differences were found in the amino acid composition of native and reduced protein. The S-carboxymethylcysteine content of the reduced, alkylated human γ-globulin was 2.86 gm./100 gm. of protein corresponding to 24 half-cystine residues per mole of protein. Half-cystine was not detected, suggesting either that both reduction and coverage of free SH groups were complete, or that any residual half-cystine was destroyed in the hydrolysis. The iodoacetamide reacted with methionine and lysine, as indicated by the presence of S-carboxymethylhomocysteine and carboxymethyllysine. These

products resulting from the reaction of iodoacetic acid with proteins have been described (29).

A comparison of the amino acid content of materials from the first and second peaks of chromatographically separated reduced and alkylated γ-globulin is given in Table IV. The results have been referred to an alanine content of 1.00 in each case since the products contained different residual

TABLE IV

*Relative Amino Acid Content of Chromatographically Separated Components of Reduced Human γ-Globulin**

Amino acid	Reduced alkylated γ-globulin‡	Material from first peak§	Material from second peak‖
Lysine	1.29	0.75	1.76
Histidine	0.28	0.13	0.43
Arginine	0.77	0.46	0.81
Aspartic acid	1.47	1.08	1.76
S-carboxymethylcysteine	0.38	0.28	0.41
Threonine	1.50	1.21	1.67
Serine	2.16	1.93	2.38
Glutamic acid	1.83	1.59	1.99
Proline	1.42	0.92	1.71
Glycine	1.32	1.03	1.67
Alanine	1.00	1.00	1.00
Half-cystine	0.12	0.10	0.27
Valine	1.64	1.06	2.18
Methionine	0.08	0.03	0.12
Isoleucine	0.41	0.38	0.41
Leucine	1.39	1.07	1.61
Tyrosine	0.78	0.61	0.84
Phenylalanine	0.63	0.45	0.70

* All values calculated relative to alanine = 1.00.

‡ C-583 fraction II γ-globulin reduced by procedure 3.

§ Area *A* in Fig. 3.

‖ Area *C* in Fig. 3.

amounts of urea. Material from the first peak which behaved as a single broad band on starch gel electrophoresis had an amino acid composition that was markedly different from both that of the starting material and material from the second peak.

The Effect of Reduction on the Carbohydrate Moiety of Human γ-Globulin

Reduction of γ-globulin conceivably might remove part or all of the carbohydrate portion of the molecule. To test this possibility, the fraction II human γ-globulin and the material recovered from the solutions inside and outside

the dialysis sac in the bulk dialysis procedure (procedure 2) were examined for carbohydrate.

Unreduced γ-globulin contained 1.4 per cent hexose, 0.8 per cent hexosamine, 0.17 per cent fucose, and 0.2 per cent sialic acid. The reduced γ-globulin from the inside of the dialysis sac showed hexosamine, fucose, and sialic acid contents similar to those of unreduced γ-globulin. Since the reduced carboxamidomethylated protein developed interfering color with the anthrone reagent, the hexose content could not be estimated accurately. On the other hand, the hexose content of the material from the solution outside the sac was 1.5 per cent by weight. Assuming that all of this material was derived from γ-globulin, this would represent less than 0.15 per cent of the total hexose of the starting material, since the yield of "outer" material was less than 10 per cent. No evidence, therefore, of appreciable loss of carbohydrate was detected after reduction of human 7S γ-globulin.

Attempts to Dissociate γ-Globulin by Chemical Means Other Than Reduction

Although the decrease in apparent molecular weight of 7S γ-globulin and macroglobulin was accompanied by an increase in titratable SH groups, it was not definitely established that disulfide bonds linked the fragments together. Other linkages that might be involved include thioester bonds, sugar-protein linkages of various types, and peptide bonds. A number of chemical procedures were used to survey these possibilities.

After oxidation of fraction II human γ-globulin with performic acid, the product was subjected to Archibald analysis in citrate buffer, pH 10.9. The apparent molecular weight was 32,000. Treatment of human 7S globulin with 0.2 and 0.5 M hydroxylamine·HCl adjusted to pH 8.0 with NaOH, both in 6 M urea and aqueous solutions, failed to bring about any change in the sedimentation coefficient. An active preparation of pneumococcal neuraminidase had no effect on the ultracentrifugal properties of 7S and 19S human γ-globulin when incubated with these proteins for varying periods of time.

An unlikely possibility is that reduction in 6 M urea by mercaptan at neutral pH might lead to rupture of sensitive peptide bonds. To check for the possible appearance of new N-terminal amino acids, qualitative N-terminal amino acid analyses were done on fraction II human γ-globulin and on the reduced S-carboxamidomethylated derivative. No DNP-amino acids other than DNP-aspartic acid, DNP-glutamic acid, and a trace of DNP-serine were detected in either type of sample.

Finally, attempts to activate any plasminogen that might contaminate fraction II γ-globulin failed. Chloroform and streptokinase in aqueous and in 6 M urea solutions had no discernible effect on the molecular weight of human γ-globulin. It seems improbable that a proteolytic enzyme would retain activity in the presence of both urea and mercaptan.

DISCUSSION

Previous approaches to the problem of the multiplicity of chains in γ-globulin molecules have relied largely upon the results of end group determinations. The data obtained for γ-globulins of different animal species are difficult to reconcile. Human 7S γ-globulin has been found to contain as many as 3 moles of N-terminal amino acids per mole of protein (30, 31) consistent with the presence of at least this number of chains in the molecule. Similar studies with rabbit γ-globulin have led to the proposal that this protein is a single long polypeptide chain (32, 33). Because of the possibility that γ-globulin may contain masked or blocked N-terminal groups not detectable by end group methods (34), other approaches were explored in the present work.

Chemical cleavage of disulfide bonds, followed by molecular weight analyses and separation of the products indicated that γ-globulins are composed of discrete subunits. Separation of these structural units was facilitated by the use of urea solutions which appeared to prevent aggregation of the cleavage products. Thus, ultracentrifugation, chromatography, and starch gel electrophoresis were all done in strong urea solutions.

The choice of solvents containing urea affects certain parameters which must be considered in interpreting the ultracentrifugal data. A decrease in the value of M_{app} $(1 - \bar{V}\rho)$ after chemical treatment may be due to an increase in partial specific volume, to an increase in the activity correction term (16), to preferential binding of one of the components of the complex solvent (35), or to an actual decrease in the molecular weight of the solute. Estimates of the order of magnitude of each of these parameters have been made (36). It appears that the apparent molecular weights of γ-globulins in urea solutions do not deviate greatly from those obtained in aqueous solutions.

The evidence suggests that the average molecular weight of reduced γ-globulin is ⅓ to ¼ that of the unreduced protein. In the case of the pathological macroglobulin, the molecular weight of the derivative resulting from reduction in urea was about $\frac{1}{20}$ that of the 19S protein (1). It is notable that the size of these derivatives is in the same range as that of Bence-Jones proteins (37). The dissociation of γ-globulin by reduction in the presence of denaturing agents has recently been confirmed by Phelps *et al.* (38) for human 7S γ-globulin, by Franěk (39) for 7S γ-globulins of various animal species, and by Jirgensons *et al.* for a pathological macroglobulin (40).

An exact determination of the types and number of bonds holding the various subunits together will require more extensive chemical studies. From the data presented here, the disulfide bond emerges as the most likely possibility. Dissociation of γ-globulin by reduction in urea was accompanied by the appearance of titratable SH groups, and oxidation of the protein caused a drop in molecular weight. The failure of hydroxylamine to cause dissociation suggests

that thioester linkages are not involved. In most cases, reduction of the disulfide bonds was incomplete. Dissociation occurred, despite this fact, suggesting that only certain disulfide bonds serve to link the subunits. In the case of the pathological macroglobulin tested here, for example, an average of no more than 2 to 3 bonds would link each 7S subunit to the next. Weak forces may also help to link the subunits. This seems to be suggested by the recent finding of Deutsch and Greenwood (41), that the 7S units of reduced alkylated macroglobulin dissociate reversibly to smaller subunits when treated with low ionic strength buffer at acid pH. As noted above, human fraction II γ-globulin that had been treated with mercaptoethanol and alkylated in the absence of urea was partially dissociated when placed in this solvent. Thus, weak forces may also be involved in linking subunits of 7S γ-globulin.

The possibility that carbohydrate-protein bonds play a role in linking the subunits remains to be explored. It is probable, however, that the carbohydrate moiety is a prosthetic group with no linkage function (42). The present findings suggest that little or no carbohydrate is removed by reduction of human 7S γ-globulin. Similar results have recently been obtained for a reduced carboxymethylated pathological macroglobulin (40).

Peptide bonds do not seem to be involved in holding the subunits together. Despite the fact that increased amounts of DNP-aspartic or DNP-glutamic acid would not have been detected by the qualitative methods used here, it is significant that no new N-terminal amino acids appeared after reduction. The subunits are probably separate polypeptide chains, an idea in accord with the results of the N-terminal amino acid analyses of human γ-globulin and myeloma proteins (30, 31, 43). Presumably, the N-terminal amino acids of certain subunits are masked; this may account for the results of the N-terminal amino acid analyses of rabbit, equine, and bovine γ-globulins (31, 32, 34). The exact number of polypeptide chains possessed by each type of γ-globulin cannot be determined from the present evidence, although an estimate of 3 to 5 chains per 7S γ-globulin molecule is consistent with the data.

Separation of the products of reduced alkylated γ-globulins on carboxymethyl cellulose in urea produced two fractions. The first of these had an amino acid composition distinctly different from that of either the starting material or the second fraction. When reduced, or reduced carboxamidomethylated human γ-globulin was separated in urea-formate buffer by starch gel electrophoresis, a pattern of at least 4 well defined bands emerged. The fastest moving band was identifiable with material from the first peak of the chromatography experiments. The second peak contained all the other bands but only small amounts of the fastest band. Control preparations of untreated γ-globulin as well as of γ-globulin which was carboxamidomethylated without having been reduced, showed only a very diffuse slow band in the gel. Immunoelectrophoretic and Ouchterlony plate analyses have revealed that material from each peak in the

chromatogram of γ-globulin reduced in the absence of urea gave precipitin lines with several specific anti-γ-globulin sera (44). This finding indicates that these chromatographic components were derived from γ-globulin and not from contaminants in the starting material. The immunologic relationships among these components are being investigated.

Myeloma proteins are members of the γ-globulin family that are distinguished by their abnormality, their individuality, and their extreme homogeneity by a number of chemical tests (37). Starch gel electrophoresis of several reduced myeloma globulins appeared to reflect the individuality of each of these proteins. Comparison of the patterns of the various reduced myeloma proteins nevertheless revealed similarities in the mobilities of certain bands. Furthermore, the mobilities of some of the slower bands of reduced myeloma proteins corresponded with those of similarly treated normal γ-globulin. A striking and consistent finding was the sharpness and distinctness of the fast moving bands, as contrasted with their diffuse counterparts in the patterns of reduced normal γ-globulin. More detailed studies of the myeloma globulins will be reported elsewhere.

Porter (45) has recently demonstrated that rabbit γ-globulin is cleaved by papain treatment into three fragments which together form over 90 per cent of the original molecule. These fragments have molecular weights ranging from 50,000 to 80,000. Similar fragments have been found for papain-treated human γ-globulins (46). The fragments obtained by treatment with papain do not seem to be identifiable with the subunits described above. It is likely, however, that the products of papain treatment are composed of portions of these subunits. This interpretation is strengthened by the finding that the two active antibody fragments obtained by treatment with proteolytic enzymes are linked by a single disulfide bond (47).

A unifying hypothesis may be formulated for the structure of proteins in the γ-globulin family based on the findings presented above as well as on findings of other investigators. 7S γ-globulin molecules appear to consist of several polypeptide chains linked by disulfide bonds. Bivalent antibodies may contain two chains that are similar or identical in structure. The 19S γ-globulins would be composed of 5 or 6 multichain units of the size of 7S γ-globulin. A provisional explanation for the wide molecular weight range of antigenically related globulins from Bence-Jones proteins to macroglobulins is suggested by this model. Heterogeneity and differences in isoantigenicity (48, 49) may arise from various combinations of different chains as well as from differences in the sequence of amino acids within each type of chain.

The finding that γ-globulin contains dissociable subunits has a possible bearing upon the pathogenesis of diseases of γ-globulin production. A primary defect in macroglobulinemia and multiple myeloma may be a failure of specificity and control in production and linkage of the various subunits to form

larger molecules. Bence-Jones proteins may be polypeptide chains that have not been incorporated into the myeloma globulins because of a failure in the linkage process. Myeloma globulins may consist of combinations of subunits differing from those of the normal γ-globulins, although both types of protein appear to contain subunits that are alike. This may explain in part the antigenic and chemical differences and similarities that have been found between the γ-globulins of disease and normal γ-globulin (36, 37). The hypothesis outlined above is capable of experimental test, since the products of various chemical treatments may now be separated and compared.

SUMMARY

When human and rabbit 7S γ-globulins were reduced in strong urea solutions by a number of procedures, their molecular weights fell to approximately $\frac{1}{3}$ of the original values. Partial separation of the reduction products was achieved using chromatography and starch gel electrophoresis in urea solutions. One of the components of reduced human 7S γ-globulin was isolated by chromatography, identified by starch gel electrophoresis, and subjected to amino acid analyses. The amino acid composition of this component differed from that of the starting material and also from that of the remaining components.

A reduced pathological macroglobulin dissociated to components with an average molecular weight of 41,000. Several reduced human myeloma proteins, when subjected to starch gel electrophoresis, yielded individual patterns that nevertheless had features in common with those of reduced normal γ-globulins. Reduction of normal and abnormal γ-globulins was accompanied by the appearance of titratable sulfhydryl groups. Chemical treatments other than reduction were used to determine the type of bond holding the subunits together. It was tentatively concluded that they were linked by disulfide bonds. An hypothesis is presented to relate the structural features of the various γ-globulins in terms of the multiplicity of polypeptide chains in these molecules.

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