RECONSTITUTION OF 7S MOLECULES FROM L AND H POLYPEPTIDE CHAINS OF ANTIBODIES AND γ-GLOBULINS*

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7S γ-globulin molecules contain two types of polypeptide chains linked by disulfide bonds and non-covalent interactions: L (light) chains having molecular weights of approximately 20,000 and H (heavy) chains having molecular weights of 55,000 to 60,000 (1–8). Several studies have shown that the starch gel electrophoretic behavior of L chains is associated with differences in antibody specificity (9, 10), that interactions of L and H chains are necessary for maximal antibody activity (11, 12), and that portions of both L and H chains (13–15) are situated at or near the antibody combining site. The available evidence suggests that the antibody molecule contains two L and two H chains (4, 8, 16), probably arranged as one L-H pair for each combining region (11).

Reconstitution of activity by L-H interaction indicates that the dissociation of antibodies into their polypeptide chains is in some degree reversible. In previously reported experiments (11), however, no direct information was given on the physicochemical nature of the reconstituted product, or on its resemblance to the intact and native antibody molecule. The present communication presents evidence that L and H chains form a reconstituted molecule similar to 7S γ-globulin in many of its properties.

Materials and Methods

γ-Globulins.—Human 7S γ-globulin was obtained as lyophilized Cohn fraction II (lot C-780) from Lederle Laboratories (Pearl River, New York). Rabbit and guinea pig γ-globulins were separated from pools of fresh sera by starch zone electrophoresis (17). Specifically purified guinea pig antibodies to f1 and f2 bacteriophage and to the dinitrophenyl group were isolated from individual sera as previously described (11, 9). Protein concentrations were determined by measurements of absorbancy at 280 mμ. $E_1^{1\%}_{cm}$ was assumed to be 14.0. When necessary, protein fractions were concentrated by ultrafiltration through collodion bags (Schleicher and Schuell, Keene, New Hampshire).

Reduction of γ-Globulins and Separation of L and H Chain Fractions.—Reduction in neutral buffers followed the methods of Edelman and Poulik (2). 0.02 M phosphate buffers pH 7.0 or, 0.05 M tris(hydroxymethyl)aminomethane (tris) buffers pH 7.2, were made 0.1 N in 2-mercaptoethanol. γ-Globulins labeled with radioactive iodine were reduced in 0.15 N NaCl made 0.2 M in borate buffer, pH 8.0, and 0.1 N in mercaptoethanol. Reduction proceeded for 3 hours at room temperature and was stopped when required by addition of iodoacetamide to a

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final concentration of 0.2 M. After 10 minutes at room temperature the solution was dialyzed against 0.5 N propionic acid at 4°C. For separation of the chains, 100 x 1.0 cm columns of sephadex G-100 in 0.5 N propionic acid (5) were used. From 20 to 50 mg of protein was loaded on the columns. Resolution was improved by using flow rates of less than 6 ml per hour and by collecting fractions of 1.0 ml or less.

The method of starch gel electrophoresis in 8 M urea and formate buffer has been described (2). To determine the distribution of protein labeled with radioactive iodine, the gel was sliced and one half was stained and photographed. The unstained half was sectioned serially in 5 mm segments along the axis of migration and the segments counted by methods described below.

Reconstitution and Reoxidation of Separated L and H Chains.—Fractions of L and H chains were mixed in 0.5 N propionic acid, usually at the protein concentrations obtained following gel filtration. The relative proportions of H chains to L chains in the mixtures were determined as ratios of their absorbancies at 280 mμ and varied from 0.5/1 to 6/1 in different experiments. The ratio of absorbancies at 280 mμ was interpreted as a mass ratio, assuming similar values of $E_{1\text{ cm}}^{1\text{ per cent}}$ for H and L chains. The mixtures and the individual chain fractions were dialyzed for 48 hours at 4°C against 0.02 M phosphate buffer pH 7.2 made 0.15 N in NaCl, or against 0.05 M tris buffer pH 7.2 made 0.15 N in NaCl.

Reduced, but undissociated, γ-globulins were reoxidized at 4°C by prolonged dialysis of the reduction mixture against several changes of distilled water, followed by several changes of 0.02 M phosphate buffer pH 7.0, made 0.15 N in NaCl. To reoxidize reconstituted mixtures of previously separated chains, the chains were separated from reduced γ-globulin, mixed in 0.5 N propionic acid, and dialyzed at 4°C against 0.05 M tris buffer pH 7.2 made 0.1 N in 2-mercaptoethanol. The mixtures were then diluted 10-fold (to protein concentrations of 0.1 per cent or less) with the same solvent, and dialyzed against several changes of 0.05 M tris buffer pH 7.2 in the absence of mercaptan.

Alkylation and Determination of Carboxymethylcysteine Content.—6 mg samples of unreduced γ-globulin, γ-globulin reduced and alkylated as described above, and γ-globulin reduced and reoxidized as described above were dissolved in 2 ml of 0.5 M Na_2HPO_4, pH 7.8. 10 mg of iodoacetamide was added to each sample and after 2 hours at room temperature, the samples were dialyzed against distilled water at 4°C, and lyophilized. They were then dissolved in 1 ml of 6 M HCl, and after oxygen was removed by repeated freeze-thawing under high vacuum (18), they were hydrolyzed for 48 hours at 110°C. Each hydrolyzate was analyzed for carboxymethylcysteine, using the automatic amino acid analyzer (18, 19).

Labeling with Radioactive Iodine and Measurement of Radioactivity.—I^{125} sodium iodide (Volk Radiochemical Company, Chicago) and I^{131} sodium iodide (Oak Ridge National Laboratory, Oak Ridge, Tennessee) were carrier-free solutions stabilized with sodium thiosulfate. Iodination followed the procedure of McFarlane (20) except that the protein was dissolved in 0.15 M NaCl brought to pH 8.0 with 0.2 M borate buffer as suggested by Helmkamp *et al.* (21). Unreacted iodide was removed by passage of the protein solutions over 6 x 1.0 cm columns of IRA-401 amberlite ion exchange resin (Mallinckrodt Chemical Works, St. Louis) equilibrated with the borate buffer. The extent of labeling averaged less than 1 atom of iodine per molecule of γ-globulin; assayed under the conditions described below, the preparations had specific activities of 10^6 to 10^7 CPM per unit absorbancy at 280 mμ.

The labeled γ-globulins were concentrated by ultrafiltration, reduced in borate buffer, and separated into L and H chains as described above. The specific activities of L chain fractions were generally 25 per cent lower than those of H chain fractions. Samples were also labeled after partial reduction with similar results, except that L and H chains had the same specific activities. Separated isotopically labeled H and L chain fractions are assigned subscripts to designate the particular isotope used (*e.g.* $H_{I^{125}}$ refers to H chains labeled with I^{125} and $H_{I^{125}}L_{I^{131}}$ designates a mixture of the appropriately labeled chains).

The radioactivity was assayed in a dual channel well-type scintillation counter (Nuclear-Chicago, Des Plaines, Illinois). The data were corrected for background, natural decay, and interference of I^{125} CPM by I^{131}. The data are presented as per cent of total recovered counts per minute or as the equivalent absorbancy at 280 mμ (assuming that protein samples were dissolved in 1 ml) calculated from the corrected counts per minute and the known specific activities.

Ultracentrifugation in Sucrose Density Gradients.—The procedure followed that of Martin and Ames (22). A linear gradient of sucrose (5 to 20 per cent) in 0.05 M tris buffer, pH 7.2 was employed. Protein samples were concentrated to 0.2 ml by ultrafiltration, layered on the gradients, and centrifuged for 15 hours using the SW-39 swinging bucket rotor and model L ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, California). In some experiments the protein solutions were not concentrated and only 0.1 ml of solution was layered on the gradient, resulting in slightly improved resolution. Fractions containing 0.18 ml were collected by punching a hole in the bottom of the cell with a No. 25 gauge needle. For determination of radioactivity, the fractions were collected directly in counting vials; the bottom of the centrifuge tube, the concentrating sacs, and dialysis sacs were also counted. In studies of the distribution of isotopic labels in reconstituted material, the separate H and L chain fractions and their mixture were layered onto parallel sucrose density gradients and centrifuged simultaneously.

Approximate sedimentation rates were calculated as suggested by Martin and Ames (22). Alkaline phosphatase from *Escherichia coli*, kindly supplied by Dr. James Schwartz, was used as a reference standard; as determined by analytical ultracentrifugation, the sample used had an uncorrected sedimentation coefficient of 6.1S in tris 0.1 M, pH 7.0 at 25°C. The activity of the enzyme was assayed using *p*-nitrophenyl phosphate (NPP, Sigma Chemical Company, St. Louis) as substrate (23, 24). Approximately 10^{-3} mg of purified enzyme was added to each protein sample prior to concentration and density gradient centrifugation. 10 μl of each fraction from the gradient was added to 0.1 ml of 10^{-3} M NPP in 1.0 tris buffer, pH 8.0 distributed in porcelain spot plates. The peak activity was estimated from the depth of the yellow color developed after 10 to 20 minutes at 37°C.

Analytical Ultracentrifugation.—A Spinco model E ultracentrifuge equipped with phase plate schlieren optics and Rayleigh interference optics was used. Sedimentation velocity experiments were done using 12 mm double sector cells; when two cells were used, one was fitted with a wedge quartz window. Sedimentation coefficients were not extrapolated to zero concentration but were corrected to $s_{20,w}$ using η and ρ values obtained from Svedberg (25), and assuming $\bar{v} = 0.73$. Equilibrium ultracentrifugation was performed as described by Yphantis (26).

Gel Filtration.—Parallel columns of sephadex G-200 (Pharmacia, Upsala, Sweden) measuring 50 x 1.0 cm and equilibrated with 0.05 M tris pH 7.2 had flow rates of 3.0 ml per hour. 10^{-3} mg of alkaline phosphatase was added to the protein samples to be loaded on each column, and the ratio of the volumes at which the peak of activity emerged was used to correct for fluctuations in the total volume between any two columns.

Immunologic Methods.—The method of double diffusion in agar has been previously described (13). Antisera to human 7S γ-globulin and antisera to human L chains were used. The sera were obtained from rabbits immunized with antigen in complete Freund's adjuvant.

Assay of Bacteriophage Neutralization.—The methods have been described in a previous publication (11).

Zone Electrophoresis on Cellulose Acetate.—2.0 x 6.5 inch strips of cellulose acetate (Consolidated Laboratories, Chicago Heights, Illinois) were soaked in 0.05 M barbital buffer, pH 8.6, blotted, and suspended in an electrophoresis chamber equilibrated with buffer. Electrophoresis proceeded for 2 hours at a potential gradient of 10 v/cm. The strips were stained for 10 minutes with 0.2 per cent ponceau S in 3 per cent trichloracetic acid, decolorized in

distilled water, dried, sliced into 5 mm segments, and assayed for radioactivity. The ponceau S stain on each strip was eluted and the absorbancy at 535 mμ measured as described by Carpenter and Hayes (27).

Hydrolysis with Papain.—The methods have been described previously (28).

RESULTS

Reversible Cleavage of Interchain Disulfide Bonds in the Absence of a Dissociating Solvent.—Reduction of mammalian 7S γ-globulins in the absence of a dissociating solvent does not cause a drop in molecular weight, even though interchain disulfide bonds are cleaved (2). This implies that these bonds are not essential for stability of the molecule in neutral aqueous solvents, and suggests that the conformation of the chains in the partially reduced molecule might permit reformation of the interchain disulfide bonds.

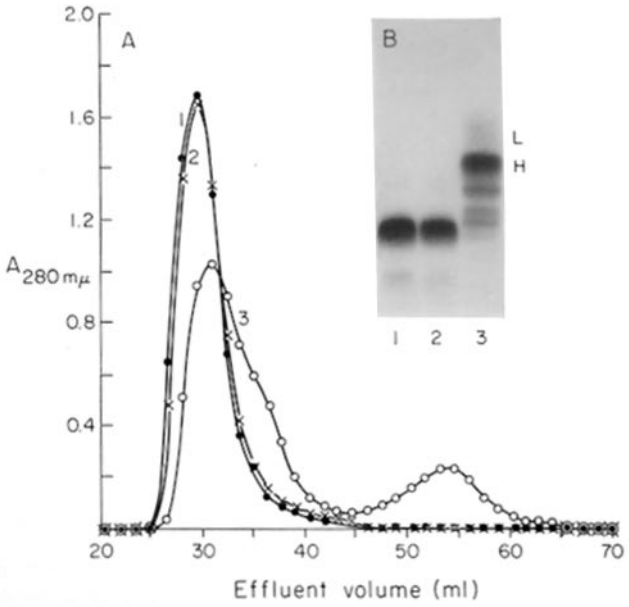

FIG. 1 *A*. Gel filtration on sephadex G-100 in 0.5 N propionic acid comparing unreduced human γ-globulin (curve 1), reduced reoxidized human γ-globulin (curve 2), and reduced alkylated human γ-globulin (curve 3). $A_{280\,m\mu}$, absorbancy at 280 mμ.

FIG. 1 *B*. Comparison by starch gel electrophoresis in urea of unreduced γ-globulin (sample 1), reduced reoxidized γ-globulin (sample 2), and reduced alkylated γ-globulin (sample 3). *L* and *H*, L and H polypeptide chains. Bands moving more slowly than H chains probably represent incompletely dissociated material.

Human 7S γ-globulin was reduced for 3 hours at room temperature in 0.05 M phosphate buffer, pH 7.0, and an aliquot was alkylated with iodoacetamide. This aliquot and the un-

alkylated remainder were dialyzed separately against distilled water at 4°C, followed by 0.02 M phosphate buffer, pH 7.0 made 0.15 N in NaCl.

Upon sedimentation velocity ultracentrifugation the two samples could not be distinguished from native γ-globulin ($s_{20,w}$ = 6.6S). A comparison by gel filtration on the same column of sephadex G-100 in 0.5 N propionic acid showed the same elution patterns for the reoxidized material and native γ-globulin; whereas the reduced alkylated material dissociated into L and H polypeptide chains (Fig. 1 *A*). Similar results were obtained by starch gel electrophoresis in urea (Fig. 1 *B*). Determination of carboxymethylcysteine after alkylation showed that the samples of native γ-globulin had 1.2 moles, reoxidized γ-globulin had 0.7 moles, and reduced alkylated γ-globulin had 5.1 moles per 160,000

TABLE I

Neutralization of f2 Bacteriophage by Purified Guinea Pig Antibody Preparations

Sample of guinea pig Ab(f2)*	K‡
Unreduced	315; 327
Reduced and alkylated	250; 308
Reduced and reoxidized	315; 293
Alkylated§	325; 256

* Ab(f2), antibody produced against f2 bacteriophage.

‡ K, first order constant for phage neutralization, expressed per minute per unit absorbancy at 280 mμ. The values of K given represent duplicate determinations.

§ Alkylated, an aliquot of unreduced Ab(f2) was alkylated under conditions identical with those for the alkylation of reduced antibody.

gms of protein. This suggested that disruption and reoxidation of an average of two disulfide bonds per molecule had taken place. As shown in Table I, reduction and alkylation, and reoxidation had no significant effect on the activity of purified guinea pig 7S antibodies to the bacteriophage f2. In addition, unreduced human γ-globulin, reduced and reoxidized, and reduced and alkylated γ-globulin were found to possess immunologic and immunoelectrophoretic identity, employing rabbit antiserum to whole human γ-globulin.

Dissociation and Reassociation of L and H Chain Fractions.—Gel filtration of reduced alkylated human 7S γ-globulin resolved the L chain fraction from the more complex first peak containing the H chains (Fig. 2). To avoid possible contamination with undissociated materials only the latter part of the first peak was pooled to form the H chain fraction. After another passage through the same column, the L chain fraction was more retarded, and the H chain fraction emerged earlier than in the initial separation. These results suggested that even in 0.5 N propionic acid, some degree of association might be present between the different chain fractions.

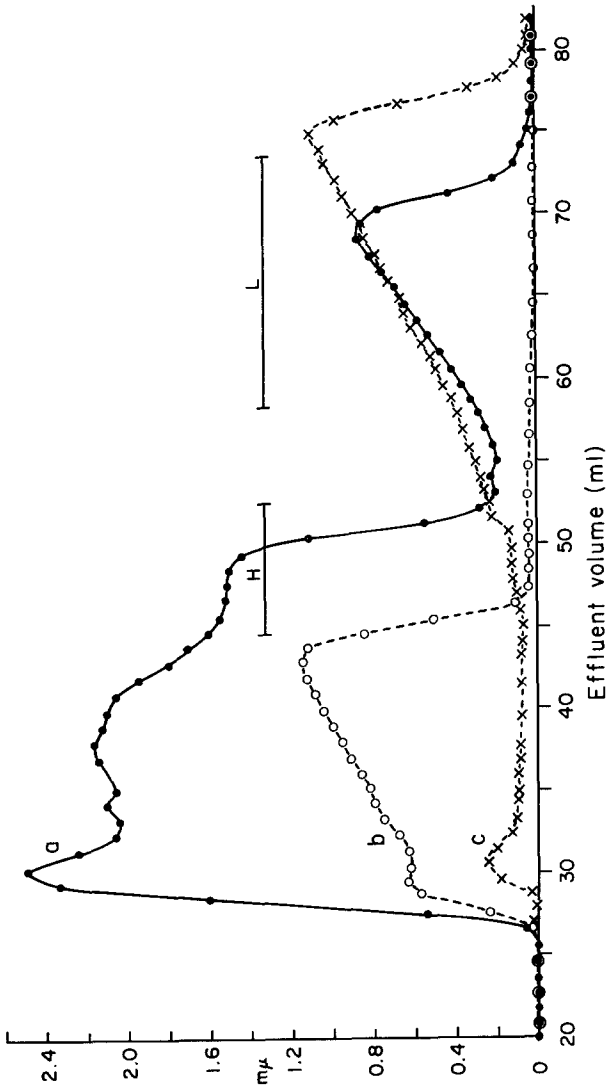

FIG. 2. Gel filtration of reduced alkylated γ-globulin and chain fractions on the same column of sephadex G-100 in 0.5 N propionic acid. (*a*) reduced alkylated human γ-globulin: *H* and *L*, fractions taken for repeated filtration. The H and L chain fractions were obtained from two identical separations, and concentrated by ultrafiltration in 0.5 *N* propionic acid prior to refiltration. (*b*) gel filtration of H chain fraction. (*c*) gel filtration of L chain fraction. $A_{280\ m\mu}$, absorbancy at 280 mμ.

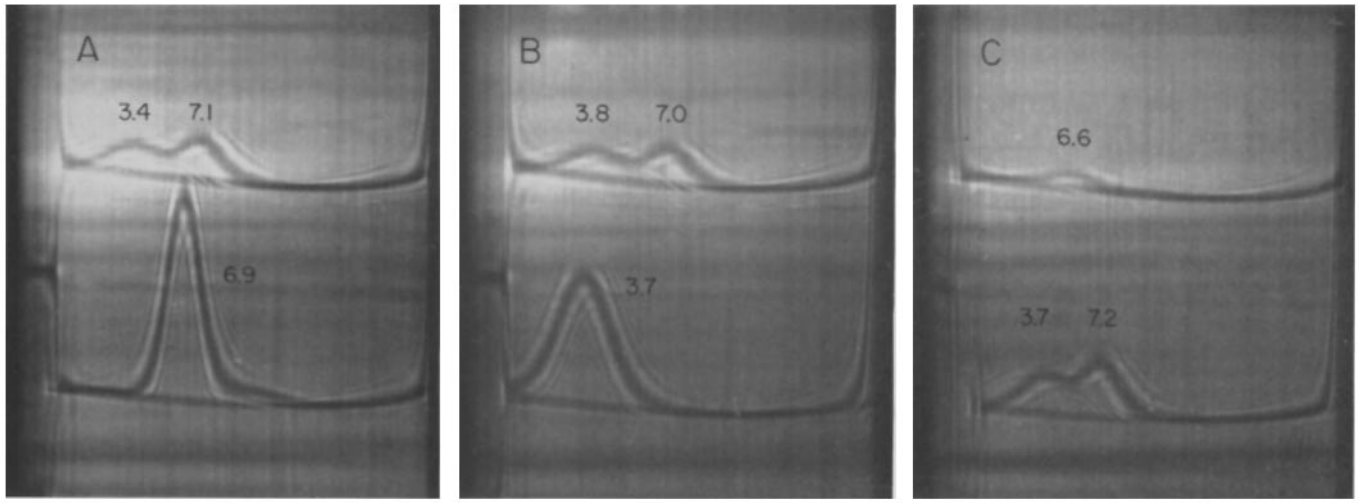

FIG. 3. Ultracentrifugal patterns of human γ-globulin chain fractions and their mixtures. (The sedimenting peaks are travelling from left to right. Each comparison represents a simultaneous sedimentation velocity experiment.) The numbers adjacent to each sedimenting peak are the calculated values of $s_{20,w}$ (see Table II).

A. Unreduced fraction II human γ-globulin (lower frame) compared with a reconstituted mixture of L and H chain fractions (upper frame). Time of photograph: 58 minutes.

B. Human γ-globulin L chain fraction (lower frame) compared with a reconstituted mixture of L and H chains (upper frame). Time of photograph: 68 minutes.

C. Reconstituted mixture of L and H chains (lower frame) compared with soluble portion of H chain fraction (upper frame). Time of photograph: 53 minutes.

Solvent: 0.02 M sodium phosphate buffer pH 7.0 with 0.15 M NaCl. Speed: 50,740. Phase plate angle: 50°.

TABLE II

$s_{20,w}$ *Values Obtained for Unreduced γ-Globulins, L and H Polypeptide Chain Fractions, and for Reconstituted Mixtures of L and H Chains*

Sample	$s_{20,w}$*
Human γ-globulin	
Unreduced	6.6[a], 7.2[b]
Reduced and alkylated	7.2[b]
Reduced and reoxidized	6.6[a]
Unreduced	6.9
L chain fraction	3.7
H chain fraction	6.6
L + H reconstituted mixtures‡	
1st peak	7.1[c]
2nd peak	3.6[c]
Guinea pig γ-globulin	
Unreduced	6.7
L chain fraction	3.7
H chain fraction	4.3
L + H reconstituted mixture	6.4[c]
Guinea pig anti-DNP antibody	
Unreduced	—
L chain fraction	3.6
H chain fraction	5.2
L + H reconstituted mixture	6.5[c]

* $s_{20,w}$, sedimentation coefficients corrected to water at 20°C. The values given represent a compilation of individual determinations. The protein concentrations used varied from 0.3 to 1 per cent. The sedimentation coefficients were not corrected for concentration differences. [a] values determined simultaneously. [b] values determined simultaneously. [c] values given represent averages from several runs.

‡ See Fig. 3.

H and L chain fractions and a mixture of the fractions made in 0.5 M propionic acid at a mass ratio of H/L = 0.75/1.0 were dialyzed for 48 hours against 0.15 N NaCl buffered with 0.02 M phosphate, pH 7.0. Each fraction and the mixture were centrifuged to remove insoluble material, and concentrated to 0.5 to 1 per cent solutions of protein. After centrifugation to remove additional insoluble material (particularly noticeable in H chain fractions), the solutions were studied by ultracentrifugation and by gel filtration on sephadex G-200.

In Fig. 3 the ultracentrifugal patterns of the soluble material in L and H chain fractions and in the reconstituted mixture are compared with those of normal 7S γ-globulin. $s_{20,w}$ values for each sample are presented in Table II which also contains data from similar studies on guinea pig 7S γ-globulin and

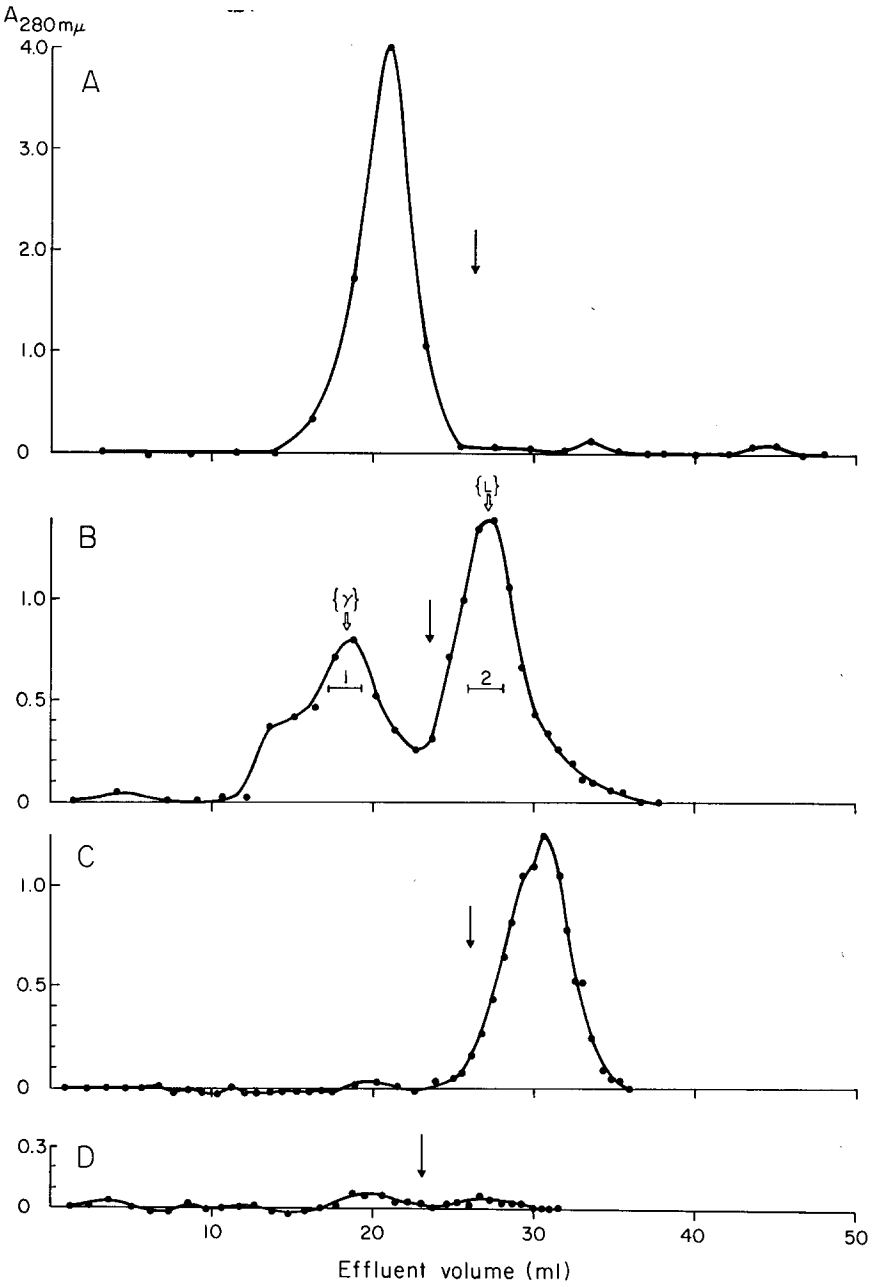

FIG. 4. Gel filtration of polypeptide chains and the reconstituted mixture on sephadex G-200 in 0.05 M tris-HCl buffer pH 8.0.

A. Unreduced human γ-globulin.

B. Reconstituted mixture of L and H chain fractions from reduced alkylated human γ-globulin. *1* and *2*, fractions used for immunologic analyses. {γ} and {*L*}, approximate positions for γ-globulin and L chain peaks calculated from curves *A* and *C* using the alkaline phosphatase peaks as reference (see Materials and Methods).

C. L chain fraction from reduced alkylated γ-globulin.

D. H chain fraction from reduced alkylated γ-globulin.

$A_{280\,m\mu}$, absorbancy at 280 mμ. The initial 2.0 ml was used as a blank; certain fractions had slightly lower absorbancies than that of the blank as shown in curves *C* and *D*. ↓, position of alkaline phosphatase peak.

anti-DNP antibodies. The reconstituted mixture of chains from human γ-globulin showed two peaks, one with a sedimentation coefficient of approximately 7S and the other of about 3.6S. The L chain fraction showed a single peak of 3.7S and the H chain fraction contained a small amount of 7S material, which possibly represents contaminating whole γ-globulin. Most of the material in the H

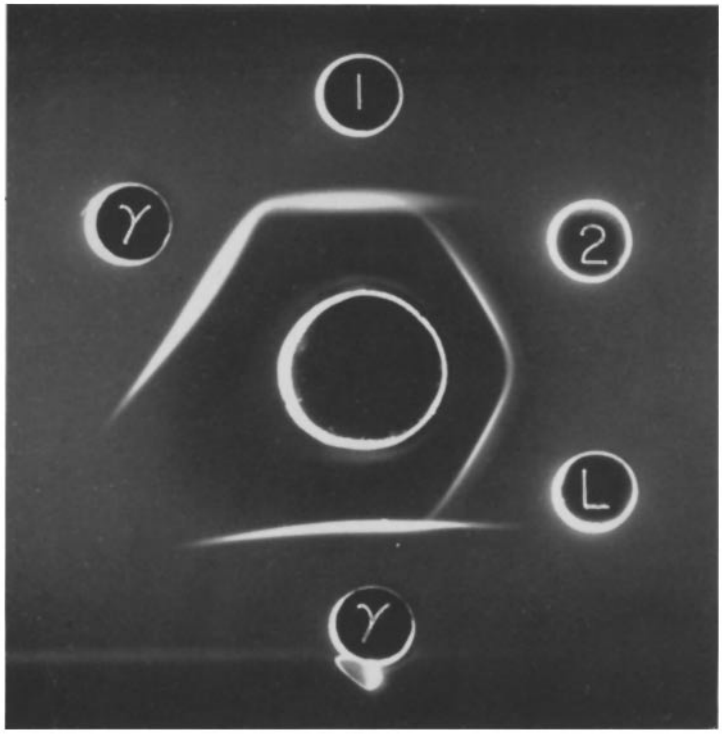

FIG. 5. Comparisons by immune diffusion of unreduced γ-globulin, L chain fraction, and fractions 1 and 2 obtained after gel filtration of a reconstituted L and H chain mixture on sephadex G-200 (see Fig. 4 *B*). Rabbit antiserum to human 7S γ-globulin was placed in the central well.

chain fraction was insoluble, however. The results obtained with guinea pig material were similar, except that the H chain fractions appeared to be more soluble than human H chain fractions and had $s_{20,w}$ values between 4.3S and 5.2S (Table II).

A comparison of unreduced human γ-globulin, L and H chain fractions, and reconstituted mixtures was made on four columns of sephadex G-200 in 0.05 M tris buffer, pH 8.0. As shown in Fig. 4, the reconstituted mixture yielded two peaks, the first eluting in the position of normal 7S γ-globulin and the second in the position of L chains. As in the ultracentrifugal experiments, less than 10

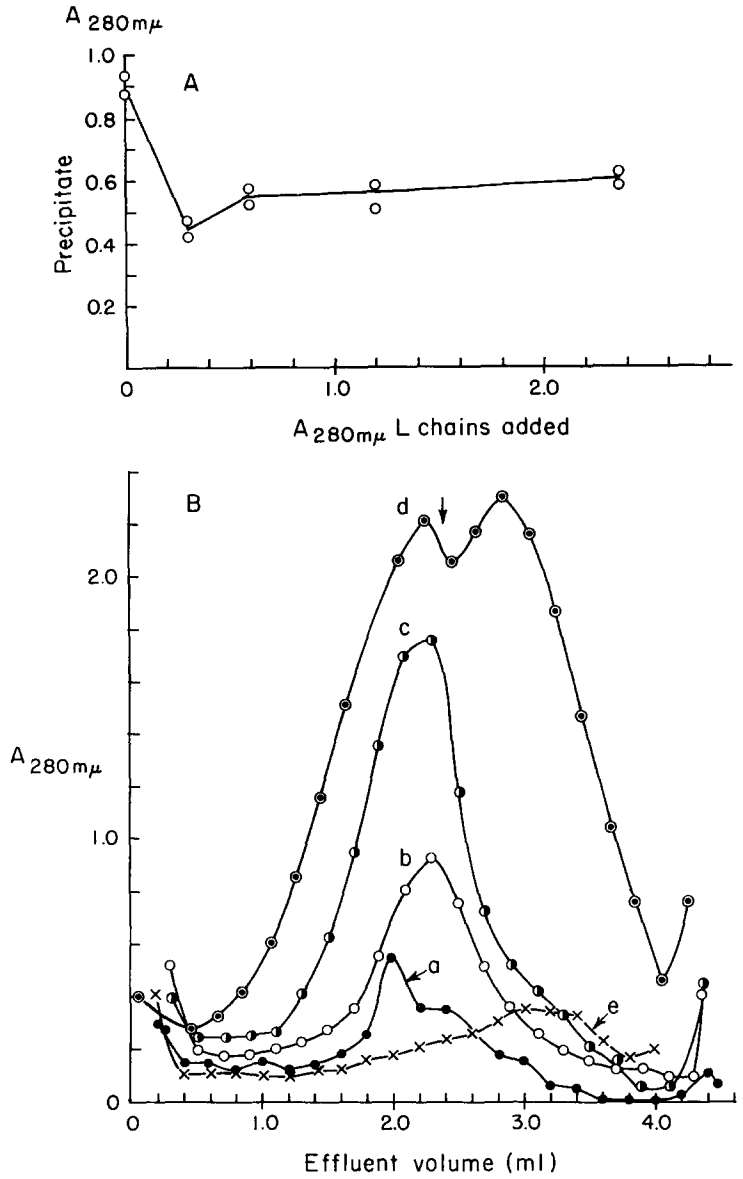

FIG. 6. Inhibition of precipitation of H chain fraction by added L chain fraction.

A. Increasing amounts of human L chain fraction were added to a constant amount of H chain fraction (absorbancy at 280 mμ = 2.0) in 0.5 N propionic acid, and the amounts of insoluble material obtained after dialysis into phosphate buffered saline were measured. $A_{280\,m\mu}$ precipitate, absorbancy at 280 mμ of precipitates solubilized in 1 ml of 0.1 N NaOH containing 2 per cent Na_2CO_3. $A_{280\,m\mu}$ L chains added, absorbancy at 280 mμ of L chain fraction in 0.5 N propionic acid.

B. Comparisons by sucrose density gradient ultracentrifugation of the concentrated supernatants obtained from material used in *A*. (*a*) soluble portion of H chains fraction alone. (*b*) H chain fraction mixed with 0.30 A_{280} units of L chain fraction. (*c*) H chains plus 0.60 A_{280} units of L chain fraction. (*d*) H chains mixed with 2.4 A_{280} units of L chains. (*e*) 0.60 A_{280} units of L chain fraction alone. The bottom of each centrifuge cell is at extreme left. $A_{280\,m\mu}$, absorbancy measured at 280 mμ. ↓, position of alkaline phosphatase peaks (see Materials and Methods).

per cent of the H chain fraction was recovered in soluble form, over 80 per cent having precipitated before application to the column. Antigenic analysis by double diffusion in agar (Fig. 5) revealed a reaction of identity between the material from the first peak of the reconstituted mixture and normal γ-globulin;

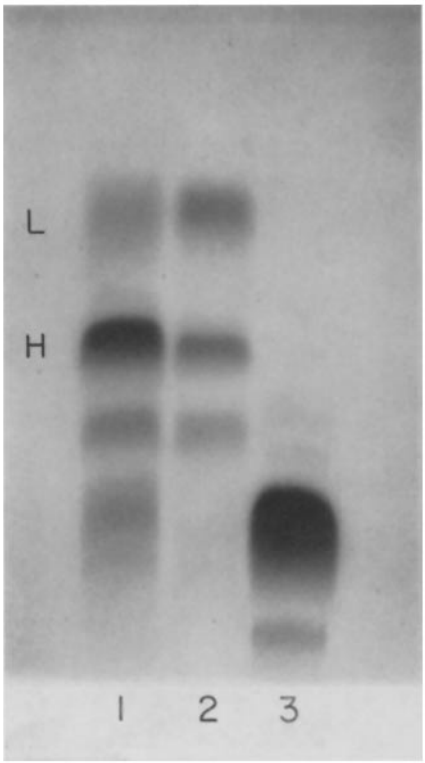

FIG. 7. Starch gel electrophoresis in urea of reduced alkylated human 7S γ-globulin (sample 1), reconstituted 7S mixture (sample 2), and unreduced fraction II human γ-globulin (sample 3). *L* and *H*, L and H polypeptide chains.

whereas material from the second peak was antigenically identical with L chains, using anti-7S γ-globulin sera. Rabbit antisera to human L chains revealed similar reactions of identity.

The foregoing results suggested that some of the otherwise insoluble H chain fraction was "solubilized" in the presence of L chains, with an apparent increase of 7S material in the mixture. In the ratios used, an excess of free L chains was found in the mixtures. An attempt was made to determine both the optimal ratio of H and L chains for formation of the reconstituted complex and the corresponding yields of reconstituted 7S material (Fig. 6).

Increasing amounts of L chains were added to a constant amount of H chains in 0.5 N propionic acid, and the mixtures were dialyzed against phosphate-saline buffer. The samples were centrifuged free of visible precipitates, the supernates were carefully collected, and the pellets dissolved in 0.1 N NaOH made 2 per cent in Na_2CO_3. In Fig. 6 *A* the absorbancy at 280 mμ of the dissolved pellets is plotted against that of added L chains. Maximal dissolution of the pellet was achieved by addition of 0.30 absorbancy units of L chains to 2.0 absorbancy units of the H chain fraction.

After concentration of the supernates of the original mixtures, they were subjected to density gradient centrifugation (Fig. 6 *B*). The soluble portion of the H chain fraction contained material migrating in the 7S region. Addition of 0.3 or 0.6 absorbancy units of L chains produced an increase of 7S material. When larger amounts of L chains were added (*i.e.*, 2.4 A_{280} units), a 4S peak appeared in addition to the 7S peak. Reconstituted material from the 7S peak (curve *b*, Fig. 6 *B*) was subjected to equilibrium centrifugation in 0.05 M tris buffer, pH 7.2 made 0.1 N in NaCl and 2 per cent in sucrose. Approximately 88 per cent of the material had a weight average molecular weight of 160,000 ± 7000; the remainder consisted of material of lower molecular weight. The molecular weight of the heavier species did not change over the concentration range of 0.1 to 0.01 per cent.

The material used for these molecular weight analyses was compared to normal γ-globulin and reduced alkylated γ-globulin by means of starch gel electrophoresis in urea (Fig. 7). Reconstituted material dissociated to L and H chain bands, and showed no residual material corresponding to intact γ-globulin. This suggested that reassociation was effected through non-covalent interactions.

Chain fractions labeled with I^{125} and I^{131} were used to obtain a more accurate estimation of the yields of reconstituted 7S product and the ratio of masses of chains in the product. In Fig. 8 *A* the patterns of $H_{I^{125}}L_{I^{131}}$ mixtures (see Materials and Methods) are compared with those of the separate chain fractions. Identical results were obtained with $H_{I^{131}}L_{I^{125}}$ mixtures. The mass ratio of H to L chains used in the original mixture was 3.3/1. In both experiments, 80 per cent of the isolated H chain fractions and 20 per cent of the isolated L chain fractions were insoluble. In mixtures of L and H chains 50 per cent of the H chain fraction was insoluble and most of the soluble portion migrated in the 7S region. Approximately 40 per cent of the soluble L chains in the mixture were present in the 7S region. In a number of such experiments, the mass ratio of H chains to L chains in the 7S material varied from 2.4/1 to 5.4/1 (see Discussion). When the original mixture in propionic acid contained six times as much H chain fraction as L chain fraction by absorbancy at 280 mμ, almost all of the soluble labeled L chains were bound to H chains in the 7S region, and the mass ratio of H to L chains in the 7S peak was 2.8/1 (Fig. 8 *B*). As calculated by the method of Martin and Ames (22), the uncorrected sedimentation coefficients of the two sedimenting peaks in each experiment were 6.6S and 3.8S.

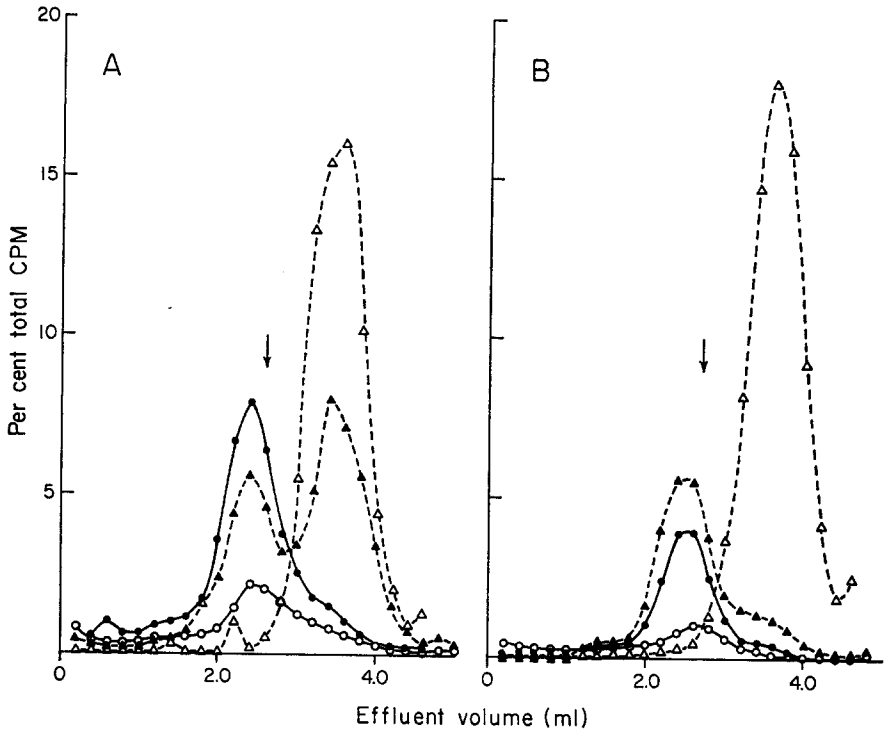

FIG. 8. Distribution of isotopic labels after density gradient centrifugation of $H_{I^{125}}L_{I^{131}}$ mixtures. Human H chain fractions labeled with I^{125}, L chain fractions labeled with I^{131}, and the products of their reconstituted mixture, $H_{I^{125}}L_{I^{131}}$ were each analyzed separately by density gradient centrifugation.

A. Mass ratio of H to L chains during reconstitution in 0.5 N propionic acid: 3.3/1.

B. Mass ratio of H to L chains during reconstitution: 6/1. △------△, labeled L chain centrifuged alone; ○——○, labeled H chain fraction centrifuged alone; ▲------▲, L chain label in reconstituted mixture; ●——●, labeled H chain fraction in mixture. Sedimentation proceeded from right to left. Per cent total CPM, counts per minute in each fraction expressed as per cent of total counts recovered from cell contents, pellet, and concentrating sac (see Materials and Methods). This total accounts for soluble and insoluble material. ↓, position of alkaline phosphatase marker.

Admixture of the separated H and L chain fractions after dialysis against neutral buffers did not result in an increase of material in the 7S region.

$H_{I^{125}}L_{I^{131}}$ reconstituted fractions mixed with reduced alkylated carrier γ-globulin were subjected to starch gel electrophoresis in urea. After staining, the gel was cut into small segments and analyzed for each isotope (see Fig. 12 *D*). Over 80 per cent of the I^{131} was found in the L chain band and 60 per cent of the I^{125} was found in the H chain band. The remainder of both isotopes was found in bands migrating more slowly than H or L chains, although a small

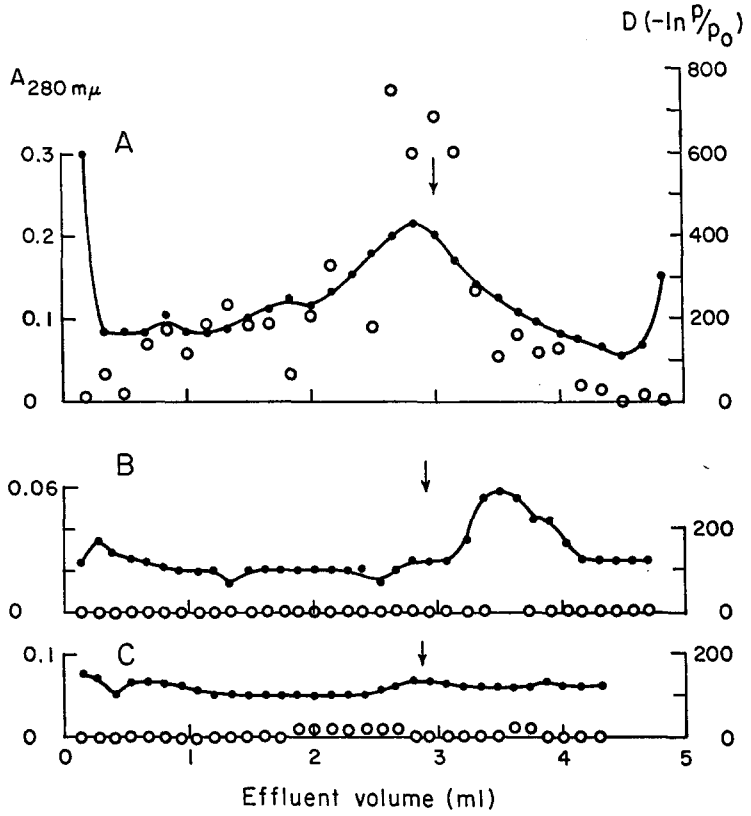

FIG. 9. Distribution of f1 phage neutralizing activity after sucrose density gradient centrifugation of L and H chains and of a mixture of L and H chains. Chains were obtained from specifically purified guinea pig antibodies to f1 bacteriophage.

A. Reconstituted mixture of H and L chain fractions.

B. L chain fraction.

C. H chain fraction.

●——●, measurements of absorbancy at 280 mμ ($A_{280\ m\mu}$). ○, measurements of phage neutralization expressed as $D(-\ln p/p_o)$, where D represents the dilution of each fraction from the gradient prior to phage assay, and p/p_o represents the fraction of phage neutralized after 30 minutes at 37°C. Sedimentation proceeded from right to left. ↓, position of alkaline phosphatase peak.

amount of I^{125} was seen in the L band. The reconstituted product could also be dissociated with recovery of the original labeled L and H chains by employing gel filtration in 0.5 N propionic acid (see Fig. 13).

In order to test whether the reconstituted product regained the specific functional properties of native antibodies, purified guinea pig antibodies to f1 bacteriophage were separated into L and H chains and reconstituted (11). A

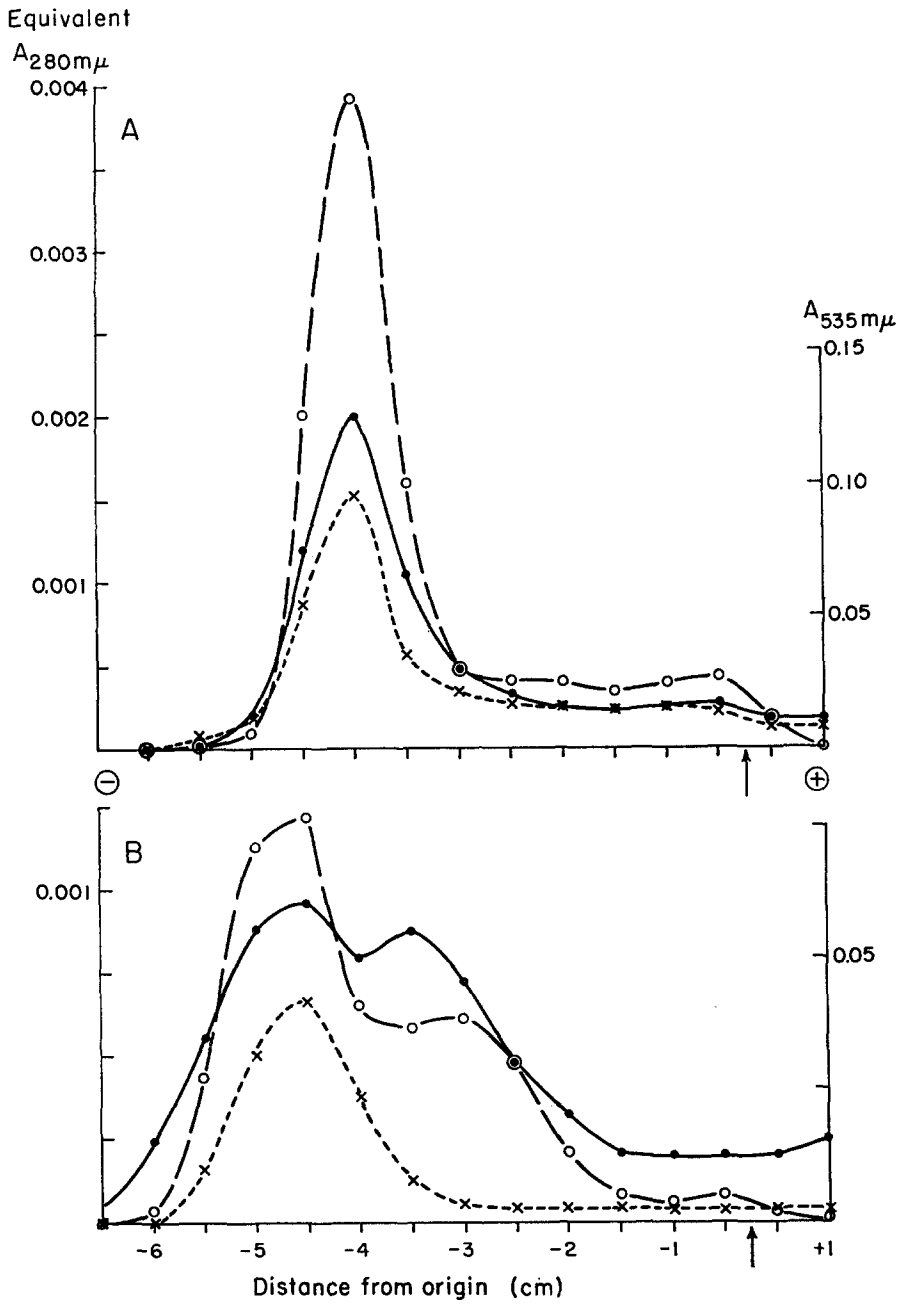

FIG. 10. Electrophoretic separation on cellulose acetate of reconstituted products before and after treatment with papain.

A. Reconstituted 7S human $H_{I^{131}}L_{I^{125}}$ mixed with unreduced carrier γ-globulin.

B. Aliquot of sample A after 3 hour digestion with papain at 37°C.

○——○ and x------x, equivalent absorbancy at 280 mμ ($A_{280\,m\mu}$), calculated from the I^{131} (○——○) and I^{125} (x------x) counts per minute and the known specific activities for the H and L chain fractions. ●——●, absorbancy at 535 mμ ($A_{535\,m\mu}$) of eluted ponceau S stain. ↑, origin. ⊕, anode. ⊖, cathode. Buffer, 0.05 M barbital pH 8.6.

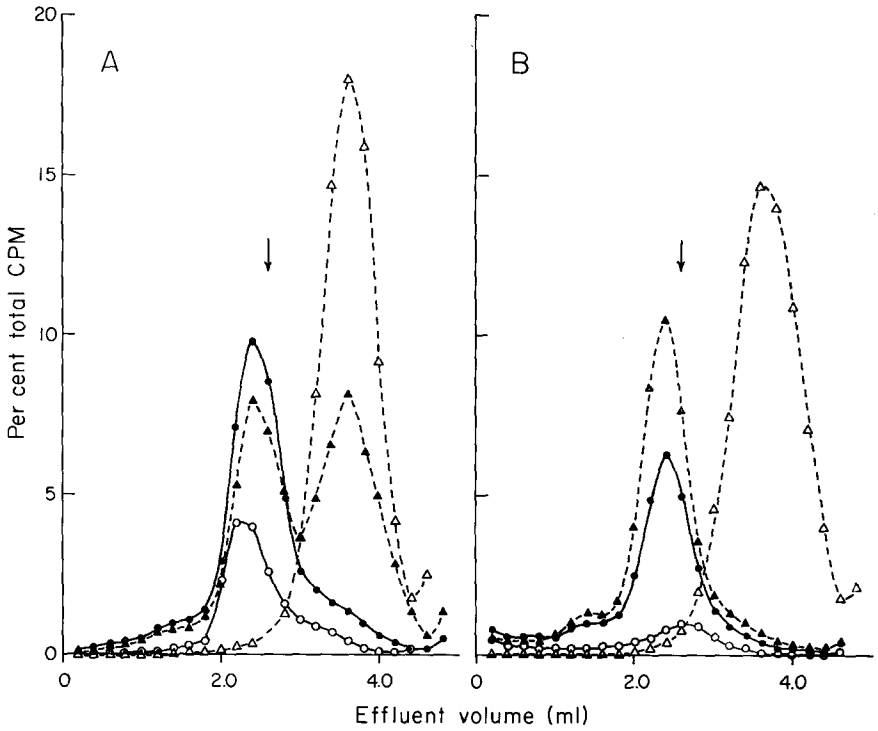

FIG. 11. Density gradient centrifugation of hybrid mixtures of H and L chains from γ-globulins of different animal species.

A. Rabbit H chains labeled with I^{131} (●——●) reconstituted with human L chains labeled with I^{125} (▲------▲), compared with unmixed H chain fraction (○——○) and unmixed L chains (△------△).

B. Human H chains labeled with I^{125} (●——●) reconstituted with rabbit L chains labeled with I^{131} (▲------▲), compared with unmixed H chain fraction (○——○) and unmixed L chains (△------△).

Sedimentation proceeded from right to left. Per cent total CPM, per cent of total recovered counts per minute (soluble and insoluble). ↓, position of alkaline phosphatase peak.

comparison of absorbancy at 280 mμ with phage neutralization in fractions obtained from the density gradients showed that the reconstituted product had maximal activity in the 7S region (Fig. 9). L chains showed no activity and the soluble portions of H chain fractions showed minimal activity in the 7S region.

If 7S material reconstituted from separated L and H chains resembles γ-globulin, it would be expected that hydrolysis with papain would yield S and F fragments (28). Doubly labeled material reconstituted from human $L_{I^{125}}$ and $H_{I^{131}}$ chains was obtained from the 7S region of the density gradient, mixed with unreduced and unlabeled 7S γ-globulin as carrier and hydrolyzed with papain. This product and unhydrolyzed material were subjected to electrophoresis on

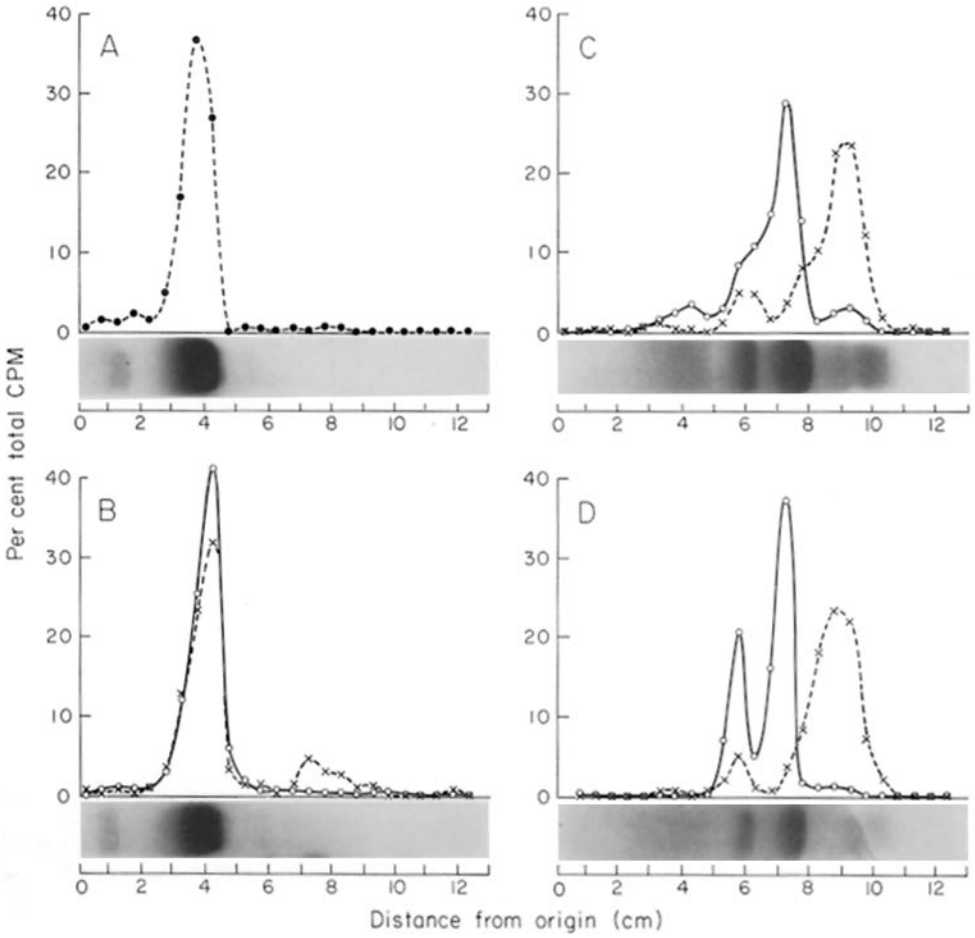

FIG. 12. Comparisons of doubly labeled reconstituted materials by starch gel electrophoresis in urea. The distributions of radioactivity obtained from sections of the unstained portion of the gel are placed above the corresponding segment of the stained portion, and distances along the gel made equivalent. H chain bands migrated between 7 and 8 cm from the origin; L chain bands migrated between 8 and 10 cm from the origin.

A. Unreduced human 7S γ-globulin, labeled with I^{125}.

B. Reconstituted and reoxidized 7S material ($H_{I^{125}}L_{I^{131}}$) containing 1 mg of unreduced carrier 7S γ-globulin.

C. Sample B reduced and alkylated before loading on the gel.

D. Material reconstituted from reduced alkylated chain fractions ($H_{I^{125}}L_{I^{131}}$) mixed with 1 mg of carrier reduced alkylated γ-globulin.

●——●, I^{125}-labeled 7S γ-globulin. ○——○, I^{125} labeled H chain fraction. x------x, I^{131} labeled L chains. Per cent total CPM, per cent of total counts per minute recovered from the gel along the entire axis of migration. The faint lines at 9 cm in *C* and *D* are cracks in the starch gel.

cellulose acetate (Fig. 10). The unhydrolyzed material contained both isotopes in a peak with the same mobility as native γ-globulin. After hydrolysis, peaks corresponding to S and F fragments were partially separated. I^{125} L chain material was present only in the region of S fragments; and I^{131} H chain material was present in both S and F fragments.

Following the same procedures of reconstitution described, it was possible to obtain hybrid 7S molecules containing the complementary chains from 7S γ-globulins of two animal species (Fig. 11). Rabbit H chains mixed with human L chains, and human H chains mixed with rabbit L chains yielded 7S products with mass ratios of the chains similar to those found in reconstitution of chains from a single species.

Reformation of Interchain Disulfide Bonds in Reassociated γ-Globulin.—It was shown above that the interchain disulfide bonds of native 7S γ-globulin could be reversibly cleaved. This finding prompted attempts to reform interchain bonds in reassociated material.

Aliquots of 50 mg of I^{131}-labeled human γ-globulin and I^{125}-labeled human γ-globulin were separately reduced without alkylation, dialyzed against 0.5 N propionic acid, and separated into L and H chains on columns of sephadex G-100. Mixtures were made as described above. The mixtures were dialyzed first against 0.05 M tris buffer, pH 7.2, made 0.1 N in 2-mercaptoethanol, in order to allow reassociation to occur in the absence of oxidation. The mixtures were then diluted to a final protein concentration of 0.01 per cent and dialyzed exhaustively at 4°C against multiple changes of 0.05 M tris buffer, pH 7.2, which was free of mercaptan. The samples were concentrated and separated by density gradient centrifugation.

A large amount of doubly labeled 7S material was recovered and the yields (30 per cent) were comparable with those of previous experiments on reduced alkylated chains. By equilibrium centrifugation 90 per cent of the 7S product had a weight average molecular weight of 160,000.

A sample of $H_{I^{125}}L_{I^{131}}$ 7S reconstituted and reoxidized material was compared to normal 7S γ-globulin, using starch gel electrophoresis in urea. Doubly labeled reoxidized product was added to 2 mg of normal 7S γ-globulin carrier, and one half of the mixture was reduced for 2 hours and alkylated with iodoacetamide. These two samples were compared with I^{125}-labeled normal γ-globulin, and 7S material reconstituted from reduced alkylated chains (Fig. 12). The reassociated reoxidized sample showed both isotopes in the region corresponding to normal γ-globulin (Figs. 12 *A* and 12 *B*). After reduction and alkylation, the reoxidized material again dissociated into $L_{I^{131}}$ and $H_{I^{125}}$ chains, yielding a pattern similar to that of material reconstituted from reduced alkylated chains (Figs. 12 *C* and 12 *D*). In addition to L and H chain bands, a slow band (at 6 cm) containing both labels was noted (Figs. 12 *C* and 12 *D*); this material possibly consists of half molecules.

Gel filtration in propionic acid (Fig. 13) gave similar results. Reconstituted and reoxidized 7S material could not be distinguished from 7S γ-globulin;

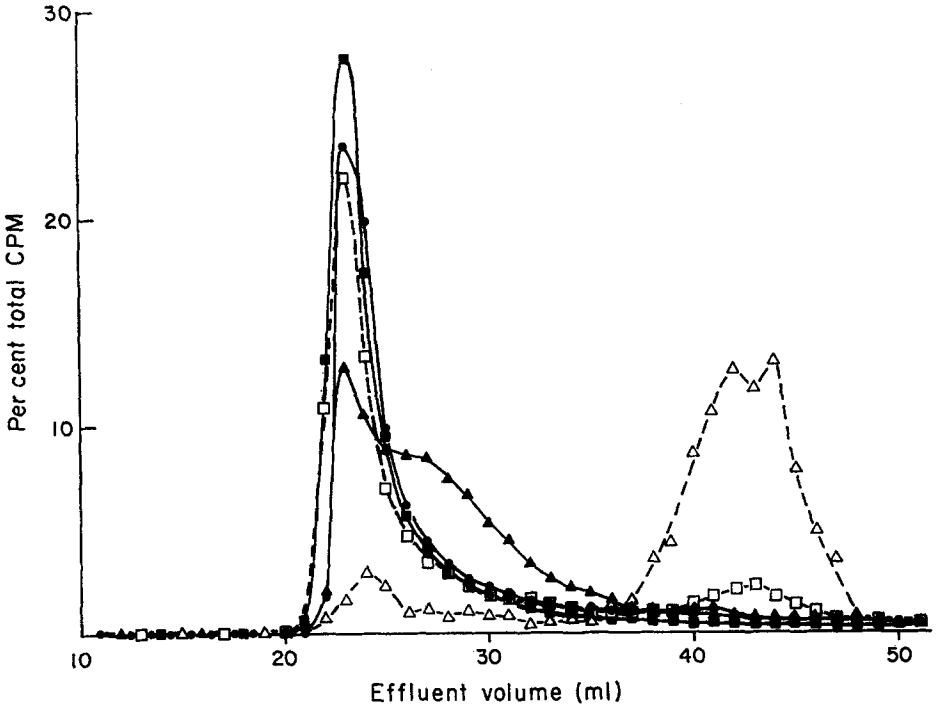

FIG. 13. Gel filtration on sephadex G-100 in 0.5 N propionic acid comparing doubly labeled reconstituted materials.

Unreduced I^{125} labeled γ-globulin (●——●).

Reconstituted and reoxidized 7S material composed of $H_I{}^{131}$ (■——■) and $L_I{}^{125}$ (□------□).

Reconstituted 7S material composed of reduced alkylated chain fractions $H_I{}^{125}$ (▲——▲) and $L_I{}^{131}$ (△------△).

Per cent total CPM, per cent of total counts recovered from the columns.

whereas 7S material reconstituted from reduced alkylated chains was redissociated in this solvent.

DISCUSSION

Under appropriate conditions of mixing, the separated polypeptide chains of γ-globulins and antibodies were found to form molecules resembling 7S γ-globulin in many of their properties. The experiments described here were based on several previously reported observations. Admixture of L and H chains of antibodies resulted in partial recovery of the capacity to combine specifically with homologous antigen (11, 12), suggesting that the chains were interacting with each other in conformations similar to those in the native 7S molecules. The molecular weight of 7S γ-globulin was found to be unchanged after reduction of

interchain disulfide bonds in the absence of urea (2), but upon subsequent exposure to urea (1, 2), acids (2, 29, 5), or elevated temperature (30), the molecule was found to be dissociated. Dissociation therefore depends upon cleavage of disulfide bonds and upon subsequent weakening of non-covalent interactions between the chains. These two steps have now been shown to be independently reversible; the reversal of both steps in the proper sequence leads to the formation of reconstituted molecules which are stable in dissociating solvents.

After cleavage of interchain disulfide bonds by 2-mercaptoethanol, the bonds may be reformed before dissociation of the molecule by removal of the mercaptan. This process appears to be highly efficient, as suggested by the almost complete recovery of 7S material which cannot be dissociated in solutions of 8 M urea and formate buffer, or in propionic acid. The antigenic properties, solubility, electrophoretic properties, and ultracentrifugal behavior of the reoxidized material were the same as those of native γ-globulin. These results, and the maintenance of the specific combining activity of antibodies treated in this manner, suggest that there is little or no rearrangement among the chains after the cleavage of interchain disulfide bonds, and no steric interference with reformation of the bonds. In the particular experiments described here, an average of two interchain bonds were broken. This is less than would be expected for the complete dissociation of four chains linked by a minimum of three disulfide bonds. The presence of aggregates migrating more slowly than H chains in the starch gel is consistent with the fact that not all molecules were completely dissociated. Nevertheless, a considerable proportion of the molecules were dissociated, as judged by the deeply staining L and H bands on the starch gel (Fig. 1 *B*) and the yield of L chains after gel filtration (Fig. 1 *A*).

Mixture of separated reduced alkylated L and H chains at concentrations of 0.01 to 1.0 per cent in 0.5 M propionic acid followed by dialysis against neutral buffers led to the reformation of 7S molecules in greater than 30 per cent yield. The reconstituted product had a sedimentation coefficient of 6.0S to 7.0S, a molecular weight of 160,000, and was shown to be composed of the originally separated L and H chains by the use of two different iodine isotopes as markers. By the use of specific antisera to whole 7S γ-globulin and to L chains, it was confirmed that this product contained both L and H antigenic determinants. The reconstituted molecules were shown to contain chains linked only by non-covalent interactions since they could by dissociated readily by gel filtration in propionic acid and by starch gel electrophoresis in urea. In these respects, and in the properties described above, the reconstituted products resembled 7S γ-globulin that has been reduced and alkylated in the absence of urea.

The approximate mass ratio of the two types of chains in the reconstituted product was calculated for the peak fractions of the 7S region of the sucrose density gradient. An example of the calculation for H chains labeled with I^{125}

and L chains labeled with I^{131} is as follows:

$$\text{mass ratio H/L in 7S region} = \frac{\frac{\text{CPM}}{\text{S.A.}}\ H_I^{125} \text{ in reconstituted mixture} - \frac{\text{CPM}}{\text{S.A.}}\ H_I^{125} \text{ in H fraction alone}}{\frac{\text{CPM}}{\text{S.A.}}\ L_I^{131} \text{ in reconstituted mixture}}$$

where CPM = corrected counts per minute and *S.A.* = specific activities of chain fractions (*i.e.* CPM/unit absorbancy at 280 mμ).

In different experiments, the mass ratios varied from 2.4/1 to 5.4/1. Assuming a molecular weight of 60,000 for H chains (31) and 20,000 for L chains (16, 7), the corresponding molar ratios H/L varied from 0.8/1 to 1.8/1. Available estimates suggest that the molar ratio of the two types of chains in whole 7S γ-globulin is unity (6). Considering the possibility that H chain fractions may contain L chains (13, 32) which would have been labeled as H chains, the present findings are consistent with a molar ratio of unity in the reconstituted product. The possibility must also be considered, however, that some reconstituted molecules contain two H chains and one L chain.

Starting with reduced L and H chains and combining the procedures of reconstitution and reoxidation in sequence, a stable 7S molecule was formed. This material was found to be dissociable only after a second reduction, and it resembled normal γ-globulin in immunologic properties, electrophoretic behavior, behavior upon gel filtration, and molecular weight. In forming this reconstituted, reoxidized product, it was found that high yields (about 30 per cent) were obtained if recombination occurred at protein concentrations of 0.1 per cent and reoxidation proceeded at concentrations of 0.01 per cent. Reoxidation at concentrations of 1 per cent or higher led to the formation of large amounts of aggregated material. It appears that, once formed, reconstituted material does not dissociate appreciably upon dilution even prior to formation of disulfide bonds. This is consistent with the observation that the molecular weights of material formed from reduced alkylated chains did not fall upon dilution.

It is premature to conclude that the reconstituted reoxidized 7S material is identical with native 7S γ-globulin, particularly since the polypeptide chains of which the reconstituted material was formed were exposed to propionic acid. Moreover, it has not been established that the number and pairing of interchain disulfide bonds in the reconstituted product are the same as in the native molecule. Studies of these properties, and of other physicochemical characteristics of the reconstituted molecule, should establish the degree of structural homology to native γ-globulin.

The results of the experiments on the hydrolysis of reconstituted molecules by papain are consistent with the view that the gross arrangement of L and H

chains is the same as that in native γ-globulin. Products resembling S and F fragments (28) resulted from hydrolysis of reconstituted molecules, and additional studies of such products have shown (30) that they were immunoelectrophoretically similar to those derived from native γ-globulin. In agreement with the results of previous studies (13, 10, 8), specifically labeled L chains were found only in the products corresponding to S fragments, and label from H chain fractions was found in the products corresponding to both S and F fragments.

As proposed previously (4, 10, 13) the S fragment appears to contain the L chain and a portion of the H chain; the remaining portion of the H chain comprises the F fragment. This evidence is supported by recovery of the L chains complete from S fragments (10, 13, 8) and by results of mapping of tryptic peptides of the chains and fragments (33). In the intact divalent antibody molecule each combining region would be subserved by an L-H pair (11) and two such pairs would be linked by weak forces and at least one disulfide bond (34). This arrangement of chains in the whole molecule raises the possibility that L chains might exist as disulfide-bonded dimers in the whole γ-globulin molecule. An examination of conformational requirements for reconstitution (30) has indicated that disulfide-bonded dimers of L chains are not incorporated with H chains into 7S molecules. Only dimers of L chains that are convertible to monomers in dissociating solvents were incorporated. These findings suggest that it is unlikely that the two L chains are present as disulfide-bonded dimers in the whole molecule.

As indicated above, the present data on reconstitution of 7S molecules are consistent with previously proposed models (4, 8) for the structure of 7S γ-globulin (4). The results of experiments on the reconstitution of activity (11, 12), and on labeling of the active site (14, 15) are not compatible, however, with the suggestion (5, 8, 29) that the H chain alone is concerned with specificity and combining site. Instead, it appears that through interaction, both L and H chains contribute to the specific conformation of antibodies (11). In the present experiments on zone centrifugation of anti-f1 antibodies, most of the activity was associated with reconstituted 7S material. Failure to reconstitute activity from heterologous chains (*i.e.* chains from unrelated antibodies) may result from failure to reconstitute 7S molecules. Preliminary experiments (35) have indicated, however, that mixtures of L chains from non-specific guinea pig γ-globulin and H chains from anti-DNP antibodies of guinea pigs would reconstitute 7S molecules, although there was no reconstitution of activity. Homologous L and H chains from anti-DNP antibodies showed reconstitution of both activity (11) and 7S molecules. Failure to reconstitute specific combining activity probably results, therefore, from inappropriate arrangements of the chains in the reconstituted molecule. This is consistent with the notion that of the many structurally different L and H chains, only subsets of each type having

the appropriate amino acid sequence will pair and form a specific combining region (4). The capacity of the chains to interact to form 7S molecules appears to be a general one, however, and is not as strictly dependent upon specific structural requirements as the reconstitution of activity. Thus, mixed molecules may be formed even between the complementary chains of two different animal species as shown for rabbit and human L and H chains.

The similarity between the behavior of the chains of γ-globulin and hemoglobulin (36–38), aldolase (39–41), and other multichain proteins is striking. γ-Globulin differs from these proteins in possessing interchain disulfide bonds, however, and the reconstitution of disulfide bonded molecules is more closely parallel to that reported for insulin (42). The experiments reported here suggest that the chains interact through weak forces, adopt the proper conformation, and then may be disulfide bonded. This sequence of events is possibly parallel to that described for the reformation of intrachain disulfide bonds of ribonuclease (43). It is remarkable that the separated chains of heterogeneous γ-globulins readily reform 7S molecules in high yield. The possibility should be entertained that in the antibody-producing cell, the appropriate L and H chains are synthesized and form antibody molecules by self-assembly; *i.e.*, by direct interaction without intervention of any additional or special mechanisms.

SUMMARY

Admixture of separated L and H polypeptide chains of 7S γ-globulins under appropriate conditions has been found to result in the reconstitution of 7S molecules. The chains were mixed in 0.5 N propionic acid and when dialyzed into neutral aqueous buffers interacted to form reconstituted material in greater than 30 per cent yield. This material had sedimentation coefficients of 6S to 7S, a weight average molecular weight of 160,000, and its antigenic structure and electrophoretic properties were the same as those of 7S γ-globulin.

By the use of I^{131} and I^{125} labels on the different types of chains, combined with ultracentrifugation of chain mixtures in sucrose density gradients, the 7S product was found to contain both isotopes in ratios consistent with the presence of two L and two H chains. After hydrolysis with papain, the reconstituted material yielded products resembling S and F fragments. All of the isotope corresponding to L chains was found in the counterpart of the S fragment; the isotope corresponding to the H chain fraction was present in both fragments. The activity reconstituted from chains of a purified guinea pig antibody to f1 phage was found to be associated mainly with the 7S material. Hybrid molecules containing rabbit L chains and human H chains and of human L chains and rabbit H chains were formed by the same techniques of reconstitution.

It was found that the interchain disulfide bonds of native 7S γ-globulins could be broken and reoxidized, as could those of reconstituted 7S material. Reduced L and H chains mixed in propionic acid, dialyzed against neutral

buffers containing mercaptan, then against neutral buffers in the absence of mercaptan, formed stable 7S molecules of molecular weight 160,000, which were dissociable only after reduction.

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