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Resemblance of the Gross Arrangement of Polypeptide Chains in Reconstituted and Native γ-Globulins*

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A study has been made of the conditions of reconstitution of 7S γ-globulin molecules from separated polypeptide chains and of the arrangement of chains in the reconstituted molecules. The molar ratios of H and L chains in the reconstituted molecules approached those of native γ-globulin when a molar excess of L chains was mixed with H chains. Hydrolysis of reconstituted molecules with papain produced S and F fragments in yields similar to those obtained with native γ-globulins. The contribution of the chains to the fragments was assessed by the use of reconstituted molecules formed from L chains and H chains labeled with different iodine isotopes (^{125}I and ^{131}I). The S fragment contained material from H and L chains; F fragments consisted largely of H-chain material. The results indicate that the gross arrangement of chains is the same in reconstituted and native γ-globulin molecules, and support the notion that the 7S γ-globulin molecule consists of two L chains and two H chains arranged in two interacting L·H pairs.

It has recently been shown that the separated H and L polypeptide chains of 7S γ-globulin will form reconstituted 7S molecules (Edelman *et al.*, 1963b; Olins and Edelman, 1964; Roholt *et al.*, 1964; Gally and Edelman, 1964; Fougereau *et al.*, 1964). This was achieved by mixing the chains in a dissociating solvent (0.5 N propionic acid) and dialyzing the mixture against neutral aqueous buffers. Interchain disulfide bonds could also be re-formed, if partially reduced chains which had not been alkylated were used as starting materials (Olins and Edelman, 1964). Reconstituted γ-globulins closely resembled native 7S γ-globulin in their electrophoretic, antigenic, and physicochemical properties. In addition it was found that the phage-neutralizing activity recovered (Edelman *et al.*, 1963b) after reassociation of chains from antibodies to f1 phage was present mainly in the 7S reconstituted fraction (Olins and Edelman, 1964).

The present communication provides additional information both on the conditions of reconstitution of H and L chains of human γ-globulin and on the similarity of the product to native 7S γ-globulin. Fragments obtained from reconstituted 7S material and native 7S γ-globulin after hydrolysis with papain have been found to be similar in their antigenic and electrophoretic properties. The data are compatible with models of the molecule consisting of two H chains and two L chains (Edelman *et al.*, 1963b; Fleischman *et al.*, 1963; Edelman and Gally, 1964) and suggest that the gross arrangement of the chains is the same in native and reconstituted γ-globulin.

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Materials and Methods

Human γ-globulin, lyophilized Cohn fraction II (lot C780) from Lederle Laboratories (Pearl River, N. Y.) was used.

Labeling of γ-Globulin with Radioactive Iodine.—Isotopic labeling of H and L chains with ^{131}I and ^{125}I was accomplished as previously described (Olins and Edelman, 1964). The iodination with carrier-free solutions of sodium [^{125}I]iodide (Volk Radiochemical Co., Chicago, Ill.) and sodium [^{131}I]iodide (Oak Ridge National Laboratory, Tennessee), followed the procedure of McFarlane (1963) except that the protein was dissolved in 0.15 M NaCl brought to *p*H 8.0 with 0.2 M sodium borate buffer as suggested by Helmkamp *et al.* (1960). Unreacted iodide was removed by passage of the protein solutions through 5 × 1.0-cm columns of Amberlite ion-exchange resin IRA-401 (Mallinckrodt Chemical Works, St. Louis, Mo.) equilibrated with the borate buffer. The specific activity of the labeled proteins ranged between 5×10^5 and 3×10^6 cpm per unit absorbancy at 280 mμ.

Reduction of Labeled γ-Globulin and Separation of H and L Polypeptide Chains.—The ^{125}I- and ^{131}I-labeled γ-globulins to be used in the same sequence of experiments were treated simultaneously. After labeling with isotope, the γ-globulin solutions were concentrated by ultrafiltration and then made 0.1 N in mercaptoethanol. Reduction proceeded in the absence of urea (Edelman and Poulik, 1961) for 2 hours and was stopped by addition of iodoacetamide to a final concen-

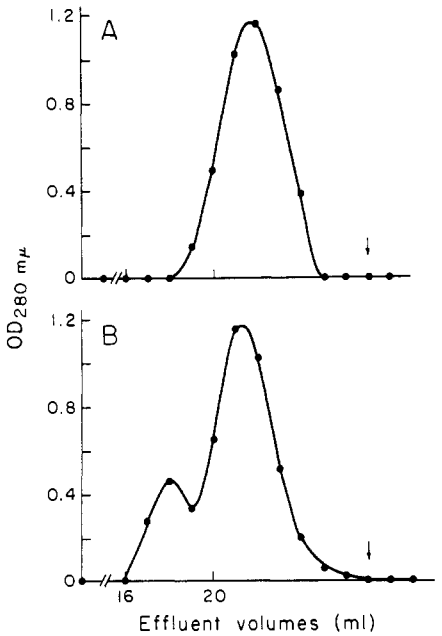

FIG. 1.—Gel filtration of native 7S γ-globulin and reconstituted material on Sephadex G-200 in 0.05 M Tris-HCl buffer containing 0.15 M NaCl, pH 8.0. (A) Native 7S γ-globulin; (B) material reconstituted from reduced and alkylated chains. The mass ratio of H-L in the original mixture was 6:1. The arrow indicates the peak activity of alkaline phosphatase added to the mixtures prior to gel filtration.

tration of 0.2 M. After 10 minutes at room temperature, the solutions were dialyzed overnight at 4° against 0.5 N propionic acid. The H and L chains were separated by gel filtration on Sephadex G-100 (Pharmacia, Uppsala, Sweden) in 0.5 N propionic acid (Fleischman *et al.*, 1962). A load of 20 mg of reduced alkylated protein was applied to a column having dimensions of 100 × 1.0 cm; the flow rate averaged 6 ml/hour and 1.0-ml fractions were collected. Columns measuring 100 × 4 cm were also used, in which case the load was 80 mg, the flow rate was 20 ml/hour, and 4-ml fractions were collected. In some instances prior dialysis against propionic acid was omitted, and reduction and alkylation were immediately followed by gel filtration.

In several experiments the H-chain fractions were individually submitted to a second gel filtration under the same conditions used for the initial separation. The fractions constituting each peak were pooled and the protein concentration was estimated from the absorbancy at 280 mμ. An aliquot of each pool was assayed for radioactivity in a dual-channel well-type scintillation counter (Nuclear Chicago, Des Plaines, Ill.) and the specific activity was calculated (Olins and Edelman, 1964).

In two instances the reduction of labeled γ-globulin was not followed by alkylation. The solutions containing the reduced polypeptide chains were loaded directly onto the Sephadex columns and were separated as described.

Reconstitution of Doubly Labeled 7S Molecules from Separated Chains.—Reduced alkylated L chains and H chains having different isotopic labels were mixed while still in 0.5 N propionic acid and dialyzed against three successive 4-liter portions of 0.05 M Tris-HCl buffer, pH 8.0, for a least 72 hours at 4°. In different experiments the relative proportions of H chains to L chains in the mixtures ranged from 1:1 to 6:1 (expressed as the ratios of their absorbancies at 280 mμ).

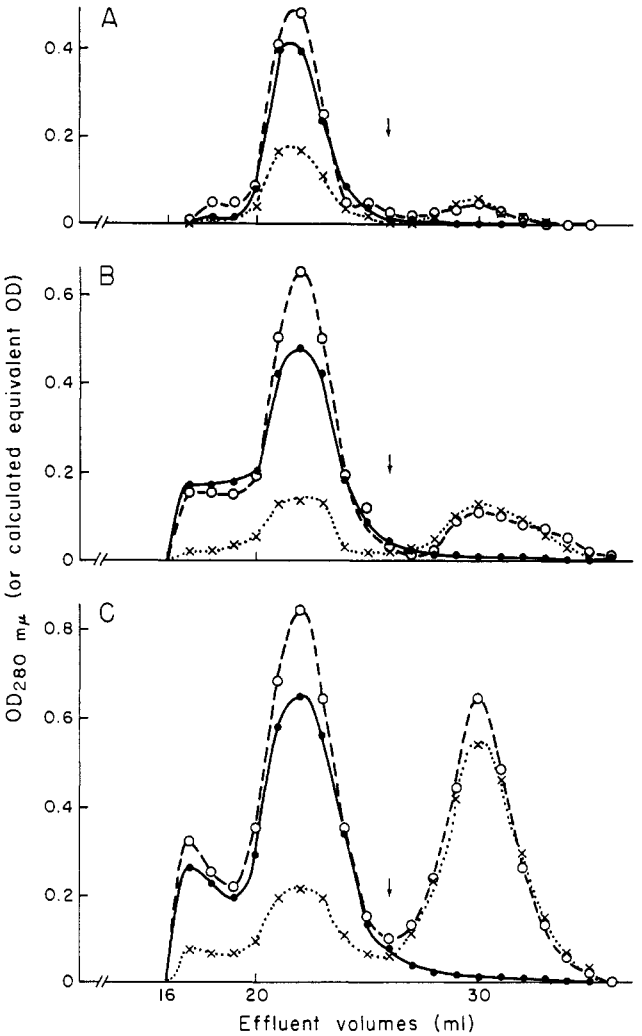

FIG. 2.—Separation upon Sephadex G-200 of reconstituted molecules prepared from reduced and alkylated H and L chains mixed at three different mass ratios. (A) Mass ratio of H-L = 3:1 in the original mixture; (B) mass ratio of H-L = 2:1 in the original mixture; (C) mass ratio of H-L = 1:1 in the original mixture. - - ○ - - ○ - -, OD 280 mμ (absorbancy at 280 mμ); —●—●—, amount of ^{125}I-labeled H-chain fraction; and - - × - - × - -, amount of ^{131}I-labeled L-chain fraction estimated by assay of radioactivity and converted into equivalent absorbancy values by the use of the known specific activities of the chains. The arrow indicates the peak of activity of alkaline phosphatase.

When reduced polypeptide chains were prepared without subsequent alkylation, the mixtures were dialyzed overnight against 0.05 M Tris, pH 8.0, made 0.1 N in mercaptoethanol. The disulfide bonds were then allowed to re-form (Olins and Edelman, 1964) by reoxidation during dialysis against three successive 4-liter portions of Tris-HCl buffer, pH 8.0, for a period of 3–4 days.

Separation of 7S Reconstituted Materials.—After dialysis the mixtures were concentrated by ultrafiltration and centrifuged in a clinical centrifuge at 500 *g* for 15 minutes. The supernatant, containing 2–10 mg of protein per ml, was then loaded on a column (50 × 1 cm) of Sephadex G-200 in 0.05 M Tris-HCl, pH 8.0, 0.15 M NaCl. The flow rate averaged 3.0 ml/hour. In order to serve as a marker for emergence of 6S-7S protein, 1 μg of alkaline phosphatase (s_{20} = 6.1 S) was loaded simultaneously (Olins and Edelman, 1964). The peak of phosphatase activity was used as a reference in comparing several experiments. Mass ratios

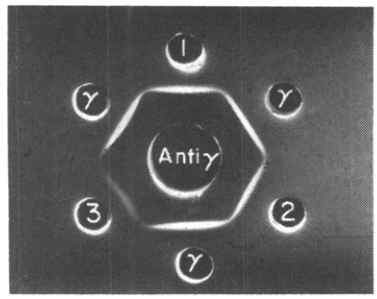

FIG. 3.—Comparison by immune diffusion of native γ-globulin (γ) and three different preparations of 7S molecules (1,2,3) obtained by reconstitution from reduced and alkylated chains mixed at a mass ratio of H-L = 6:1. Anti-γ, rabbit antiserum against human γ-globulin.

of H chains to L chains in each peak were calculated from the following equation:

$$A = \frac{\text{counts per minute}}{\text{sp. act.}}$$

where A is the estimated absorbancy and sp. act. is the specific activity of each chain fraction (i.e., cpm/unit absorbancy at 280 mμ) (Olins and Edelman, 1964).

Separation of F and S Fragments.—The reconstituted 7S = labeled material was hydrolyzed with papain. The conditions were those defined by Porter (1959) except that the hydrolysis was allowed to proceed for only 1 hour. Normal human γ-globulin was also hydrolyzed under the same conditions in order to provide S and F fragments (Edelman *et al.*, 1960) for immunological comparison. In both cases, the products were separated by starch-block electrophoresis (Olins and Edelman, 1962) at a potential gradient of 8 v/cm for 20 hours. The block was cut into half-inch segments which were eluted with normal saline. Each fraction was then assayed for radioactivity in the dual-channel well-type scintillation counter.

Immunological Methods.—Ouchterlony double diffusion in agar was modified as previously described (Olins and Edelman, 1962). Immunoelectrophoresis was performed according to the micromethod of Scheidegger (1955). Rabbit antisera against human γ-globulin, S fragments, and L chains were used.

RESULTS

Isolation and Identification of Reconstituted 7S Material.—An example of the separation of reconstituted material by gel filtration on Sephadex G-200 in Tris-NaCl (see Materials and Methods) is presented in Figure 1. The mass ratio of H chains to L chains in the initial mixture made in propionic acid was 6:1. Two peaks were observed. The second peak emerged at the same position as native 7S human γ-globulin, as shown by a control experiment (Fig. 1A) in which the peak activity of alkaline phosphatase was used as a reference. The H-L mass ratio (see Materials and Methods) in the second peak of the reconstituted mixture was 3.9:1. The proportion of H chains in the first peak was much higher, the H-L mass ratio being 9:1. Mixtures both of reduced and alkylated chains and of chains reduced without subsequent alkylation were submitted to gel filtration under the above conditions. The results were the same in either case.

In Figure 2 are shown the patterns obtained using mixtures prepared with different proportions of reduced alkylated H and L chains. Different amounts of ^{131}I-labeled L chains were added to the same amount of ^{125}I-labeled H chains and gel filtrations of the three

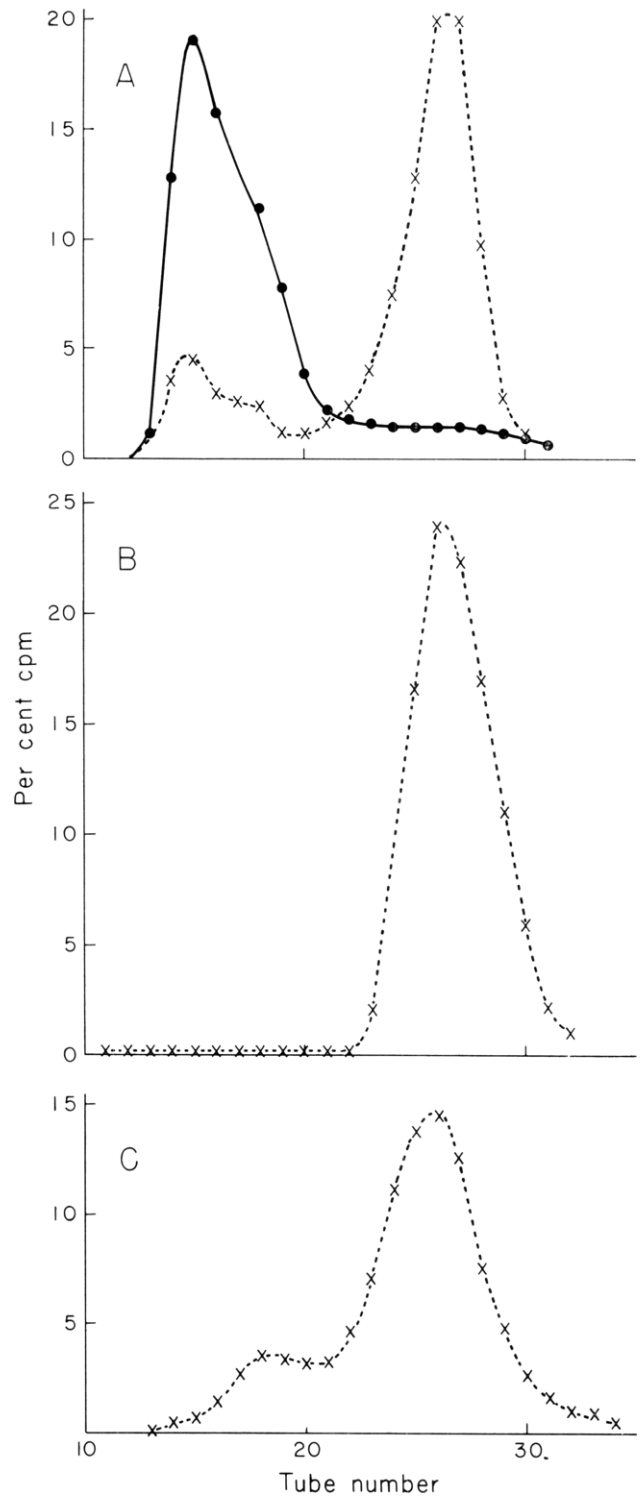

FIG. 4.—Gel filtration on Sephadex G-100 in 0.5 N propionic acid. Comparing (A) 7S material reconstituted from reduced and alkylated chains, (B) L-chain fraction kept in 0.5 N propionic acid after initial isolation, and (C) L-chain fraction after dialysis over a period of 72 hours against a neutral buffer. —●—, ^{131}I-labeled H chains; - - - × - - -, ^{125}I-labeled L chains. Per cent cpm, counts per minute in each fraction expressed as per cent of total counts for each isotope recovered in all fractions after gel filtration.

preparations were performed simultaneously. In the experiments illustrated in Figure 2A,B,C the mass ratios of H-L chains in the starting mixtures were 3:1, 2:1, 1:1 respectively.

As estimated by measurements of the absorbancy at 280 mμ, more 7S material was recovered when the

TABLE I
CONTRIBUTION OF H AND L CHAINS TO WHOLE RECONSTITUTED 7S γ-GLOBULIN AND TO FRAGMENTS PRODUCED BY HYDROLYSIS WITH PAPAIN

	H-L Mass Ratio in Original Mixture	H-L Mass Ratio in 7S Reconstituted Material	H-L Mass Ratio in S Fragment[a]	H-L Mass Ratio in F Fragment[a]	Mass Ratio S-F	Per Cent of H-labeled Material in Fragments S	F	Per Cent of L-labeled Material in Fragments S	F
Experiment 1									
H(^{131}I)L(^{125}I)	6:1	4.8:1	2.8:1	10:1	1.6:1	57	43	80	20
Experiment 2[b]									
H(^{131}I)L(^{125}I)	6:1	3.9:1	2.4:1	10:1	1.71:1	58	42	82	18
Experiment 3									
H(^{125}I)L^{1}(^{31}I)	6:1	3.7:1	1.83:1	7:1	1.9:1	62	38	79	21
Experiment 4									
H(^{125}I)L(^{131}I)	3:1	3.52:1							
	2:1	3.12:1							
	1:1	2.95:1	1.95:1	7:1	1.85:1	59	41	82	18
Experiment 5									
H(^{131}I)L(^{125}I)	3:1	3.9:1							
	2:1	3.76:1							
	1:1	3.46:1							
Experiment 6 (reoxidized material)									
H(^{125}I)L(^{131}I)	1:1	3.3:1	2:1	10:1	1.65:1	55	45	88	12
H(^{131}I)L(^{125}I)	1:1	3.4:1	2.1:1	8.5:1	1.7:1	57	43	83	17

[a] The material migrating toward the cathode was referred to as the S fragment. The remainder was considered to be the F fragment. [b] The H chains were submitted to a second gel filtration before mixture with L chains.

proportion of L chains in the starting mixture was increased. Mass ratios of H to L chains in the pooled material of the 7S peak were calculated from the known specific activities of the starting material. The results, presented in Table I (expt. 4), showed that the calculated H-L mass ratio in the reconstituted product approached the lowest values when an excess of L chains was added to the initial mixture.

When the H-L mass ratio in the original mixture was 3:1, 65–70% of the L chains were incorporated into the 7S material (second peak); the remainder was eluted in a third peak after gel filtration on Sephadex G-200 (Fig. 2A). The calculated H-L mass ratio in the second peak was 3.52:1. With a mass ratio of 2:1 in the starting material, 40–45% of the L chains were incorporated (Fig. 2B). When a 1:1 starting mass ratio was used, the H-L mass ratio in 7S peak was close to the value of 3:1 expected for the native molecule assuming a molecular weight of 60,000 for the H chains and 20,000 for the L chains (Edelman, 1963b); 30% of the L chains were incorporated in reconstituted 7S material (Fig. 2C).

Other experiments similar to that shown in Figure 1, indicated that when the original mixture was prepared with an excess of H chains (6:1 mass ratio) almost 100% of L chains were recovered in the 7S reconstituted material, in agreement with previous results (Olins and Edelman, 1964). As emphasized above, however, when an excess of L chains was added to the starting mixture, the total recovery of reconstituted 7S material was higher, and the H-L ratio of the 7S peak was closer to the ratio expected to be present in the native molecule. In Table I (expt. 5) are presented the results of the same sequence of experiments performed with alternate labels. The results were similar except that the H-L ratios were slightly higher.

The reconstituted 7S materials were tested for their antigenic behavior; analyses of three different preparations are shown in Figure 3. Complete antigenic identity was observed between the native γ-globulin and the reconstituted materials when rabbit antiserum to human γ-globulin was employed.

It has been shown in a previous communication (Olins and Edelman, 1964) that 7S material reconstituted from alkylated chains was redissociated into its components when placed in a dissociating solvent or when examined by starch-gel electrophoresis in urea. In the present experiments an aliquot of reconstituted 7S material was filtered through a column of Sephadex G-100 in 0.5 N propionic acid. As shown by the elution curve presented in Figure 4, the dissociation was almost complete. The L chains were well resolved and the H chains did not show further dissociation, as indicated by the very small amount of H-labeled material present in the L-chain peak (Fig. 4A). Although a small amount of L chains was present in the first peak, isolated L chains were shown to be free of H contaminant when submitted to a second gel filtration before mixing (Fig. 4B). When isolated L chains were dialyzed against neutral aqueous buffer over a period of several days and then subjected to gel filtration in 0.5 N propionic acid (Fig. 4C), their behavior was similar to that found for L chains of the reconstituted globulins (Fig. 4A).

Hydrolysis of Reconstituted 7S Material with Papain.—About 3 mg of reconstituted 7S material was digested with papain in each different experiment. Because of the small amount of material used, the mass of protein in each fraction eluted from the starch block was determined by assay of radioactivity (see Materials and Methods). Typical separations of F and S fragments of 7S material reconstituted from reduced alkylated and reduced reoxidized chains appear on Figure 5. All of the material that migrated toward the cathode was considered to be S fragment; the remainder represented the F fragment. The patterns were similar, although the yield of F fragments from the reduced reoxidized preparation was higher. The calculated S-F mass ratios ranged from 1.6:1 to 1.9:1. Label associated with L chains migrated mainly with the slowly moving fraction; in different experiments, the percentage recovered in the S fragment varied from 79% to more than 88%. The remainder was found partly in the peak corresponding to F fragments and partly in faster-moving degradation products. Isotope associated with H chains was distributed between S and F fragments in the relative amounts of 58% and 42%, respectively. Calculated mass ratios of labeled

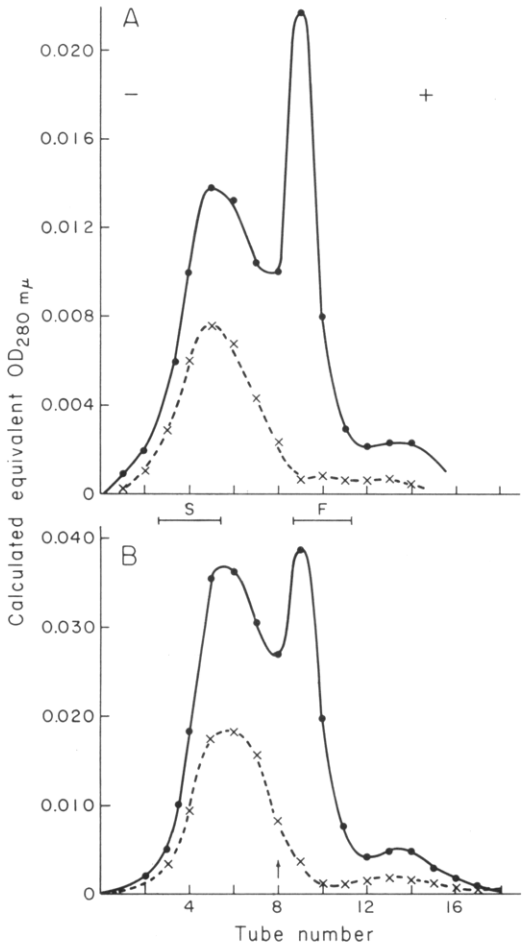

FIG. 5.—Isolation by zone electrophoresis on starch of S and F fragments from reconstituted 7S material hydrolyzed with papain. (A) 7S material obtained by reassociation of reduced chains; (B) 7S material obtained by reassociation of reduced alkylated chains. —●—, H-chain material labeled with ^{125}I; - - - × - - - × - - -, L-chain material labeled with ^{131}I. The arrow indicates the origin; (−) = cathode, (+) = anode.

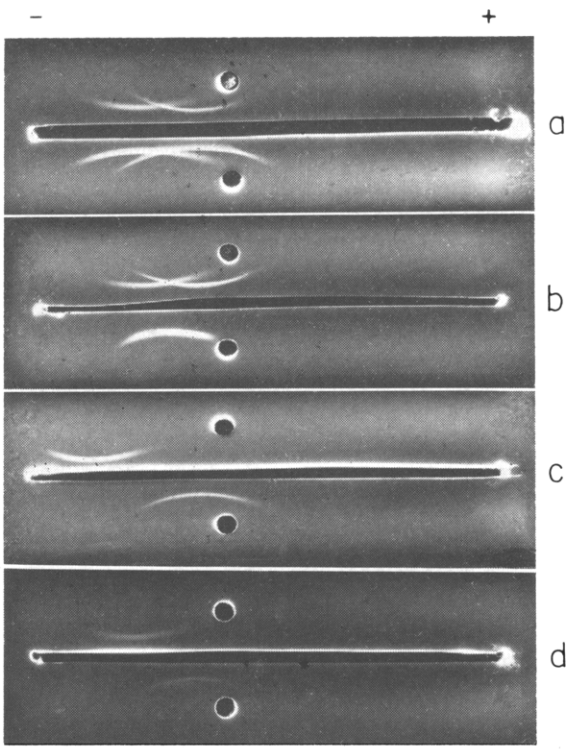

FIG. 6.—Identification of fragments of reconstituted material by immunoelectrophoresis. (a) 7S material reconstituted from reduced and alkylated chains (top) and native 7S γ-globulin (bottom) after hydrolysis with papain; (b) reconstituted reoxidized 7S molecules (bottom) and fragments obtained from this material after hydrolysis with papain (top); (c) S fragment (top) and F fragment (bottom) isolated from native γ-globulin after hydrolysis with papain; (d) S fragment (top) and F fragment (bottom) isolated from 7S material reconstituted from reduced and alkylated chains. Rabbit antiserum against human γ-globulin was used.

H-chain material to labeled L chains in the S fragments of reconstituted molecules varied from 2.8:1 to 1.8:1 (Table I). In the F fragment, H-chain material was predominant (7:1 to 10:1). All these values are summarized in Table I.

The separation of S and F fragments was clearly demonstrated by immunoelectrophoresis as shown in Figure 6a. There was a close resemblance to the patterns given by the fragments of native human γ-globulin and no cross reaction was observed between the two components. Similar separation was observed with reconstituted and reoxidized material (Fig. 6b). In Figure 6c,d are shown the patterns obtained by immunoelectrophoresis of F and S fragments isolated by starch-block electrophoresis. In this case the fragments were isolated as indicated on the elution curves shown on Figure 5. The mobility of the S fragment of reconstituted γ-globulin was slightly different from the S fragment of native γ-globulin. Similar patterns were found after immunoelectrophoresis of different preparations.

The antigenic identity of S and F fragments from normal and reconstituted γ-globulin was established by double diffusion in agar. A reaction of identity was obtained between normal S fragment and its counterpart from reconstituted γ-globulin, as tested with a rabbit antiserum to human γ-globulin (Fig. 7). Identity was also found for the respective F fragments (Fig. 7). An additional faint line was seen in the F fragments of reconstituted material.

DISCUSSION

It has been suggested (Edelman and Benacerraf, 1962; Edelman *et al.*, 1963a,b) that both H and L polypeptide chains are involved in the antibody activity of 7S γ-globulin molecules. Recovery of specific combining activity toward antigen following admixture of separated chains (Edelman *et al.*, 1963b; Edelman, 1963a; Franěk and Nezlin, 1963a,b) has strengthened this hypothesis and has prompted a search for active reconstituted 7S material. Evidence for the recovery of such reconstituted material has been recently reported (Olins and Edelman, 1964; Roholt *et al.*, 1964). Although reconstituted molecules have not been shown to be identical to 7S γ-globulin, there was close resemblance in their sedimentation coefficients, molecular weights, electrophoretic mobility, and immunological properties. The present studies provide additional evidence of a close resemblance between 7S material reconstituted from separated H and L chains and native 7S γ-globulin. Both materials had the same behavior upon gel filtration on Sephadex G-200, their immunological properties were similar, the number of chains appears to be the same, and the arrangement of chains is alike in each.

The values of the H-L mass ratio in the 7S reconstituted material are particularly important in assessing

the degree of resemblance between native and reconstituted molecules. The best available estimates (Edelman, 1963a,b; Pain, 1963; Small *et al.*, 1963) indicate that the molecule of 7S γ-globulin is constituted of two H chains (each having a molecular weight of 55,000–60,000) and two L chains (each having a molecular weight of 20,000–25,000). As a consequence, the mass ratio of H chains to L chains must be within the range of 2.5:1 to 3:1. The values of this ratio differed appreciably in the different experiments on reconstituted materials. Nevertheless, when mixtures were prepared simultaneously from the same pool of chains, the mass ratio approached theoretical values when an excess of L chains was added. It seems reasonable to propose that in this case the H chains were saturated with L chains. At lower L-chain concentrations the population of molecules in the 7S zone would be heterogeneous, an appreciable proportion of them possibly containing only one L chain. The above assumptions are supported by experiments on the "solubilization" of the H chains by the L chains (Olins and Edelman, 1964). Deviations in the H-L ratio might also result from contamination of H-chain fractions with L chains prior to mixing, leading to labeling of L chains as H material. The relative contribution of contaminating L chains would be decreased by dilution when an excess of distinctively labeled L chains was added. This interpretation is supported by the data indicating that the H-L mass ratio was lower when H chains which had been subjected twice to gel filtration were used. (Table I, compare expts. 1 and 2.)

Redissociation of the 7S material used in the latter experiments (Fig. 4) confirmed that the H chains did not contain large amounts of free L chains. It should be emphasized that 15–20% of the isolated L chains remained aggregated when submitted to gel filtration in 0.5 propionic acid after exhaustive dialysis against neutral buffer. The presence of this reaggregated material suggests that dimerization of L chains might have occurred (Gally and Edelman, 1964). The L material in the H-chain peak (Fig. 4A) probably represents the counterpart of the aggregated L chains observed in Figure 4C; it could also represent a fraction of L chains that remained associated with the H chains. In either case, the reconstituted 7S material would not completely redissociate in 0.5 N propionic acid.

The gross arrangement of polypeptide chains in 7S material reconstituted both from alkylated and nonalkylated chains was similar to that of native 7S γ-globulin, as shown by the fact that after cleavage of the two kinds of molecules with papain the fragments obtained had similar properties. The electrophoretic behavior appeared to be similar, although the fragments derived from reconstituted material showed a slightly lower mobility than the native F and S fragments. It is possible that the chains present in the reconstituted molecules may have been slightly altered in tertiary structure during the reduction and separation procedures so that a change in net charge may have resulted upon reconstitution. S fragments and F fragments of native and reconstituted material were antigenically identical. In accord with earlier findings (Edelman *et al.*, 1960) there was also evidence that hydrolysis with papain produced minor components of higher mobility than the F fragment.

Hydrolysis of native γ-globulin by papain yields twice as much of the S fragment as of the F fragment on a mass basis (Edelman *et al.*, 1960). The same relative yield of S and F fragments was obtained after hydrolysis of 7S reconstituted material having an H-L mass ratio of 3:1 (i.e., an approximate mole ratio of unity). Analysis of the labeled material from the H chain indicated that the S fragment contained slightly more of this material than did the F fragment. The best estimation for the mass ratio of H chains to L chains in the S fragment was 1.83:1. This ratio appears to be slightly higher than the ratio expected if one assumes molecular weights of 55,000 for the H chains, 20,000 for the L chains, and 50,000 for the S fragment. Since the S fragment appears to contain one L chain, the contribution of material from the H chain in the S fragment would be 30,000, leading to a theoretical ratio of H-L of 1.5:1. It should be stressed, however, that the calculated values used in assessing the ratios actually reflect tyrosine residues that have been iodinated. Since there is no evidence of an even distribution of these residues along the chains, nor of an even distribution of labeling, it is conceivable that the contribution of certain portions of the chains to the fractions is over- or underestimated. Specific activities of S and F fragments obtained from singly labeled 7S γ-globulin molecules were found, however, to differ only slightly from one another in several experiments. Incomplete hydrolysis by papain might also result in overestimation of the proportion of the H-chain label present in S fragments.

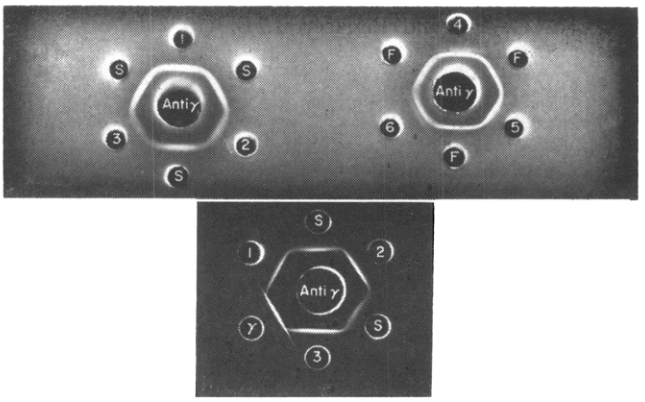

FIG. 7.—Comparison by immune diffusion of S and F fragments of native γ-globulin and products obtained by hydrolysis of reconstituted molecules with papain. (1,2,3) S fragments and (4,5,6) F fragments of three different preparations of 7S reconstituted molecules. S = S fragment and F = F fragment of native γ-globulin (γ); Anti-γ = rabbit antiserum against human γ-globulin.

Previous studies (Edelman and Benacerraf, 1962; Olins and Edelman, 1962; Fleischmann *et al.*, 1963; Small *et al.*, 1963) have already established the relationship existing between F fragments and H chains on one hand, and S fragments and L chains on the other. Additional data obtained by high-voltage electrophoresis of tryptic hydrolysates of polypeptide chains and fragments after reduction of the fragments suggest that the F fragment contains parts of H chains and that the L chains are present relatively complete in the S fragment. The present analysis of doubly labeled reconstituted molecules and their products is consistent with this assignment and implies that the 7S γ-globulin or antibody molecule contains two L chains and two H chains (Edelman and Benacerraf, 1962; Fleischman *et al.*, 1963) probably arranged as one L·H pair for each combining region (Edelman and Gally, 1964).

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Sedimentation-Equilibrium Studies of the Molecular Weight of Single and Double Chains from Rat-Skin Collagen

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Preparations of $\alpha 1$ and $\alpha 2$ single chains and the covalently linked double chain β_{12} ($\alpha 1$-$\alpha 2$) isolated by chromatography from denatured rat-skin collagen were studied by sedimentation equilibrium in *p*H 4.8 potassium acetate buffer at 40° and in 5 M guanidine at 7°. Comparison of the observed distribution of protein with the theoretical distribution calculated from the concentration dependence of molecular weight indicated that $\alpha 1$ was essentially homogeneous. Samples of $\alpha 2$ showed contamination. Although β_{12} was unstable at 40°, studies in 5 M guanidine indicated good homogeneity. It was concluded that the molecular weights of $\alpha 1$ and $\alpha 2$ are 98,000 ± 5,000. The molecular weight of β_{12} is 196,000 ± 10,000, a value consistent with its structure. Sedimentation coefficients and intrinsic viscosities of the various components are consistent with the random-coil structure. These results support the proposal that the collagen monomer contains three α chains (two $\alpha 1$ and one $\alpha 2$).

Chromatographic studies of denatured collagen have provided strong evidence that newly synthesized molecules consist of three chains of which two, designated $\alpha 1$, are apparently identical while the third, designated $\alpha 2$, has a different amino acid composition. In most collagen samples intramolecular cross-linking produces covalently linked double chains of two types, $\alpha 1$-$\alpha 2$ and $\alpha 1$-$\alpha 1$. The double chains are called β_{12} (for $\alpha 1$-$\alpha 2$) and β_{11} (for $\alpha 1$-$\alpha 1$((Piez *et al.*, 1961, 1963).

To support this proposed chain structure and to provide a basis for further structural studies, accurate molecular weights of these various components are necessary. Previous studies, all done with mixtures of single and double chains, have yielded results between 70,000 and 125,000 for single chains and between 160,000 and 290,000 for double chains. Many of these figures were proposed only as approximations. These data have been summarized and discussed by Hannig and Engel (1961). The work reported here was done with samples of $\alpha 1$, $\alpha 2$, and β_{12} which have been demonstrated to represent single molecular species by the criteria of chromatography, amino acid composition, sedimentation velocity (Piez *et al.*, 1963) and acrylamide-gel electrophoresis (Nagai *et al.*, 1964). Short-column sedimentation equilibrium was employed under conditions where the chains would be fully denatured as measured by changes in viscosity and optical rotation (see the review by Harrington and Von Hippel, 1961).

METHODS

Samples.—$\alpha 1$, $\alpha 2$, and β_{12} were isolated from rat-skin collagen by chromatography on CM-cellulose as previously described (Piez *et al.*, 1963). The collagen was prepared either by 1 M salt or 0.5 M acetic acid extraction. Preparations of $\alpha 1$ routinely showed no evidence of other components by the criteria of chromatography, sedimentation velocity (Piez *et al.*, 1963), and gel electrophoresis (Nagai *et al.*, 1964). The $\alpha 2$ samples usually had small amounts of heavier material (presumably β_{12}) which could not be removed by rechromatography. The amount present could not usually be seen by sedimentation velocity in the ultracentrifuge but could be visualized by the more sensitive technique of electrophoresis on acrylamide gel (Nagai *et al.*, 1964). The amount of contamination could not be measured quantitatively but was estimated to be about 5%. The samples of β_{12} sometimes contained a small amount of lighter material but the degree of purity was difficult to evaluate because of the instability of this component. It has not yet been possible to prepare β_{11} in pure enough form to warrant study by the procedures used here.

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