The Subunits of Purified Rabbit Antibody*

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Received April 13, 1964

A method has been developed for the separation of mildly reduced and alkylated rabbit 7S γ-globulin into its subunits. The fractionation was carried out by filtration at room temperature through Sephadex G-200 in a neutral solvent containing 0.03 to 0.05 M sodium decylsulfate. In the analytical ultracentrifuge mildly reduced and alkylated rabbit 7S γ-globulin showed the same sedimentation behavior as the original protein. In detergent solution, however, it exhibited fragmentation at a rate and to an extent that were dependent on the concentration of decylsulfate and the extent of reduction. The splitting of four disulfides was sufficient to provide the maximum yield by gel filtration of the separated components A and B. A second filtration of these components and the further extensive reduction of component A indicated that components A and B were distinctive components. Immunochemical analysis by the Ouchterlony method with monospecific goat anti-A and anti-B sera showed that A and B were antigenically distinct and unrelated components. Their antigenic relationship to papain pieces II and III as shown by these antisera is in accord with the scheme proposed by Porter. Purified rabbit antihapten antibody was separated into components A and B and immunologic activity was evaluated by the binding of a homologous hapten using the method of equilibrium dialysis. The unitary free energy for the binding of the hapten by the A component was 87% of that of the mildly reduced and alkylated antibody (−8.28 kcal/mole versus −9.55 kcal/mole). It was concluded tentatively that the antibody-combining region is exclusively associated with the A chain and that the stated difference was probably due to aggregation and/or denaturation of the A chain. It is suggested that the B chain serves to prevent these effects and thereby indirectly contributes to the immunologic reactivity. The apparent partial specificity of the B chain in enhancing the activity of the isolated A chain, reported by others, is attributed to a selection among the varieties of B chains of those which are most appropriate for complex formation with that portion of the A chain whose conformation is associated with its specific reactivity.

Recent studies of the products of the reductive cleavage of 7S γ-globulin from several species have demonstrated that these molecules are composed of several polypeptide chains held together in covalent linkage by disulfide bonds (Edelman, 1959; Edelman and Poulik, 1961; Franěk, 1961; Ramel *et al.*, 1961). The observations of Edelman and Poulik (1961) have indicated, furthermore, that at least two types of chains are present in γ-globulin. More recently, the important discovery was made by Fleischman *et al.* (1962) that 7S γ-globulin could be fractionated by gel filtration into two components following reduction under conditions which led to the breakage of a maximum of five disulfide bonds. This extent of reduction is in excellent agreement with observations made earlier with bovine γ-globulin (Markus and Karush, 1957; cf. Katchalski *et al.*, 1957) and rabbit γ-globulin (Karush, 1958) reduced under similar conditions. The fast-moving component, called A, has a molecular weight of about 50,000 (Pain, 1963; Small *et al.*, 1963) and retained some of the immunologic activity of the antibody from which it was derived (Fleischman *et al.*, 1963). The slow-moving component, called B, has a molecular weight of about 20,000–25,000 (Pain, 1963; Small *et al.*, 1963) and was devoid of immunologic activity (Fleischman *et al.*, 1963). Each component appeared to be a single polypeptide chain. The A and B chains correspond, respectively, to the H and L chains defined by Edelman and Benacerraf (1962) following the earlier work of Edelman and Poulik (1961). On the basis of the yields of components A and B and their antigenic relationship to the papain-digestion pieces I (II) and III, Porter (1962) has proposed a symmetrical four-chain structure for 7S γ-globulin in which the chains are crosslinked by disulfide groups.

These findings have posed the question of the functional significance of these components in determining the specific immunologic behavior of the intact antibody molecule. Although the A component appears to be essential for activity, the role of the B component remains to be clarified. We have endeavored to answer this question with the use of specifically purified antihapten antibody. In such a system it is not only possible to measure the extent of retention or recovery of combining sites, but it is also possible to evaluate the affinity of the specific interaction. Such information is essential in order to recognize a possible contribution of the B chain to these sites.

In this paper an alternative, convenient, and possibly superior method is described for the fractionation of reduced and alkylated γ-globulin into its A and B components. This method differs from that originally employed (Fleischman *et al.*, 1962) primarily by the use of detergent instead of acid to effect the dissociation and solubilization of the subunits. The evaluation of their activity was made by working with rabbit antibody (anti-Lac)[1] specific for the haptenic group *p*-

* This investigation was supported in part by a Public Health Service Research grant (H-6293) from the National Heart Institute and in part by a Public Health Service Training grant (5 Tl-Al-204) from the National Institute of Allergy and Infectious Diseases. Presented in part before the American Association of Immunology, Atlantic City, April 16–20, 1963.

† Recipient of a Public Health Service Research Career Award (5-K6-Al-14,012) from the National Institute of Allergy and Infectious Diseases.

[1] Abbreviations used in this work: anti-Lac, rabbit antibody specific for the haptenic group *p*-azophenyl-β-lactoside; Lac dye, the dye *p*-dimethylaminobenzeneazophenyl-β-lactoside; MRA-RGG, mildly reduced and alkylated rabbit 7S γ-globulin; ERA-RGG, extensively reduced and alkylated rabbit 7S γ-globulin; RGG, normal rabbit γ-globulin; Lac-BγG, immunizing antigen (diazotized inter-

(*Footnote continued on next page*)

azophenyl-β-lactoside, and by measuring the binding of the structurally related dye *p*-dimethylaminobenzeneazophenyl-β-lactoside (Lac dye). The interaction of Lac dye with purified rabbit anti-Lac antibody has been studied previously (Karush, 1957).

Experimental Procedures

Rabbit γ-Pseudoglobulin.—γ-Globulin was obtained from a pool of rabbit serum by precipitation three times at neutral *p*H with ammonium sulfate at a final concentration of 1.35 M. The pseudoglobulin was taken as the supernatant protein after exhaustive dialysis against distilled water in the cold room. The γ-pseudoglobulin was further purified by chromatography with DEAE-cellulose in 0.02 M potassium phosphate buffer, *p*H 7.20. This procedure was carried out with 1.5–2 g of sample on a 50-g column at room temperature. Usually the first major peak was separated from the second peak, which appeared as a shoulder, and from the extended tail to provide the purified globulin. By the criterion of immunoelectrophoresis such preparations contained no detectable γ_1A-globulin. Sedimentation analysis showed a single 7S component, except that occasionally barely detectable levels of a 9S component were present.

Immunizing and Precipitating Antigens.—These antigens were prepared by diazotizing the intermediate *p*-aminophenyl-β-lactoside (Karush, 1957) and coupling it to bovine γ-globulin (Armour Pharmaceutical Co., Kankakee, Ill.) and human serum albumin (Cutter Laboratories, Berkeley, Calif.). The coupling was done at 0–5° and the *p*H was maintained at 9.5 with a *p*H-stat. For the preparation of the immunizing antigen, Lac-BγG, 1 mmole of the amine was used for 2 g of BγG and for the precipitating antigen, Lac-HSA, 4 mmoles of the amine per 5 g of protein. These azo proteins were freed of noncovalently linked azo constituents by extensive dialysis followed by passage through a column of Sephadex G-25. On the basis of a molar extinction coefficient of 2.48×10^4 at 450 mμ derived from the Lac dye (Karush, 1957), Lac-BγG was found to contain 20.4 haptenic groups per protein molecule and Lac-HSA from 15 to 20 groups per molecule for several preparations.

Preparation of Antisera.—The antigen was administered in complete Freund's adjuvant to male albino rabbits weighing from 4 to 5 kg. Each foot pad received a subcutaneous injection of 0.4 ml of the emulsion, amounting to an initial dose of 8 mg of Lac-BγG per animal. On day 21, a second dose was given intravenously in the form of 0.5 ml of a solution of Lac-BγG in physiological saline at a concentration of 5 mg protein per ml. The animals were bled on days 28, 30, and 32 by cardiac puncture and exsanguinated during the final bleeding. The sera from the individual animals were tested for antibody content by the visual precipitin test with Lac-HSA, and those showing substantial antibody were pooled. Usually seven or eight rabbits of weekly groups of eight animals were usefully positive.

Column Fractionation Procedures.—The separation of the subunits of γ-globulin was carried out by gel filtration through columns of Sephadex G-200 (Pharmacia, Uppsala, Sweden). The column sizes employed were 3 × 45 cm, 3.5 × 50 cm, and 5 × 75 cm. The flow rate was controlled by a Kinetic Clamp pump (Sigmamotor, Middleport, N.Y.). connected to the output end of the column. The flow rates in different experiments ranged from 15 to 30 ml/hour. The effluent was collected with a fraction collector in 5-ml fractions and in some runs was passed through an ultraviolet analyzer (Vanguard Instrument Co., LaGrange, Ill.) to locate the protein peaks. With preparative quantities of protein it was found useful to set the analyzer at 290 mμ rather than at 280 mμ.

Sodium Decylsulfate (SDeS).—This detergent was synthesized by the procedure described by Dreger *et al.* (1944) and recrystallized twice from 95% ethanol.

Purification of Antihapten Antibody.—For the purification of anti-Lac antibody, the protein was precipitated by the addition to 2 liters of antiserum of an amount of Lac-HSA corresponding to slight antigen excess. A typical example is the use of 20 μg of Lac-HSA N per ml of an antiserum containing 107 μg of antibody N. The collected specific precipitate was washed at least five times with cold saline followed by a wash with 0.02 M sodium phosphate buffer, *p*H 7.2, and suspended in 40 ml of 0.5 M lactose in 0.02 M sodium phosphate buffer, *p*H 7.2, including 0.01% merthiolate. The mixture was incubated at 37° until complete solution of the precipitates occurred (1–2 hours).

Removal of the antigen from the clarified solution was achieved by the use of a column of DEAE-cellulose (0.9 meq/g, Bio-Rad Laboratories, Richmond, Calif.). The column (3 × 40 cm) was equilibrated with a 0.5 M lactose solution including 0.02 M sodium phosphate buffer, *p*H 7.2, and 0.01% merthiolate. The chromatography was carried out at 37° with the same solvent at a flow rate of 1.5 ml/minute. The antigen was retained in the column and the antibody was collected in a 200-ml volume virtually free of antigen.

For the separation of lactose from the antibody, two alternative procedures were employed. In the first, the antibody solution was simply dialyzed in the cold room against a large volume of buffered saline for 4 days with daily changes of the external solution. The second procedure was designed to assure complete removal of lactose, both free and antibody-bound. It is based on the displacement of lactose bound to antibody by a hapten which exhibits lower affinity and which also can be readily dissociated from the antibody under practical conditions. The hapten galactose is suitable for this purpose since it is bound about 200-fold less strongly than lactose and has an association constant in the neighborhood of 46 l/m (Karush, 1957).

After overnight dialysis of the antibody solution to remove the bulk of the lactose, the protein was precipitated at room temperature by 40% saturation with ammonium sulfate. The precipitate was dissolved at room temperature with 100 ml. of 0.1 M galactose in 0.02 M sodium phosphate buffer, *p*H 7. The antibody was again similarly precipitated and the cycle was carried out two more times. The precipitate was finally dissolved in buffered saline and the solution was dialyzed in the cold room against neutral buffer followed by distilled water. A small quantity of gelatinous precipitate formed during the last step. The average recovery of purified antibody from the quantity originally precipitated was about 60% in both procedures. A recovery as high as 70% has also been attained. The purity of the antibody preparations was demonstrated by the results of hapten-binding experiments which showed two binding sites per mole of protein. In the analytical ultracentrifuge the preparation showed a single 7S component and gave evidence of only γ_2-

mediate Lac coupled with bovine γ-globulin); Lac-HSA, precipitating antigen (diazotized intermediate Lac coupled with human serum albumin); SDeS, sodium decylsulfate; MRA-anti-Lac, mildly reduced and alkylated anti-Lac antibody; Plac, untreated anti-Lac antibody; MRA-Plac, mildly reduced and alkylated anti-Lac; A-Plac, B-Plac, components A and B, respectively, derived from MRA-Plac; A-RGG, B-RGG, components A and B, respectively, derived from RGG.

globulin when examined by immunoelectrophoresis with sheep antiserum to rabbit serum.

Sulfhydryl Assay.—The sulfhydryl content of samples of reduced protein was measured by the amperometric titration method with silver nitrate (Benesch *et al.*, 1955) in the presence of 1% sodium "Lorol" sulfate (Du Pont).

Reduction and Alkylation.—The preparation of reduced and alkylated derivatives of γ-globulin and antibody was carried out under a range of conditions resulting in a wide span in the extent of reduction. Mercaptoethanol was employed as the reducing agent at concentrations from 0.02 to 1.4 M with urea (recrystallized) ranging from 0 to 12 M. The reduction and alkylation reactions were carried out in Tris buffer at *p*H 8.0, usually at room temperature. At mercaptoethanol concentrations beyond 0.2 M a *p*H-stat was employed with 1 N sodium hydroxide. A reaction vessel with two side arms was used and was operated in an atmosphere of nitrogen. The protein solution, in buffer and urea when necessary, at a concentration between 1 and 2%, was contained in the main chamber and the mercaptoethanol and the iodoacetate were in the side chambers. After passage of nitrogen through the system for 20–30 minutes, the reducing agent was reacted with the protein for 1 hour. The solution in the second side arm, containing sodium iodoacetate and EDTA in Tris buffer, was then added. The amount of iodoacetate was equivalent to 1.5 times the quantity of mercaptoethanol and the final concentration of EDTA was 0.002 M. After a 90-minute reaction time, 0.1 M iodoacetamide (recrystallized) was added with a syringe at a final concentration equal to 10-fold the anticipated concentration of protein sulfhydryl groups. An incubation period of 60 minutes followed this addition.

The alkylated protein was freed of low-molecular-weight components by passage of the reaction mixture through a column of Sephadex G-25 (20 g) previously equilibrated with 0.002 M Tris-HCl buffer, *p*H 8.0, and the protein-containing effluent was lyophilized. For the measurement of protein SH groups formed, a sample of the reaction mixture was removed anaerobically. It was adjusted immediately with 2 N HCl to *p*H 2–3 and EDTA was added to a concentration of 0.002 M. The sample was then passed through a column of Sephadex G-25 previously equilibrated with a solvent at *p*H 2.4 containing 0.010 N HCl and 0.002 M EDTA. This operation was carried out in the cold room with a column large enough to allow nearly quantitative recovery of protein without contamination with reagents.

Sedimentation Analysis.—This was done with a Spinco Model E ultracentrifuge at 59,780 rpm in the temperature range 20–25°. Many of the runs were made in the presence of sodium decylsulfate. Two such solvents were employed: (I) 0.040 M SDeS, 0.20 M NaCl, 0.010 M sodium phosphate buffer, *p*H 7.7, and (II) 0.0125 M SDeS, 0.20 M NaCl, 0.010 M sodium phosphate buffer, *p*H 7.7. For some analyses in solvent II the protein sample was pretreated with 0.050 M SDeS either at room temperature or 37° for 60 minutes, and the solvent composition then was adjusted. The factors used to convert the observed sedimentation values to $s_{20,w}$ are 1.105 for solvent I and 1.067 for solvent II.

Protein Determinations.—Both the micro-Kjeldahl procedure and the Biuret method were used for this purpose (Kabat, 1961).

Hapten Binding.—Measurements of the binding of Lac dye by anti-Lac antibody and by the subunits derived from it were made by the method of equilibrium dialysis at 25° as previously described (Karush, 1957).

Removal of Detergent.—Following the separation of the A and B components by gel filtration, described later, it was usually necessary to remove the sodium decylsulfate contained in the solutions of the fractions. This has been done by the use of ion-exchange resin Rexn-Rg-1 (Fisher Scientific Co.) in the chloride form. Rapid removal was achieved by passage of the protein solution at room temperature through a resin column equilibrated with 0.010 M Tris-HCl buffer, *p*H 7.60, or 0.020 M Tris-HCl buffer, *p*H 8.50. For slow removal of the detergent, the protein solution with SDeS was dialyzed in the cold room against 6 liters of 0.002 M Tris-HCl buffer, *p*H 8.0, in which was suspended 10 g of the resin per 100 ml of protein solution. Several daily changes of the external solvent were made. The concentration of residual detergent was measured by a colorimetric method previously described (Karush and Sonenberg, 1950). A concentration of 1×10^{-5} M was readily detectable by this procedure.

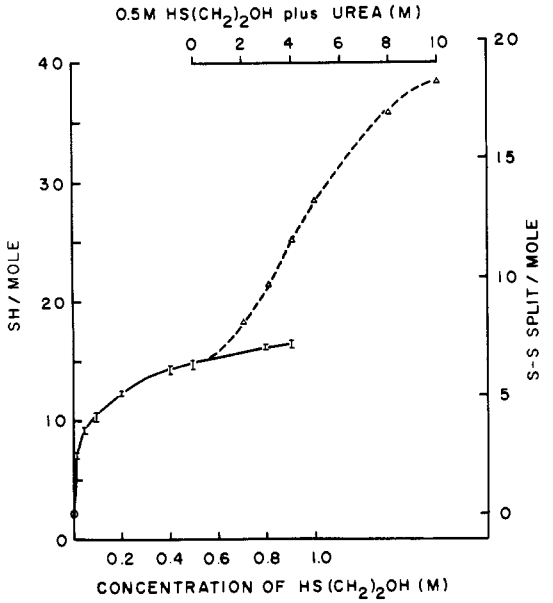

FIG. 1.—Extent of reduction as a function of the concentrations of mercaptoethanol and urea.

Dialysis of the solutions of component A did not lead to significant precipitation until the measurable detergent concentration in this solution fell below 1×10^{-5} M. The precipitate formed after more extensive dialysis was largely insoluble in neutral solvents. Following lyophilization of a solution dialyzed without precipitation, generally from 50 to 80% of the protein was retained in the supernatant of an extract with a neutral solvent after low-speed centrifugation. The opalescent appearance of the extract indicated the existence of aggregates. In the case of the preparation of the A component used for the binding studies, involving reduction with 0.05 M mercaptoethanol, 80% of the lyophilized preparation was retained in the supernatant after the extraction.

Papain Digest of γ-Globulin.—The enzymatic fragmentation of γ-globulin was carried out by digestion with 1% of its weight of crystallized papain (Worthington Biochemical Corp., Freehold, N. J.) at 37° for 10 hours in a solvent containing 0.01 M cysteine, 0.002 M EDTA, and 0.1 M sodium acetate buffer, *p*H 5.5, according to the procedure of Putnam *et al.* (1962). The separation of the reaction products into three fractions (pieces I, II, and III) was effected by chromatography on CM-cellulose following the method described by Porter (1959).

Preparation of Goat Antisera against Components A and B.—Two pairs of goats were immunized with A and

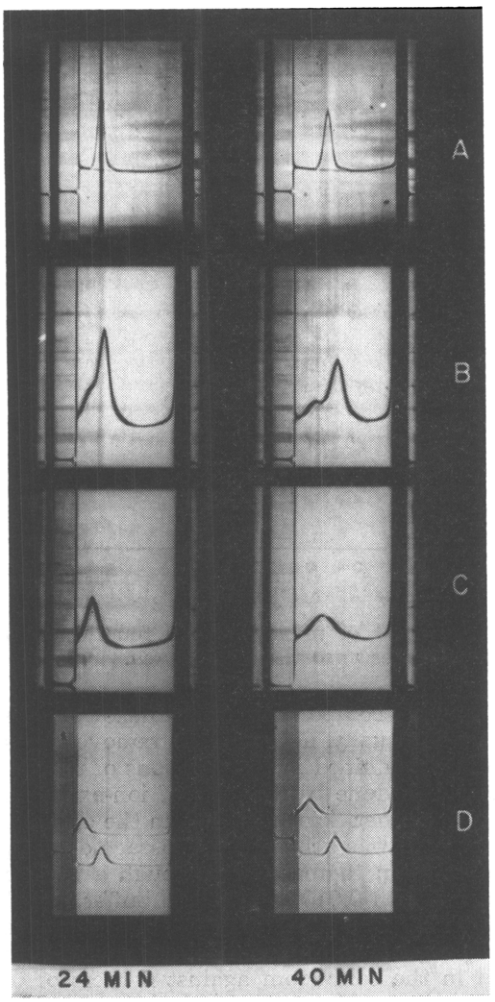

FIG. 2.—Sedimentation of mildly reduced and alkylated γ-globulin (MRA-RGG) and extensively reduced and alkylated γ-globulin (ERA-RGG). Sedimentation is from left to right. A, MRA-RGG in 0.2 M NaCl, 0.01 M phosphate, *p*H 7.7; B, MRA-RGG in 0.2 M NaCl, 0.01 M phosphate, 0.0125 M SDeS, *p*H 7.7; C, MRA-RGG in 0.2 M NaCl, 0.01 M phosphate, 0,0125 M SDeS, *p*H 7.7, after exposure to 0.05 M SDeS; D (upper), ERA-RGG in 0.2 M NaCl, 0.01 M phosphate, 0.0125 M SDeS, *p*H 7.7; D (lower), 0.5% RGG in same solvent.

B, respectively, prepared by mild reduction and alkylation of normal rabbit γ-globulin followed by gel filtration in Sephadex G-200 with SDeS. The initial injection (intramuscular) was made in complete Freund's adjuvant with 20 mg per animal of A and 13 mg per animal of B. Subsequent injections were made at biweekly intervals with 5 mg of protein per animal in incomplete Freund's adjuvant. Bleedings were made usually 10 days following an injection. The antisera were found to contain antibodies to the contaminating component and were rendered monospecific by absorption with an appropriate amount of the contaminant.

Immunochemical Analysis by Gel Diffusion.—Antigenic analysis was carried out by the Ouchterlony (1953) method with microscope slides (2 × 3 in.). A solution of approximately 1% agar in a solvent containing 0.15 M NaCl, 0.005 M EDTA, and 0.02 M potassium phosphate, *p*H 8.0, was used. The shortest distance between the center well and the peripheral wells was 8–9 mm.

RESULTS

Reduction of γ-Globulin.—The extent of reduction of rabbit γ-globuin in the absence of a denaturing agent

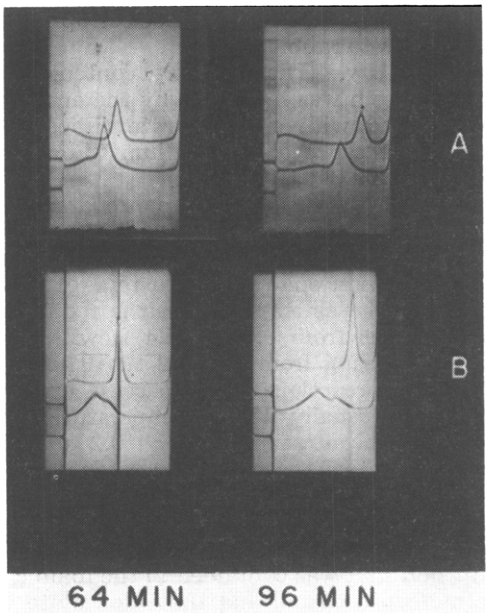

FIG. 3.—Sedimentation of anti-Lac and MRA-anti-Lac in 0.04 M SDeS, 0.2 M NaCl, 0.01 M phosphate, *p*H 7.7. A (upper), 0.5% anti-Lac; (lower), MRA-anti-Lac immediately after solution; B (upper), 1% anti-Lac; (lower), MRA-anti-Lac after incubation in solvent for 2 hours. Sedimentation is from left to right.

was studied as a function of the concentration of the reducing agent. The results are shown in Fig. 1, from which it is evident that a plateau exists corresponding to about 17 or 18 titratable SH groups per molecule of protein based on a molecular weight of 160,000. At a constant concentration of 0.50 M mercaptoethanol, more extensive reduction was achieved by increasing concentrations of urea. The shape of the urea curve indicates, however, that complete reduction had not resulted even at 10 M urea. More direct evidence for this conclusion will be presented shortly.

The estimation from the SH assay of the number of disulfides split required correction for 2.2 SH groups which were found by amperometric titration of untreated γ-globulin. In Figure 1, and in additional results to be presented, this correction has been made. A similar value has been previously reported by Markus *et al.* (1962) who also showed that these apparent SH groups are unusually unreactive towards sulfhydryl reagents, although not completely so.

Because the amperometric titration method is subject to some uncertainty in interpretation (Cecil and McPhee, 1959), further efforts were made to substantiate the reality of the actual or potential SH groups in native rabbit γ-globulin. In Table I are shown the results of treatment of this protein with reagents which might lead to the destruction of SH groups. The incubations were done in air and the disappearance of SH groups might have been due in part to oxidation. In addition, the formation of cyanate from the decomposition of urea could lead to reaction of SH (Christensen, 1952). It is particularly significant that titratable SH is largely destroyed by a 2-hour treatment with a mixture of iodoacetate and iodoacetamide in detergent. We may conclude, therefore, that these apparent SH groups do in fact exist, either as such in the inaccessible interior of the molecule or in a chemically modified but potentially available form such as a thioester.

More extensive reduction than that indicated in Figure 1 can be effected by more extreme conditions. Several experiments were carried out with 10 M urea and 1.40 M mercaptoethanol at an apparent *p*H of 8.5.

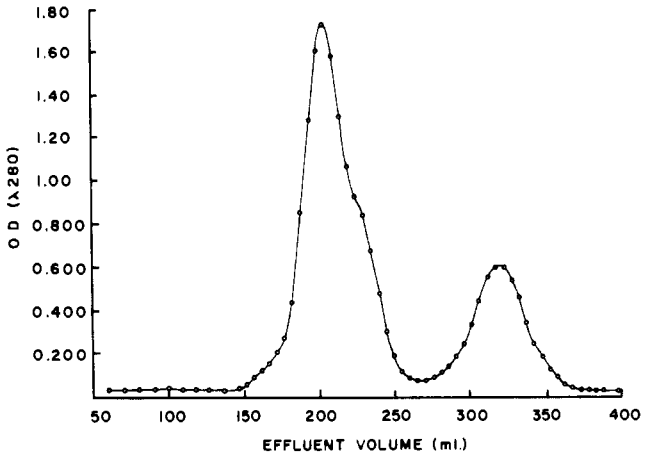

FIG. 4.—Separation by gel filtration with Sephadex G-200 of components A and B from mildly reduced and alkylated rabbit anti-Lac antibody. The first peak is A and second B. Solvent: 0.05 M SDeS, 0.02 M Tris-HCl, *p*H 8.

TABLE I

DESTRUCTION OF THE SH GROUPS OF RABBIT γ-GLOBULIN

Treatment[a]	Time (hr)	SH Groups[b]
None		2.2
Iodoacetate (0.02 M)	2	1.7
Iodoacetate (0.02 M)	20	1.3
Urea (8 M)	2.5	0.7
Urea (8 M)	20	0.6
Iodoacetate (0.01 M), urea (8 M)	20	0.7
Iodoacetate (0.014 M), SDeS (0.033 M)	2	1.5
Iodoacetate (0.007), iodocetamide (0.007 M), SDeS (0.033 M)	2	0.5
Same	20	0.3

[a] Incubations were carried out at room temperature in Tris buffer, *p*H 8.0, with protein concentrations between 1 and 2%. [b] These are the number of sulfhydryl groups per molecule by amperometric titration based on a molecular weight of 160,000.

These gave SH values of about 42 per mole of protein. The maximum extent of reduction was observed when the protein was treated at 40° for 45 minutes with 12 M urea and 0.54 M mercaptoethanol. The titration showed 43.7 SH groups per mole, corresponding to the splitting of 21 disulfides.

Sedimentation Properties of Reduced γ-Globulin.—The effects of limited and extensive reduction of γ-globulin on its sedimentation behavior are shown in Figure 2. In runs A, B, and C the protein (MRA-RGG) had been reduced with 0.1 M mercaptoethanol and alkylated as described. As seen in A this treatment had no apparent effect on the sedimentation of the protein since its behavior was identical with that of the untreated protein. Thus the scission of four disulfide bonds did not lead to the fragmentation of the original molecules. On the other hand, as is evident from B and C, in the presence of 0.0125 M decylsulfate slower-moving components derived from MRA-RGG made their appearance. The dissociation process was relatively slow and could be accelerated by exposure of the altered protein to 0.05 M detergent. When γ-globulin was extensively reduced with 0.5 M mercaptoethanol and 8 M urea, the resulting derivative showed ready and extensive fragmentation in the detergent. A single peak with an $s_{20,w}$ of 2.9 S was observed (run D) compared to 6.5 S for the untreated protein in the same solvent. It may be noted that the detergent did not alter the sedimentation coefficient of γ-globulin.

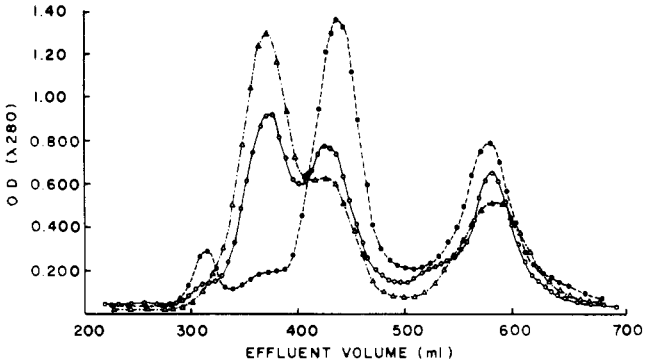

FIG. 5.—Separation by gel filtration with Sephadex G-200 of components derived from reduced and alkylated γ-globulin. The curves have been normalized with respect to the position of component B. Δ···Δ···Δ···Δ, 4 disulfide bonds split; O–O–O–O, 11 disulfide bonds split; ●--●--●--●, 18 disulfide bonds split.

The behavior of mildly reduced (0.1 M) mercaptoethanol) and alkylated anti-Lac antibody (MRA-anti-Lac) in detergent solution is shown in Figure 3. Run A was made immediately after the MRA-anti-Lac was dissolved in the solvent containing 0.04 M decylsulfate and showed a single peak with a reduced sedimentation coefficient ($s_{20,w}$ of 4.8 S) for the altered protein. Continued exposure to the detergent, consisting of a 2-hour incubation in the solvent prior to the sedimentation analysis, resulted in the appearance of a second and slower peak with $s_{20,w}$ equal to 3.4 S. The value of $s_{20,w}$ for the untreated anti-Lac remained 6.4 S, practically unaffected by the monomeric and micellar detergent. As was also apparent from Figure 2, the dissociation of MRA-anti-Lac was a relatively slow process even with the increased detergent concentration.

Separation of Subunits by Gel Filtration.—The separation of two components, A and B, from MRA-RGG can be achieved by gel filtration through Sephadex G-200 in the presence of sodium decylsulfate with virtually complete recovery of protein. Such a fractionation is shown in Figure 4 for purified anti-Lac antibody. The first peak is designated A and the second B. The anti-Lac antibody was reduced with 0.10 M mercaptoethanol with the cleavage of 4 disulfide groups and others with 0.40 M mercaptoethanol with the breakage of 6 disulfides. Similar patterns obtained with various preparations established the reproducibility of the procedure. It was also evident that the intercomponent disulfides are among those most readily reduced since an increase in the number of disulfides broken did not significantly alter the fractionation pattern.

In addition to the main components, there was also present a small quantity of a third component. It appeared consistently as a slight shoulder on the slow side of peak A, but could be emphasized by more extensive reduction as illustrated in Figure 5. The reduction in the relative rate of filtration of this component in the most extensively reduced sample suggest that it is a monomeric form, probably a single polypeptide chain, which exists as a dimer in the mildly reduced preparation. It may be noted that the presence of a small quantity of intact γ-globulin would result in a shoulder on the fast-moving side, but no evidence of this can be seen in Figure 4.

The distribution of protein between the two separated peaks was nearly invariant for mildly reduced samples. The average recovery of component B was 28% in terms of protein nitrogen with an observed variation of about ±10% of this value for patterns

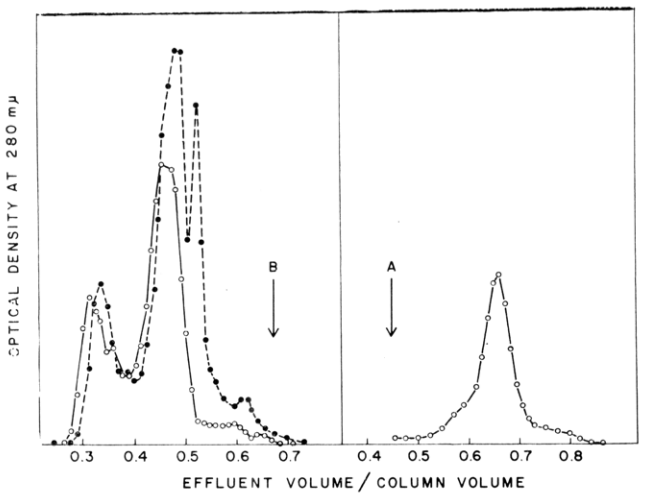

FIG. 6.—Gel filtration of separated components A and B with Sephadex G-200. Solvent: 0.04 M SDeS, 0.01 M Tris-HCl, *p*H 8.0, 0.001 M EDTA. Components A and B were derived from MRA-RGG and MRA-anti-Lac. The arrows indicate the expected positions of A and B. *Left*: O–O–O–O, rerun of component A from RGG; ●--●--●--●, after extensive reduction of component A from anti-Lac. *Right*: O–O–O–O, rerun of component B from RGG.

of the quality shown in Figure 4. The extinction coefficients of A and B were determined in 0.04 M decylsulfate, 0.01 M phosphate, *p*H 7.2. The values obtained for $E^{1\%}$ at 280 mμ were 14.5 and 13.2 for A and B, respectively. These values may be compared to the corresponding figures of 13.7 and 11.8 found by Crumpton and Wilkinson (1963) with 0.01 N HCl as the solvent.

Gel Filtration of Components A and B.—These components derived from mildly reduced and alkylated normal γ-globulin were individually subjected to a second filtration cycle under conditions similar to those employed in the initial separation. As seen in Figure 6, component B yielded a single peak which appeared at its original position. Component A also showed a reproducible peak but also gave rise to an additional peak of larger molecules. This second peak was undoubtedly due to the aggregation of component A during the procedures used for the concentration of the protein from the original separation. The tendency of this component to associate with itself and with component B appears to be a characteristic property of this substance (Fleischman *et al.*, 1962). A similar complexity of component A has been observed where 1 N propionic acid was employed as the solvent with Sephadex G-200 (Fleischman *et al.*, 1963). It is also evident from Figure 6 that the rerun of component A did not reveal the presence of component B as an impurity.

In another experiment illustrated in Figure 6, component A from MRA-anti-Lac was subjected to extensive reduction and alkylation. The purpose here was to evaluate the possibility that this component was constituted of multiple chains, held together by disulfides, of a size comparable to those of the B component. Although the resulting pattern was complex, in accord with the preceding observations, no evidence of any significant quantity of such chains was forthcoming.

Sedimentation of Isolated Components.—These were examined in the analytical ultracentrifuge in two solvents. Component A from MRA-RGG showed in solvent II a broad peak (Fig. 7, A) with an average value for $s_{20,w}$ of 4.8 S. It is evident that there was considerable aggregation of component A in this solvent. In more concentrated detergent (solvent 1) two

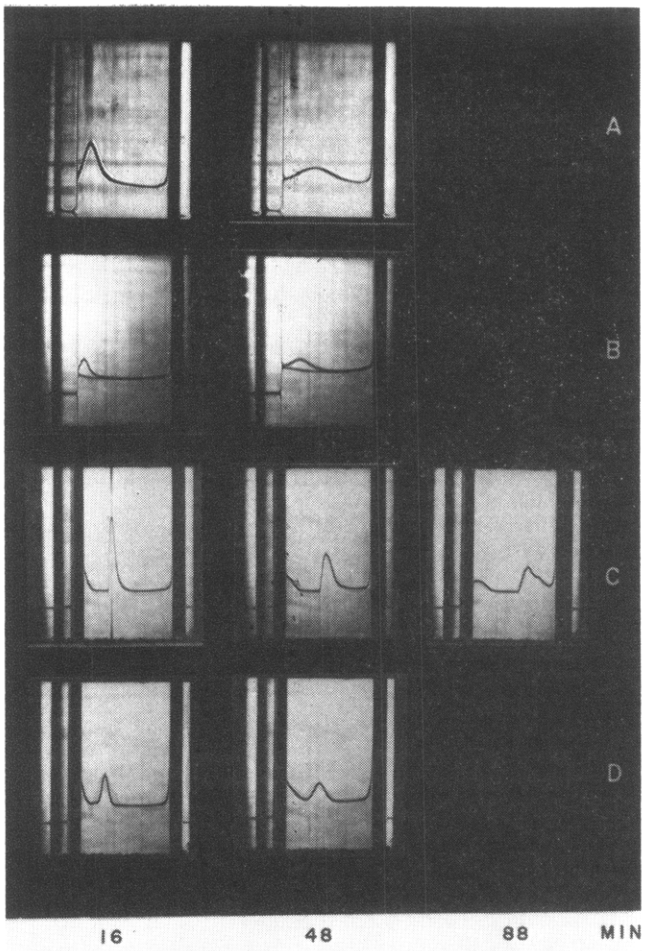

FIG. 7.—Sedimentation of separated components A and B from mildly reduced and alkylated γ-globulin. A, component A in 0.0125 M SDeS, 0.01 M phosphate, 0.2 M NaCl, *p*H 7.7; $s_{20,w}$ = 4.8 S; B, component B in same solvent as (A); $s_{20,w}$ = 2.7 S; C, component A in 0.04 M SDeS, 0.01 M phosphate, 0.2 M NaCl, *p*H 7.7; $s_{20,w}$ = 3.9 S and 2.9 S; D, component B in same solvent as (C); $s_{20,w}$ = 2.7 S.

peaks were seen (Fig. 7, C) with $s_{20,w}$ values of 3.9 and 2.9 S. Component B from MRA-RGG, on the other hand, showed a single peak in both solvents (Fig. 7, B and D) with an $s_{20,w}$ value of 2.7 S. Components A and B derived from extensively reduced and alkylated rabbit γ-globulin were characterized by fairly symmetrical single peaks in solvent II (Fig. 8), with $s_{20,w}$ values of 2.9 and 2.1 S, respectively.

Antigenic Properties of Subunits.—The immunochemical behavior of components A and B has been studied by the Ouchterlony technique using the absorbed antisera described, goat anti-A and goat anti-B. In Fig. 9 are shown the results of an experiment designed to demonstrate the utility of these antisera for the identification of the components. The samples denoted A-Plac and B-Plac were the A and B components derived from mildly reduced and alkylated anti-Lac antibody. The untreated anti-Lac antibody is designated as Plac. Samples A-RGG and B-RGG were similarly prepared from normal rabbit γ-globulin (RGG). Aside from the obvious conclusion that A and B are antigenically distinct components the expected relations were found between these components and the proteins from which they were derived. Thus all of the A-containing samples reacted almost identically with anti-A and similarly those containing B with anti-B. It is evident that the antigenic determinants

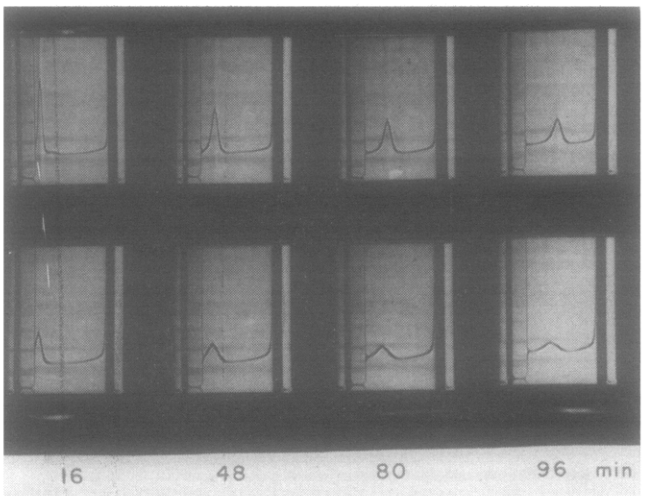

FIG. 8.—Sedimentation of separated components A and B from extensively reduced and alkylated γ-globulin. Solvent: 0.0125 M SDeS, 0.2 M NaCl, 0.01 M phosphate, pH 7.7. Top, component A, $s_{20,w}$ = 2.9 S; bottom, component B, $s_{20,w}$ = 2.1 S.

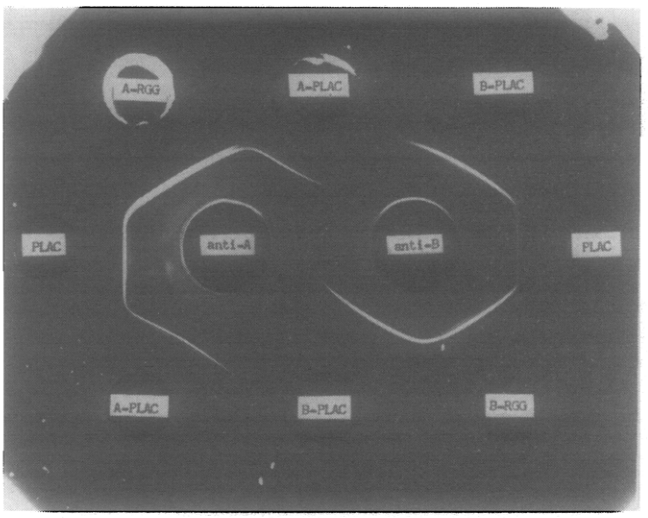

FIG. 9.—Precipitin reactions with goat anti-A and anti-B. Plac is purified anti-Lac, A-Plac and B-Plac are components A and B from anti-Lac, and A-RGG and B-RGG are derived from normal γ-globulin.

which were effective in the induction of anti-A and anti-B antibodies were contained in an accessible form in the original intact antibody.

The antigenic properties of mildly reduced and alkylated anti-Lac (MRA-Plac) with respect to anti-A and anti-B were examined as previously. Before use the preparation was exposed to 0.04 M sodium decylsulfate and diluted to yield a final concentration of 0.01 M detergent. As anticipated, the MRA-Plac showed precipitin lines with both antisera. A spur formed by the reaction with anti-B serum indicated that the dissociation of components A and B which was brought about by 0.04 M detergent was at least partially retained under the conditions of the Ouchterlony analysis.

The goat antisera were also utilized to establish the identity of component A prepared by the detergent procedure described with the fast-moving fraction (A) obtained by gel filtration in 1 N propionic acid, and similarly with component B. The corresponding components were prepared with propionic acid as described by Fleischman *et al.* (1962). The results of this comparison showed that the same kind of separation by gel filtration was effected in the two solvents. Furthermore the corresponding fractions appear to be virtually identical as judged by precipitin analysis.

The antigenic relationship between pieces II and III obtained by limited proteolytic digestion of γ-globulin and the A and B chains is shown in Figure 10. For this comparison a mixture of the goat antisera was placed in the center well. As expected, pieces II and III were antigenically distinct from each other. Of particular interest was the reaction of identity between chain A and piece III. This result implies that the antibodies which the goat formed against A were directed exclusively to antigenic determinants contained in that portion of the A chain carried by piece III. The apparent lack of immunogenicity of the remainder of the A chain, the A piece, was also indicated by the absence of a spur in the precipitin line formed by piece II relative to the precipitin line formed by component B (Fig. 10). A similar behavior was observed for the A (H) chain of human 7S γ-globulin by Olins and Edelman (1962). In this case a reaction of identity was found between the A (H) chain and the F fragment with anti-γ-globulin serum.

The reactions of identity between piece III and component A and between piece II and component B are in accord with the 4-chain structure of γ-globulin proposed by Porter (1962) and his assignments of the sites of enzymatic cleavage. In terms of this structure piece II (I) is composed of a B chain and about one-half the A chain (A piece), and piece III contains a portion of the A chain and none of the B chain.

An antigenic comparison between normal γ-globulin and components A and B was made using again a mixture of anti-A and anti-B with the results shown in Figure 11. It is evident that the normal γ-globulin was capable of precipitating all the anti-A and anti-B antibodies. We may infer therefore that the antigenic determinants of components A and B are present on the γ-globulin molecule in an accessible form. It may be noted that the pieces II and III showed the expected relationship to the original γ-globulin molecule and were indeed the antigenic equivalent of components A and B.

Binding Properties of Subunits.—The immunologic activity of mildly reduced and alkylated anti-Lac antibody (MRA-Plac) and components A(A-Plac) and B derived from it was evaluated by measurement of the specific binding of Lac dye. The MRA-Plac was prepared by reduction with 0.05 M mercaptoethanol in the usual way and fractionated by the detergent procedure. An estimate was made of the extent of contamination of component A with B by immunochemical titration in gel. As shown in Figure 12, 2-fold dilutions of stock solutions of A and B were placed in wells and reacted with goat anti-B. From the relative intensity of the precipitin bands it was estimated that the contamination of A with B did not exceed 4–5%. This value is in good agreement with the figure of 3% obtained by Fleischman *et al.* (1963). They employed a chemical method based on the analysis for N-terminal alanine and aspartic acid.

The results of the binding experiments are shown in Figure 13 in the form of a plot of r/c versus r, where r is the average number of dye molecules bound per protein molecule at the free-equilibrium dye concentration c. Calculations were made on the basis of 2 sites per 100,000 for A-Plac and 2 sites per 160,000 for MRA-Plac. Average association constants (K_A) were obtained from the values of $1/c$ corresponding to $r = 1$. A value of $2.1 \times 10^4\ l/m$ was found for A-Plac and $18 \times 10^4\ l/m$ for MRA-Plac. The affinity for the specific reaction is given by the unitary free energy ΔF_u (Karush, 1962), calculated for 25° from the equation: $\Delta F_u = -1365 \log K_A - 2.38$ in kcal per mole of bound

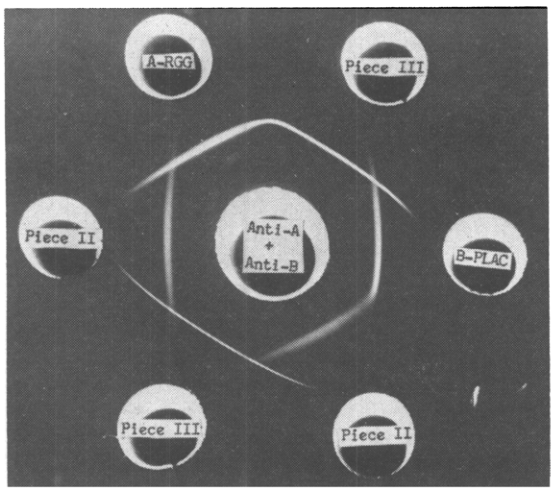

FIG. 10.—The antigenic relationship between the proteolytic pieces II and III and the components A and B. A mixture of goat anti-A and goat anti-B was placed in the center well. Pieces II and III are papain fragments derived from normal γ-globulin (RGG). A-RGG is component A from normal γ-globulin and B-Plac is component B from anti-Lac antibody.

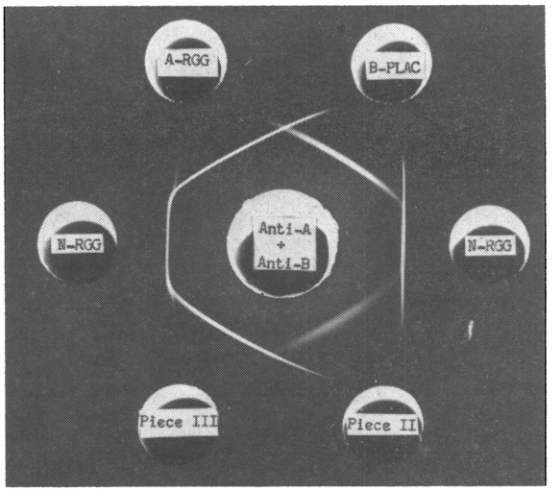

FIG. 11.—The antigenic relationship of normal γ-globulin to components A and B and to the papain fragments II and III. A mixture of goat anti-A and goat anti-B was placed in the center well. N-RGG is normal γ-globulin and the other designations are the same as in Fig. 10.

hapten. The resulting values of ΔF_u are -8.28 kcal/mole for A-Plac and -9.55 kcal/mole for MRA-Plac. There was therefore a 13% loss of affinity for component A relative to that of the parent protein. The specificity of the reactivity of component A was established by the absence of dye binding when component A derived from normal rabbit γ-globulin was used (Fig. 13). It was also found that component B from MRA-Plac was unable to bind the Lac dye (Fig. 13).

DISCUSSION

The results of this investigation demonstrate that rabbit γ-globulin can be conveniently and reproducibly fractionated into antigenically distinct components following mild reduction and alkylation. The use of a detergent-containing solvent at neutral *p*H provides an alternative procedure to that developed by Fleischman *et al.* (1962), in which 1 N propionic or acetic acids were employed. There is an apparent advantage provided by the detergent since with Sephadex G-200 only two peaks, representing A and B, were found, whereas with 1 N propionic acid four peaks were observed of which only the last contained B (Fleischman *et al.*, 1963). It appears that there is more extensive aggregation of component A in the acid solvent than in the detergent solvent.

The 4-chain structure proposed by Porter (1962) involving one pair of B chains and one pair of A chains adequately accounts for the yields of the A and B components and their antigenic dissimilarity. In addition supporting evidence has been provided by these studies for the interpretation (Fleischman *et al.*, 1963) that piece I resulting from papain digestion of γ-globulin consists of a B chain and a portion of the A chain whereas piece III contains only antigenic determinants of the A chain.

The binding results clearly demonstrate that the immunologic activity of the anti-Lac antibody is primarily associated with the A chain. This conclusion is in agreement with the observations on equine antiprotein antibody made by Fleischman *et al.* (1963). They concluded from the specific reactivity of the A chain and the inactivity of the B chain, "that in horse γ-globulin the specific antibody-combining site is associated with chain A." A similar conclusion was reached by Metzger and Singer (1963) with regard to the A chain of rabbit antihapten antibody.

The presence of component B as a contaminant to the extent of 4 or 5% can be ruled out as an important factor in the binding behavior of A-Plac. Since component B would in this case constitute not more than 15 mole %, the binding of Lac dye by an AB complex could not contribute more than 0.3 to the value of r. This may be compared to the largest value of r actually measured, 1.0, as seen in Fig. 13. It is evident, therefore, that at least 70% of the combining regions of A-Plac involve only the A chain. We may note also that even if the AB complex bound the Lac dye with relatively high affinity, because of its minor contribution to the total value of r at which K_A is taken as equal to $1/c$, i.e., $r = 1$, this fact would have little bearing on the value of K_A obtained. In other words the K_A of 2.1×10^4 for A-Plac does indeed characterize the specific affinity of the A chain for the Lac dye.

Since there is the a priori possibility that the antibody-combining region is constituted of parts of each of the two types of chains, the quantitative comparison of the values of ΔF_u for A-Plac and MRA-Plac takes on crucial significance. The binding curves for these preparations show that the affinity of A-Plac for the Lac dye is only 13% less than that of MRA-Plac (-8.28 kcal/mole versus -9.55 kcal/mole). The fact that the isolated A component retains 87% of the affinity found in the undissociated 6.5 S molecule provides cogent evidence that the B chain is not directly involved in the combining region of the antibody since the 13% loss of affinity which is observed may be the consequence of the exposure of component A to denaturing concentrations of the detergent. Furthermore, examination of A with the analytical ultracentrifuge in the same solvent as that used for the binding experiments revealed considerable aggregation. Either or both of these factors, which may be causally related, could reasonably account for the observed difference in affinities. We tentatively conclude, therefore, that the specific combining regions of the antibody molecule are exclusively associated with its A chains.

The validation of this conclusion requires, of course, that the kind of analysis described here for the anti-Lac system be extended to other systems. Experimentation along these lines is currently under way. Furthermore a rigorous evaluation has yet to be made of the possibility that the B chains participate in the combining

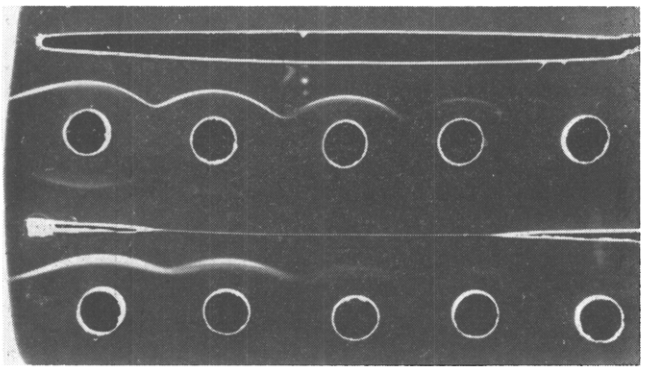

FIG. 12.—Immunochemical analysis by gel diffusion of the contamination of A-Plac with B-Plac. The upper series of wells contains A-Plac starting with 2.3 mg/ml in the left well followed by 2-fold dilutions. The lower series containing B-Plac is similarly arranged with 0.38 mg/ml in the left well. The upper slot contains goat anti-A and the lower goat anti-B.

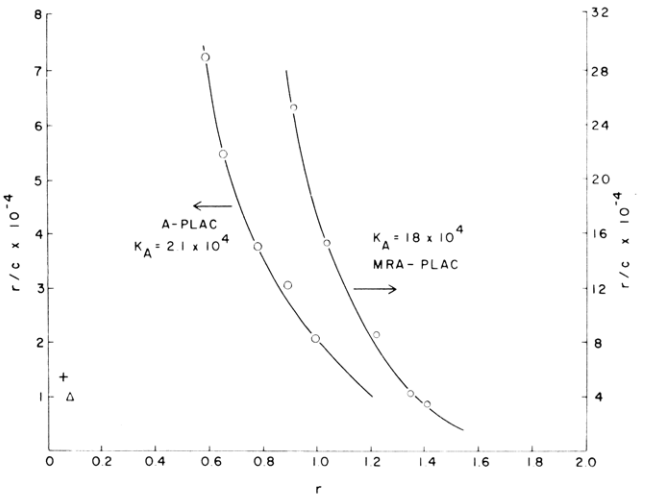

FIG. 13.—Binding of Lac dye at 25° by mildly reduced and alkylated anti-Lac (MRA-Plac) and component A (A-Plac) derived from it. Controls include component B (+) from anti-Lac and component A (Δ) prepared from normal rabbit γ-globulin.

region but their energetic contribution is so small that its expression is manifested only when this contribution supplies an incremental increase to the affinity provided by the A chains. The clarification of the role of the B chains is necessary, furthermore, in order that an unequivocal structural interpretation may be made of the apparent close alignment between combining sites and B chains which is indicated by the labeling of these chains in affinity-labeling experiments (Metzger *et al.*, 1964; Roholt *et al.*, 1963).

In contrast to the exclusive association of the combining site with the A chain a significant role for the B chain in the specific reactivity of the antibody region has been inferred from several recent investigations (Franěk and Nezlin, 1963; Edelman *et al.*, 1963; Roholt *et al.*, 1964). In each case the basis for this inference was the observation that the specific reactivity of the A chain derived from antibody was more or less specifically enhanced by the addition of the corresponding B chain. However, these studies provided neither a quantitative evaluation of the affinities of the A chain and its parent antibody nor a quantitative estimate of the contribution of the B chain to the free energy of the specific reaction. This limitation was partly a consequence of the assay method employed, such as the rate of phage neutralization (Edelman *et al.*, 1963) or the specific adsorption of antibody to a solid phase (Franěk and Nezlin, 1963; Roholt *et al.*, 1964). In the former case a kinetic measurement was obtained which cannot be simply converted into an association constant and which may, indeed, be greatly dependent on such physical factors as aggregation. In the case of antibody adsorption the complexity of the system precluded thermodynamic analysis.

Some measurements were also reported involving equilibrium dialysis with antihapten systems (Edelman *et al.*, 1963; Roholt *et al.*, 1964). Although such experiments can, in principle, provide the desired information, those reported were unable to do so because of their limited scope. Working with guinea pig antibodies against the dinitrophenyl- (DNP) group, Edelman *et al.* (1963) found that the substantial binding of dinitrophenol by the isolated H(A) chain was increased by a factor of 1.7 when the corresponding L(B) chain was present, although the effect of a nonspecific L(B) chain was not reported. A straightforward interpretation of this result is not possible because it was based on an extrapolation to infinite hapten concentration which gave a value of only 0.5 site per mole of native antibody. In the study of Roholt *et al.* (1964) an evaluation was made of the binding of *p*-iodobenzoate by H(A) chain derived from purified rabbit anti-*p*-azobenzoate antibody and the effect of L(B) chains on the binding. Again a substantial enhancement of binding was observed when the effect of the homologous L(B) chain was compared to a normal L(B) chain. Unfortunately, no result was given for the binding of the H(A) chain alone. In any case these results do not lend themselves to quantitative analysis of the affinities of the H(A) and L(B) chains.

Nevertheless in the published results discussed it is apparent that there is interaction between A and B chains in which a substantial element of specificity is involved as well as enhancement of activity. For example, in the neutralization of f1 phage by H(A) chain from guinea pig anti-f1 antibody (Edelman *et al.*, 1963), the addition of L(B) chain from anti-f1 phage antibody is more effective in enhancement than the addition of the L(B) chain from anti-f2. A parallel observation was made in the binding studies of Roholt *et al.* (1964). What is equally striking, however, is that a nonhomologous L(B) chain was also effective in enhancement as was observed when L(B) from either anti-f1 or from anti-DNP was added to H(A) from anti-f2.

A consistent explanation for the behavior of the B chain, similar to the modulation notion of Edelman *et al.* (1963), can be provided in terms of its capacity to combine with A chain and prevent the aggregation of the latter. This tendency appears to be a characteristic feature of the A chain (Fleischman *et al.*, 1962) and may indeed reflect a molecular property essential to its function. The aggregation of the A chain derived from antibody could readily influence the quantitative expression of its immunologic specificity. The recovery of activity on the addition of the homologous B chain would be expected if the experimental conditions allowed for the reversal of aggregation and formation of AB complexes. A basis for partial specificity in this interaction emerges from two considerations. In the first place antibodies of different specificity would possess different conformations in that portion of the A chain which is associated with the B chain (as in piece I). In the second place there appears to be a multiplicity of B chains (Edelman *et al.*, 1961; Fleischman *et al.*, 1963; Cohen and Porter, 1964) from which a selection would be made by the A chain. The notion

is that an A chain from a given antibody molecule would be able to interact more favorably with one or another of the variety of B chains available. This selection would be governed by the conformational relationship of the interacting portions of the two chains and would be presumed to occur during biosynthesis. The studies of Edelman *et al.* (1961) on guinea pig antibodies provide cogent evidence for such a selective process. Following subsequent dissociation the homologous B chain would be expected to form more stable complexes with the A chain than the other kinds of B chains. However, because of the limited variety of B chains and because of their similarity, interaction of the A chain with some or all of the B chains provided by a different population of γ-globulin would be expected. Thus the limited choice in the structural variants of the B chain could result in a partial specificity in the interaction of the A and B chains.

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Evidence for Linkage of Univalent Fragments or Half-Molecules of Rabbit γ-Globulin by the Same Disulfide Bond*

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Received April 30, 1964

Previous work has shown that rabbit γ-globulin dissociates into half-molecules at low *p*H after mild reduction. Also, peptic digestion of γ-globulin removes an inactive fragment, corresponding to a large part of fragment III of a papain digest, leaving a 4.6 S bivalent residue. The latter is cleaved into two univalent fragments, approximately equal in size, by reduction of one labile disulfide bond. Univalent products are similarly obtained by reduction, followed by peptic digestion. The present results indicate that the single, exceptionally labile disulfide bond which links half-molecules in a large part, if not all, of the γ-globulin population is the same bond as that which joins univalent fragments after peptic digestion. The results are also consistent with the hypothesis that half-molecules, consisting of an A and a B chain, are linked in the native molecule by a disulfide bond that joins two A chains. The data provide supporting evidence as to the nature of the structural relationship between the fragments liberated by pepsin and the polypeptide chains of the molecule.

The work of Edelman, Porter, and their collaborators (Edelman and Poulik, 1961; Edelman and Benacerraf, 1962; Fleischman *et al.*, 1962, 1963; Porter, 1962) indicates that the 6.5 S γ-globulin of various species consists of two types of polypeptide chain, designated as "H" (heavy) and "L" (light) (Edelman and Benacerraf, 1962) or, equivalently, as "A" and "B" chains (Fleischman *et al.*, 1962).[1] Each molecule appears to consist of two A chains, each of molecular weight

* This work was supported by grants from the National Science Foundation (GB-1563), and the National Institutes of Health (AI-03552).

[1] The symbols A and B will be used here since they were applied to rabbit γ-globulin.