Relative Labilities of Interchain Disulfide Bonds in Human Immunoglobulin M Subunits*

T. K. S. Mukkur† and F. P. Inman‡

ABSTRACT: The relative labilities of interchain disulfide bonds in the subunits (IgM_s) of human paraprotein immunoglobulin M (IgM) were determined by using mercaptoethylamine (MEA) as reducing agent. IgM was first dissociated into IgM_s in such a way that the subunits' interchain disulfide bonds remained intact. These subunits then were treated with various concentrations of MEA. In the range of 0.06–0.10 M MEA, a total of two disulfide bonds were broken per molecule of IgM_s reduced. However, only about one-half of the total light-chain population was dissociable in propionic acid. When reduction of IgM_s was carried out with 0.13–0.30 M MEA, three disulfide bonds were cleaved per molecule of IgM_s reduced, and all light chains were dissociable in propionic acid. A sample of IgM_s was reduced with 0.10 M MEA. Two interchain disulfide bonds were reduced, but only one-half of the total light-chain population was dissociated in propionic acid. The protein remaining after removal of the dissociable light chains was itself treated with 0.2 M 2-mercaptoethanol. Indirect analysis indicated a total reduction of four disulfide bonds, and all remaining light chains were dissociable in propionic acid. It was concluded that the most labile disulfide bonds in IgM molecules are those joining the subunits to each other. Within the subunit, the disulfide bond linking one of the μ-light-chain pairs was more labile to the action of MEA than that of the other μ-light-chain pair. One of the inter-μ-chain disulfide bonds had a lability similar to that of the most labile disulfide bond associated with a μ-light-chain pair.

Many sera contain one or more of the various classes of immunoglobulins,[1] all of which may possess antibody activity. Although the basic structure of the immunoglobulin now is known, additional detailed information would enhance one's understanding of structure–function relationships. One of those about which additional fine detail is needed is immunoglobulin M, IgM. The intact molecule comprises five similar subunits, each designated IgM_s (Miller and Metzger, 1965a). One subunit is covalently bound to two adjacent subunits by disulfide bridges (Miller and Metzger, 1965b; Morris and Inman, 1968).

IgM_s comprises two μ chains, and two light chains (Miller and Metzger, 1965a,b). One light chain is attached to one μ chain through a single disulfide bond (Chaplin *et al.*, 1965; Miller and Metzger, 1965b) and noncovalent interactions. Two of the light-chain–μ-chain pairs are covalently bound to form one molecule of the basic subunit, IgM_s (Miller and Metzger, 1965a,b). Noncovalent interactions are also implicated in the association of the two μ chains.

Miller and Metzger (1965a) studied IgM and reported that the μ chain of the molecule they investigated was involved with four interchain disulfide bonds. One of these linked the μ chain to a light chain. The exact placement of the other three was not clearly understood. Later, Morris and Inman (1968) presented evidence that each μ chain was joined to a μ chain of an adjacent IgM_s molecule by one disulfide bond, at least in the molecule which they examined. Additional support for this premise was obtained by the controlled use of papain (Inman and Hazen, 1968). The experimental results also indicated the cysteine involved in the intersubunit bond is near the C terminus of the μ chain. If one is permitted to integrate these data from the different laboratories, then two disulfide bonds join the two μ chains in each molecule of IgM_s. Support for this case has been presented (Miller and Metzger, 1966; Beale and Feinstein, 1969).

The similarity between the structure of IgM_s and of IgG, the best characterized of the immunoglobulins, is striking. Although one would suspect that IgM should behave functionally like IgG, that is not strictly the situation. There are several anomalies about the structure–function relationships of IgM, such as the question of whether IgM_s has one or two antigen binding sites, that encouraged one to study IgM structure in more detail. In the present work we have attempted to learn more of the fine structure of IgM_s by comparing the labilities of the various interchain disulfide bonds.

Materials and Methods

Preparation of IgM. IgM was prepared from the plasma of patient Ov. who was ill with Waldenström's Macroglobulinemia and lymphosarcoma. The precipitation and purification of the IgM was carried out essentially as described before (Morris and Inman, 1968). Approximately 40 ml of plasma

* From the Department of Microbiology, University of Georgia, Athens, Georgia 30601. *Received August 26, 1969.* This study was supported by a grant from the National Science Foundation (GB-5551). F. P. I. also received partial support from the Institute of Comparative Medicine, University of Georgia.

† Postdoctoral research associate supported by the University of Georgia and the National Science Foundation (GB-5551).

‡ To whom inquiries should be sent.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: 0.32 SB, 0.32 M NaCl solution containing 10^{-3} M sodium borate, pH 8.0; MEA, mercaptoethylamine; 2-ME, 2-mercaptoethanol, redistilled; Gd·HCl, guanidine hydrochloride. The nomenclature used is that recommended by the World Health Organization Committee on Nomenclature for Human Immunoglobulins (*Bull. World Health Organ. 30*, 477 (1964)).

was slowly dripped into 400 ml of distilled water. The precipitated euglobulin was recovered by centrifugation, and the precipitation procedure was repeated two times additionally. The precipitate finally was taken up in about 40 ml of 0.32 SB, and then was dialyzed against the same buffer. Purified IgM was recovered by filtering the euglobulin through a column (2.5 × 132 cm) of Bio-Gel P-200 equilibrated with 0.32 SB using the ratio of 1 mg of protein/5 ml of packed column material. Purity of the IgM recovered from the column was determined by immunoelectrophoresis using anti-IgM, anti-lipoprotein, and anti-whole human serum antisera (Hyland Laboratories).

When necessary, the IgM or its subunits were concentrated in a Diaflo ultrafiltration cell (Amicon Corp.). The cell utilized a UM-10 membrane and was operated with house compressed air.

Preparation of Unreduced IgM_s. Subunits (IgM_s) of IgM were prepared essentially according to the procedure of Morris and Inman (1968). IgM was suspended in 0.2 M Tris solution (pH 8) by dialysis. The protein was treated with 0.02 M MEA at 30° for 30 min under nitrogen. The reduced IgM then was alkylated with neutralized iodoacetic acid at a final concentration of 0.04 M. The subunits were separated from the unreduced IgM by gel filtration through P-200 as described above. The purity of the IgM_s was tested by ultracentrifugation in 0.32 SB. In addition, amino acid analyses for carboxymethylcysteine showed the presence of 2.2–2.4 residues, which was consistent with the continued integrity of the intrasubunit interchain disulfide bonds in almost all the molecules. The IgM_s sedimented as a single, symmetrical peak at 4.3 S in 6.7 M Gd·HCl solution (pH 8) but aggregation was noted in 4 M guanidine solution. IgM_s prepared as described above will be referred to as "unreduced IgM_s."

Conditions of Reduction. The unreduced IgM_s was incubated with various concentrations of MEA ranging from 0.01 to 1.5 M using the conditions described above. After reduction, the mixtures were treated with neutralized iodoacetic acid in 100% excess over MEA for 1 hr at ambient temperature. The reduced and alkylated IgM_s samples were dialyzed against the appropriate buffer for subsequent studies.

In some instances, reduction was carried out with 0.2 M 2-ME for 1 hr at 30° in a nitrogen atmosphere. These samples were alkylated as described above with 0.4 M iodoacetic acid.

Amino Acid Analyses. Protein samples prepared at least in duplicate were hydrolyzed in evacuated, sealed glass tubes for 18 hr at 110° in 6 N HCl. The hydrolysates were recovered quantitatively, dried *in vacuo*, and then were dissolved in approximately 1 ml of distilled water and dried again *in vacuo*. The residues were taken up in amino acid analyzer diluting buffer. Analyses were performed with a Beckman Model 120C amino acid analyzer.

The analyses for CM-cysteine were done as previously described (Morris and Inman, 1968) by slightly overloading the long column (0.9 × 69 cm) on the analyzer. For calculation purposes, the molecular weight of IgM_s was assumed to be 180,000 (Miller and Metzger, 1965a; Inman and Hazen, 1968).

Dissociation of Subunits. Unreduced IgM_s which subsequently had been treated with MEA was dialyzed against 1 M propionic acid. The protein then was applied to columns of Bio-Gel P-150 which had been equilibrated with 1 M propionic acid. A ratio of 1 mg of protein/8–10 cm^3 of packed column material was employed. The identification of μ chains and light chains was done immunologically after the eluted protein was renatured by dialysis first against distilled water, and then against 0.32 SB. Specific anti-μ chain and anti-κ chain (Cappel Laboratories) antisera were used for these analyses.

The separation of light chains from one type of subunit (defined in text) was preceded by reduction with 0.2 M 2-ME and alkylation as described above. Afterward, these subunits were dialyzed against 1 M propionic acid prior to gel filtration through the P-150 columns.

The percentage of chains dissociated from the two types of subunit was determined by the calculation of the peak areas using a planimeter. For purposes of comparison, an average value of 25% (Morris and Inman, 1968; Inman and Hazen, 1968; T. K. S. Mukkur and F. P. Inman, unpublished observations) was considered to be that fraction of the total area of elution profiles attributable to the dissociable light chains from IgM.

Analytical Ultracentrifugation. Sedimentation analyses of mixtures and sedimentation coefficients of certain proteins were determined in a Beckman Model E analytical ultracentrifuge equipped with schlieren optics and electronic speed control. Samples centrifuged in dilute neutral salt buffers were analyzed at 20° whereas samples centrifuged in Gd·HCl (Mann Laboratories, Ultra Pure) solutions were centrifuged at 25°. The rotor (An-H) was operated at 68,000 rpm for samples suspended in Gd·HCl solutions, and s_{obsd} was corrected to $s_{-5,w}$ according to Schachman (1957) and utilizing viscosity and density factors for Gd·HCl solutions interpolated from the data of Kawahara and Tanford (1966). The partial specific volume was assumed to be the same as $\bar{v}_{20}$ for IgM_s (0.724) reported by Miller and Metzger (1965a).

Immunological Analyses. Double diffusion in gel was carried out on glass plates coated with 0.85% agarose made up in 0.1 M barbital (pH 8.6). Electrophoresis was performed on glass plates (11.5 × 5.7 cm) coated with similar agarose. The current was adjusted to 1.2 mA/2.5 cm length per plate, and electrophoresis was carried out for 60 min. The developing sera were specific anti-μ chain and anti-κ (Cappel Laboratories) antisera.

Determination of Protein Concentration. Protein concentrations were determined using the following extinction coefficients ($E^{1\%}_{1cm,280\,m\mu}$): (a) IgM and IgM_s, 12.0 (Miller and Metzger, 1965a); (b) μ chains (since we were unable to determine the value for μ chains, that for rabbit γ chains was utilized), 13.7 (Crumpton and Wilkinson, 1963); and (c) the assumed value for subunits other than those listed above, 12.0.

Results

Cleavage of Interchain Disulfide Bonds in IgM_s. IgM was mildly reduced with 0.02 M MEA to prepare unreduced IgM_s. Under these conditions, only the intersubunit disulfide bonds are broken (Morris and Inman, 1968). The alkylated subunits (IgM_s) were found to carry 2.2–2.4 CM-cysteine residues, indicating that the interchain disulfide bonds remained intact in almost all the molecules.

Small portions of the unreduced IgM_s were treated with one particular concentration of MEA in the range of 0.01–1.5 M. After the appropriate treatment (see Methods), the content of CM-cysteine in each reduction mixture, and the amount

TABLE I: The Number of Disulfide Bonds Broken per Molecule of IgM_s Reduced with MEA.

Concn of MEA (M)[a]	Total IgM_s Reduced (%)[b]	Total New CM-Cysteine Residues	SH Groups/ Molecule IgM_s Reduced	SS Bonds Broken/ Molecule IgM_s Reduced
0.01	70	1.0	1.4	0.7
0.02	74	2.0	2.7	1.4
0.04	85	2.9	3.4	1.7
0.06	89	3.7	4.2	2.1
0.08	91	4.3[d]	4.7	2.4
0.10	100	4.0	4.0	2.0
0.13	100	6.2	6.2	3.1
0.16	100	6.3	6.3	3.2
0.20	100	5.9	5.9	3.0
0.25	100	6.4	6.4	3.2
0.30	100	6.8	6.8	3.4
0.50	ND[c]	10.2[e]	10.2[e]	5.1[e]
1.0	ND[c]	11.9[e]	11.9[e]	6.0[e]
1.5	ND[c]	12.1[e]	12.1[e]	6.1[e]

[a] Samples of unreduced IgM_s were treated with the indicated concentration of MEA and alkylated with iodoacetic acid as described in Materials and Methods. [b] Figures represent the proportion of the total area of the peaks observed in schlieren patterns of the reduced and alkylated protein attributable to the most slowly sedimenting component and takes into account the per cent of light chains dissociated in propionic acid. The dispersal phase was 4 M Gd·HCl solution at pH 8. [c] Not determined. [d] Single determination. [e] Assumes 100% of IgM_s population was reduced.

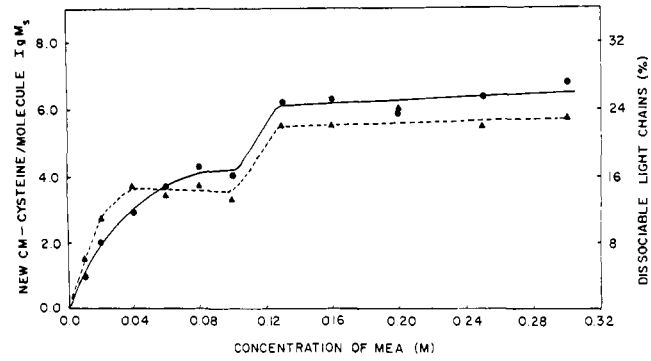

FIGURE 1: The relationship between the amount of dissociable light chains, the number of new CM-cysteine residues, and the concentration of MEA used for reduction of IgM_s. Unreduced IgM_s was treated with various concentrations of MEA and alkylated with iodoacetic acid. Part of each sample was analyzed for its content of new CM-cysteine residues. Another portion was subjected to gel filtration on Bio-Gel P-150 using 1 M propionic acid as solvent in order to determine the per cent of the eluted protein which could be attributed to light chains. (●—●) New CM-cysteine residues per molecule of IgM_s; (▲—▲) dissociable light chains.

of light chains released subsequent to gel filtration using 1 M propionic acid as solvent were determined.

The CM-cysteine contents of the samples are given in Table I and are illustrated graphically in Figure 1. The CM-cysteine residues of the unreduced IgM_s were subtracted from the total CM-cysteine residues ascertained. This allowed one to distinguish the disulfide bonds cleaved by subsequent reduction of unreduced IgM_s. In the range of 0.01–0.30 M MEA, two plateaus were observed (Figure 1). One occurred between 0.06 and 0.10 M MEA, and the other appeared with the use of 0.13–0.30 M MEA. The sensitivity of disulfide bonds to MEA was moderately strong in the concentration ranges of 0.01–0.06 M MEA and 0.10–0.13 M MEA. These results were interpreted to indicate the presence in IgM_s of interchain disulfide bonds having different labilities to MEA.

Each sample of reduced and alkylated IgM_s was gel filtered using propionic acid as solvent to separate light chains from the other constituents of the mixture. When the data were graphed, two distinct plateaus were observed (Figure 1). One lay in the relatively narrow range 0.04–0.10 M MEA, and the other was much broader, extending from 0.13 to 0.30 M MEA. The correlation between the shapes of the two curves in Figure 1 was striking. One interpretation of the results was that there were two populations of disulfide bonds covalently linking a μ chain with a light chain, each of which had a different susceptibility to reduction with MEA under the described conditions. One set of disulfide bonds was susceptible to MEA in the range of 0.04–0.10 M, and the other became susceptible at a concentration of 0.13 M MEA. In addition, there was enough difference in the shapes of the two curves in the lower concentration range of MEA to suggest adjunctive activity in cleavage of disulfide bonds.

Alternatively, the data might suggest that reduction of one μ-light-chain disulfide bond resulted in a change in the conformation of the IgM_s molecule. It is conceivable that a modification of this type could render the second μ-light-chain disulfide bond more resistant to reduction.

After removing each sample's light chains, which were separated from the other constituents by gel filtration in the presence of propionic acid, the material eluted first was put into 4 M Gd·HCl solution for analysis by analytical ultracentrifugation. The schlieren patterns of these materials are shown in Figures 2 and 3. A rapidly sedimenting constituent was evident in samples which had been reduced with 0.01–0.08 M MEA (Figure 2). However, as the concentration of MEA was increased, this heavier component gradually diminished in quantity, such that at a MEA concentration of 0.10 M, only a single constituent (3.6 S; Figure 2) was observed. Unreduced IgM_s sedimented (in 6.7 M Gd·HCl solution) at a rate of 4.3 S (Figure 2A).

The per cent of the total IgM_s reduced by each concentration of MEA is given in Table I. The CM-cysteine content of the reduced IgM_s also is proffered. From these data, we calculated the number of disulfide bonds split per molecule of IgM_s which sustained reduction. The calculations were based on the premise that the most rapidly sedimenting components in Figure 2B–F, represented intact IgM_s, and the more slowly sedimenting components (Figure 2B–G) were practically identical and represented the product of reduction. The per cent of light chains dissociated by propionic acid was taken into account for the calculations. The results, when plotted (Figure 4), again indicated a plateau in the

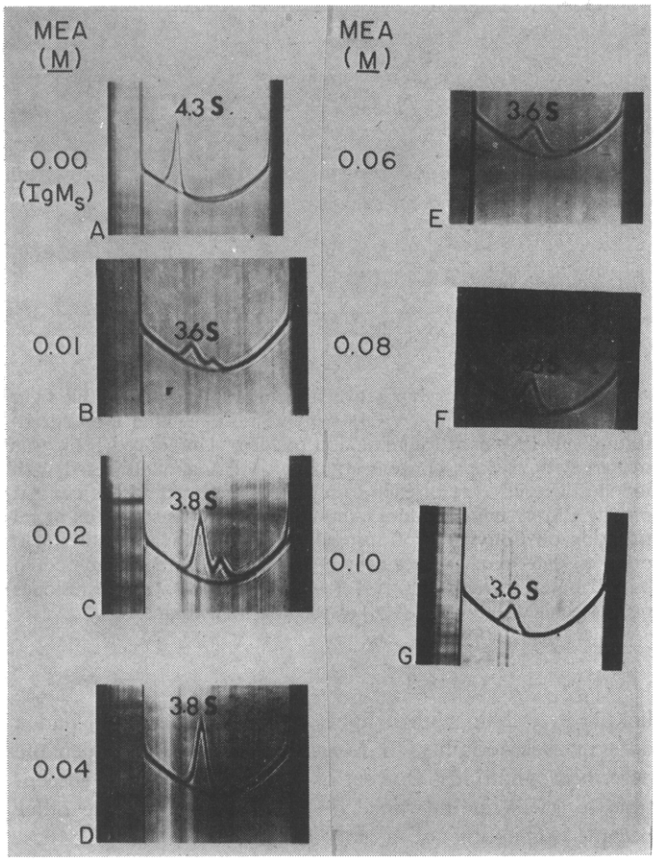

FIGURE 2: Schlieren patterns of unreduced IgM_s and the product of its reduction with MEA. Several samples of unreduced IgM_s were treated with MEA as indicated. After alkylation and gel filtration through Bio-Gel P-150 equilibrated with 1 M propionic acid, the dissociable light-chain fraction was removed. The remainder of the protein was renatured prior to ultracentrifugation in 4 M Gd·HCl solution (pH 8). Unreduced IgM_s was not gel filtered in propionic acid before dialysis against 6.7 M Gd·HCl solution (pH 8) for centrifugation. (A) Unreduced IgM_s (5.0 mg/ml); (B–G) unreduced IgM_s treated with various concentrations of MEA: B, 0.01 M (3.2 mg/ml); C, 0.02 M (7.4 mg/ml); D, 0.04 M (7.2 mg/ml); E, 0.06 M (5.0 mg/ml); F, 0.08 M (5.0 mg/ml); and G, 0.10 M (4.0 mg/ml). All photographs were made 96 min after reaching a rotor speed of 68,000 rpm at 25°.

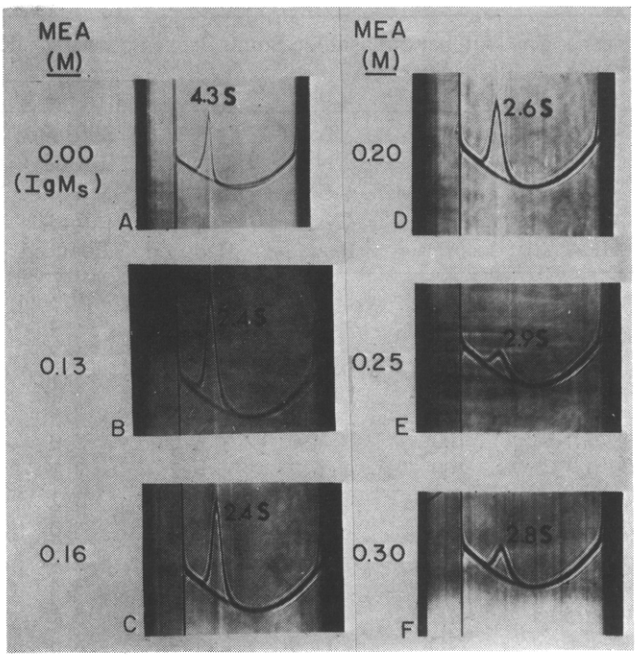

FIGURE 3: Schlieren patterns of unreduced IgM_s and the product of its reduction with MEA. Several samples of unreduced IgM_s were treated with MEA as indicated. After alkylation and gel filtration through Bio-Gel P-150 equilibrated with 1 M propionic acid, the dissociable light-chain fraction was removed. The remainder of the protein was renatured prior to ultracentrifugation in 4 M Gd·HCl solution (pH 8). Unreduced IgM_s was not gel filtered in propionic acid before dialysis against 6.7 M Gd·HCl solution (pH 8) for centrifugation. (A) Unreduced IgM_s (5.0 mg/ml); (B–F) unreduced IgM_s treated with various concentrations of MEA: B, 0.13 M (7.2 mg/ml); C, 0.16 M (7.0 mg/ml); D, 0.20 M (7.2 mg/ml); E, 0.25 M (2.8 mg/ml); and F, 0.30 M (3.0 mg/ml). All photographs were made 96 min after reaching rotor speed of 68,000 rpm at 25°.

region bounded by 0.06–0.10 M MEA. (It is suspected that the value for the number of disulfide bonds broken by 0.08 M MEA was high due to experimental error, and that the curve is correct as drawn. The values (Table I) for reduction with 0.06 and 0.10 M MEA agree well, and that for another independently performed experiment (see below) also agreed with these numbers.) Approximately two disulfide bonds were cleaved per molecule of IgM_s reduced. In this same region of MEA concentration, the data indicated that only about 58% of the available light chains in IgM_s were dissociable in propionic acid (Figure 1).

When the concentration of MEA was increased to 0.13 M, an additional disulfide bond was reduced (Figure 4), and all of the light chains were released (Figure 1). It was notable that between 0.13 M and up to 0.30 M MEA, only three disulfide bonds were reduced per molecule of IgM_s (Figure 4). Since all the light chains were dissociable at this level of reduction, at least two interchain disulfide bonds could be accounted for directly. The remaining one most likely was an inter-μ-chain disulfide bond. The lability of the latter to MEA approximated that of the first μ-light-chain disulfide bond which was reduced.

The nonlight-chain protein fractions from IgM_s treated with 0.13–0.30 M MEA were pooled individually and examined in the ultracentrifuge (Figure 3B–F). In 4 M Gd·HCl solution, their sedimentation coefficients ($s_{\pm 5,w}$) ranged from 2.4 to 2.9 S, values which apparently were dependent on concentration. Since this protein was μ-chain material almost free (antigenically) of light chains, it was considered tentatively to be μ-chain dimers. On the assumption of two inter-μ-chain disulfide bonds, the one disulfide bond which apparently was not reduced should have united the two μ chains covalently.

Based on these data, it was concluded that the more slowly sedimenting components (3.6–3.8 S) observed in Figure 2B–G, were molecules consisting of two μ chains and one light chain. It appeared that one of the two μ-light-chain disulfide bonds present in IgM_s was more susceptible to reduction with MEA than was the other. The number of disulfide bonds cleaved per molecule of IgM_s reduced was always greater than one above a MEA concentration of 0.01 M (Table I). These values represented a maximum number of bonds broken rather than a minimum. Up to a concen-

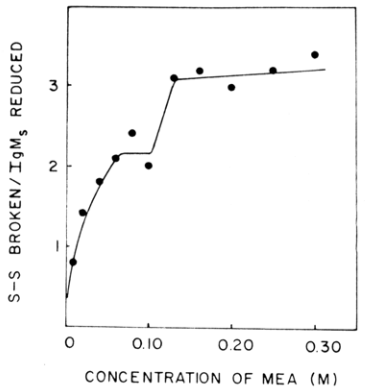

FIGURE 4: The number of disulfide bonds broken per molecule of IgM_s reduced expressed as a function of the concentration of MEA. Samples of unreduced IgM_s were treated, respectively, with various concentrations of MEA in the range of 0.01–0.30 M as indicated. The reactions were terminated by addition of iodoacetic acid. A portion of each sample was analyzed for its content of CM-cysteine. The data are tabulated in Table I.

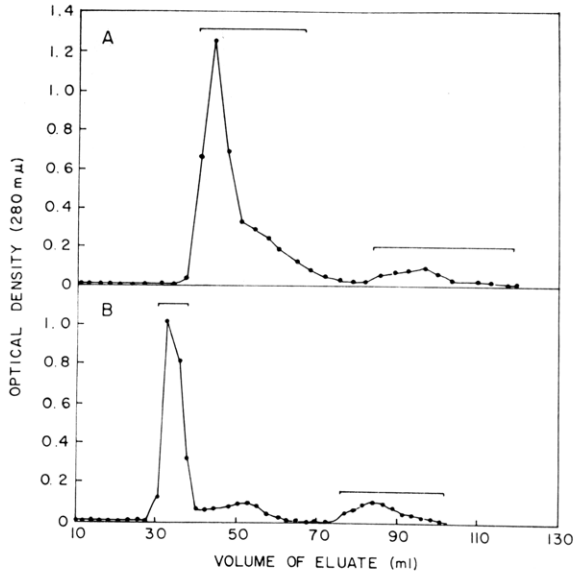

FIGURE 5: Light- and heavy-chain studies. (A) Dissociation of light chains from mildly reduced IgM_s. Unreduced IgM_s was treated with 0.10 M MEA, alkylated, and gel filtered through Bio-Gel P-150 equilibrated with 1 M propionic acid. (B) The heavier material from A (40–65 ml as indicated by horizontal bar) was renatured. It then was reduced with 0.2 M 2-ME, alkylated, and gel filtered through Bio-Gel P-150 equilibrated with 1 M propionic acid. Horizontal bars indicate regions which were pooled for various analyses (see text).

tration of 0.10 M MEA, only one of the disulfide bonds cleaved would be required for release of the light chain. The other bond probably was not an intrachain bond, because reduction was carried out in the absence of dissociating agents (Cecil and Wake, 1962). It was considered most likely that it was an inter-μ-chain disulfide bond which was also quite labile, more so even than the second μ-light-chain disulfide bond.

When higher concentrations of MEA were used (0.5–1.5 M; Table I), five to six disulfide bonds were reduced. This would account for all suspected interchain bonds in addition to some intrachain bonds.

Characterization of Partially Reduced IgM_s. Unreduced IgM_s was treated with 0.10 M MEA and alkylated. A portion of the reduced material was filtered through Bio-Gel P-150 equilibrated with 1 M propionic acid. Based on the elution profile shown in Figure 5A, light chains accounted for 13% of the eluted protein. The light-chain fraction pooled from 86–118 ml as indicated on Figure 5A was not contaminated with μ chains (Figure 7C), although in various other experiments of this type, the light-chain fraction frequently was found to be slightly contaminated.

The protein eluted between 40 and 65 ml was combined into one pool (Figure 5A). A photograph of the schlieren pattern of the pooled material is presented in Figure 6. The protein sedimented essentially as a single component in 4 M Gd·HCl solution with a $s_{25,w}$ of 3.6 S. When analyzed by double diffusion in gel (Figure 7A,B), the protein was shown to comprise both μ and light chains. Electrophoretically, the material migrated just a little faster than IgM_s, but slower than μ chains (Figure 8). Only a single arc appeared when the pooled protein was challenged with anti-μ-chain antiserum (Figure 8).

The 3.6S protein described above was reduced with 0.2 M 2-ME and alkylated. When it was passed through a column of Bio-Gel P-150 equilibrated with 1 M propionic acid, dissociation into μ and light chains occurred (Figure 5B). The light-chain fraction accounted for 14% of the recovered protein. These light chains, which were eluted between 76 and 101 ml, were pooled as shown on Figure 5B. Antigenically, they were identical with those dissociated following the initial reduction of IgM_s with MEA, but were slightly contaminated with μ chains (Figure 7C). The material which peaked at 53 ml (Figure 5B) was essentially μ chains.

The remainder of the protein, eluted with 30–38 ml of

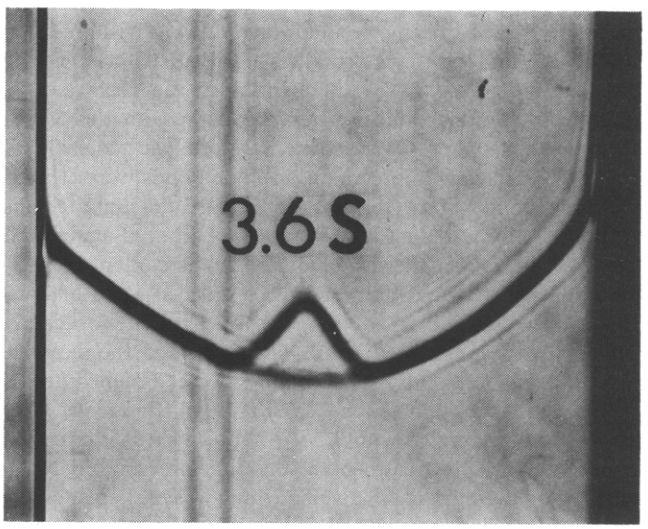

FIGURE 6: Schlieren pattern of the protein remaining after the dissociation of light chains from IgM_s which had been reduced with 0.10 M MEA. The material is that portion eluted earliest (40–65 ml) in the elution profile of Figure 5A, as indicated by the area under the appropriate horizontal bar. The photograph was made 128 min after attaining a rotor speed of 68,000 rpm at 25°. The solvent was 4 M Gd·HCl (pH 8) and the protein concentration was 5.0 mg/ml.

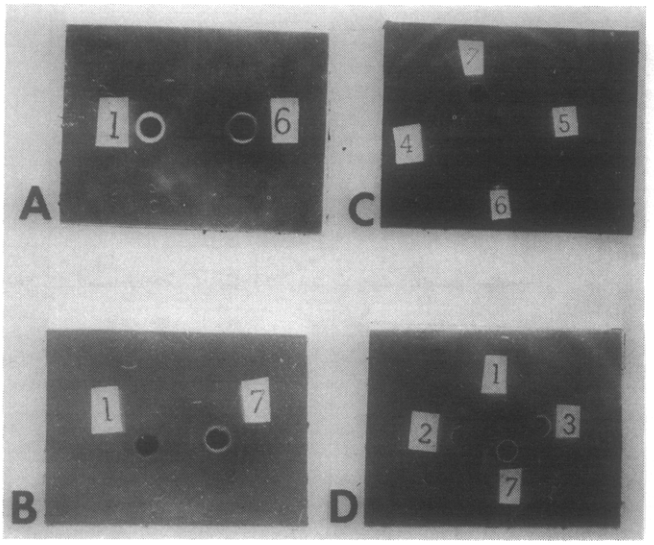

FIGURE 7: Analysis of proteins by double diffusion in agarose gel. Identification of components in wells: (1) unreduced IgM_s which had been treated with 0.10 M MEA, alkylated, and passed through Bio-Gel P-150 equilibrated with 1 M propionic acid in order to remove any dissociable light chains; (2) unreduced IgM_s; (3) the protein eluted with 30–38 ml of propionic acid, pooled as indicated in Figure 5B; (4) the protein eluted with 86–119 ml of propionic acid, pooled as indicated in Figure 5A; (5) the protein eluted with 76–101 ml of propionic acid, pooled as indicated in Figure 5B; (6) anti-κ antiserum, and (7) anti-μ-chain antiserum.

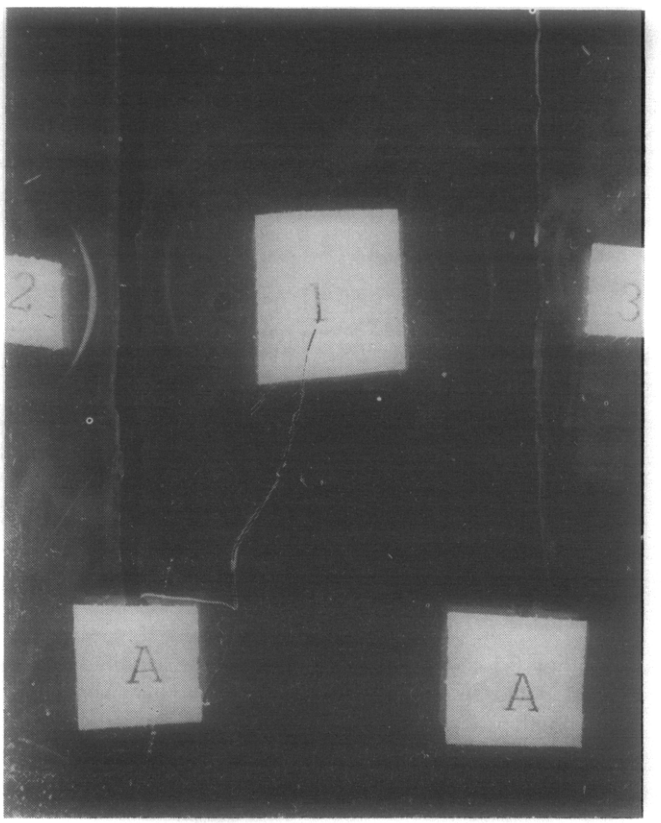

FIGURE 8: Immunoelectrophoresis patterns. Samples were electrophoresed in agarose as described in Materials and Methods. The cathode is at the top of the photograph. (1) Unreduced IgM_s which had been treated with 0.10 M MEA, alkylated, and passed through Bio-Gel P-150 equilibrated with propionic acid in order to remove any dissociable light chains; (2) unreduced IgM_s; (3) μ chains. (A) Anti-μ-chain antiserum.

solvent, was pooled (Figure 5B) and was shown to comprise μ chains (Figure 7D). These chains showed a line of identity with the 3.6S protein (Figure 6) described above and also with unreduced IgM_s (Figure 7D). Evidently, these chains were free of light chains since they failed to react with anti-κ antisera.

The number of disulfide bonds cleaved by treatment of the proteins with either MEA or 2-ME was determined by analysis for CM-cysteine residues as follows. The unreduced IgM_s was found to carry 2.2 residues of CM-cysteine indicating that the unreduced IgM_s still maintained the probity of its interchain disulfide bonds. After reduction with the MEA, there were 3.9 new CM-cysteine residues per molecule of IgM_s. Therefore, two interchain disulfide bonds were cleaved with the concomitant release of 52% of the light chains (Figure 5A). The reduced IgM_s then was reduced again with 0.2 M 2-ME, alkylated, and analyzed for its content of CM-cysteine. It was found that the twice-reduced IgM_s had 7.3 CM-cysteine residues in addition to the 2.2 residues originally present in the unreduced IgM_s. This indicated the cleavage of 3.7 disulfide bonds. These values were assumed to be representative of that in the 3.6S protein (Figure 6) after it too had been treated with 0.2 M 2-ME. This was interpreted to mean that after treatment of the 3.6S protein (Figure 6) with 2-ME, a μ-light-chain disulfide bond was broken allowing the acid dissociation of all remaining light chains (Figure 5B). In addition, one other disulfide bond, presumably interchain, was reduced.

Discussion

It is not unusual to find differing labilities among interchain disulfide bonds in immunoglobulins. The inter-γ-chain disulfide bond in rabbit IgG was shown to be more susceptible to reduction with a low concentration of MEA than the γ-light-chain disulfide bonds (Palmer *et al.*, 1963). However, Williamson and Askonas (1968) found that the heavy–light-chain disulfide bonds of mouse, human, and guinea pig IgG were more susceptible to reduction with 2-ME than the inter-heavy-chain bonds. In human IgM, the intersubunit disulfide bonds are more labile than the intrasubunit (IgM_s) bonds (Morris and Inman, 1968). The present report suggests there is varying lability among the interchain disulfide bonds in IgM_s itself.

It already has been demonstrated (Morris and Inman, 1968) that when human IgM was treated with a low concentration of MEA, the subunits (IgM_s) which were released from the parent IgM molecule retained their interchain disulfide bonds intact. When subunits of this type were reduced with various concentrations of MEA, differing degrees of lability of their disulfide bonds were noted.

When the concentration of MEA was adjusted from 0.04 to 0.10 M (Figure 1), it was sufficient to reduce one μ-light-chain disulfide bond. This was evident because even though two disulfide bonds were cleaved per molecule of IgM_s reduced (Figure 4), only about one-half of the available light chains were dissociable in propionic acid. Another disulfide bond, which almost certainly was an inter-μ-chain

bond, also was labile at this concentration of MEA. Beale and Feinstein (1969) recently reported, too, than an inter-μ-chain disulfide bond was quite labile to dithiothreitol.

After the dissociable light chain was removed, the remainder of the molecule evidently consisted of one light chain covalently bonded to one of the two μ chains. Presumably, the two μ chains were joined through one disulfide bond, the other having been reduced.

Both light chains were dissociable when IgM_s was reduced with 0.13–0.30 M MEA (Figure 1). At this concentration of mercaptan, a total of only three disulfide bonds were reduced. Two of these must have been μ-light-chain bonds. After removal of the dissociable light chains, only μ chains remained, and there was a corresponding decrease in sedimentation coefficient (Figure 3).

We did not consider it likely that the apparent reduction of only one inter-μ-chain disulfide bond was the result of disulfide interchange. Although it could explain the rather broad range of MEA concentration (0.13–0.30 M; Figure 1) where additional bonds evidently were not broken (Figure 4), one would have expected to observe evidence of heterogeneity in the ultracentrifuge. Specifically, half-molecules of IgM_s and free μ chains would result from interchange. Thus far, we have seen no evidence of this, and the symmetry of the peaks in the schlieren patterns (Figures 2 and 3) argued against the presence of half-molecules mixed with the polypeptide chains.

We attempted to study the labilities of the bonds in a slightly different manner. Unreduced IgM_s was treated with 0.10 M MEA and alkylated. This concentration of mercaptan had already been shown to reduce 100% of the IgM_s population with a concomitant release of about one-half of the total light-chain population. All of the IgM_s molecules were reduced and although two disulfide bonds were broken only 52% of the total light chains were dissociable in propionic acid (Figure 5A). Disregarding the dissociated light chains, antigenic analyses confirmed that the remaining material comprised both μ and light chains (Figure 7).

It was most likely that the latter material was constructed of two μ chains and one light chain, *i.e.*, of the IgM_s molecules reduced, each of them suffered breakage of the interchain disulfide bond between only one of the two μ-light-chain pairs. An alternative explanation was that each of the interchain disulfide bonds linking both pairs of μ and light chains was cleaved in only half the IgM_s population. If this had happened, one would have observed gross heterogeneity in the schlieren patterns of the protein due to the presence of μ-chain dimers and intact IgM_s. It was shown, however, that the schlieren pattern was that of a homogeneous protein (Figure 6).

The molecule comprising two μ chains and one light chain then was reduced with 2-ME to ensure breakage of all suspected interchain disulfide bonds. The light chains accounted only for 14% of the total eluted protein when gel filtered with propionic acid solvent. This was consonant with the initial material consiting of only one light chain. Antigenic analyses (Figure 7) indicated the larger material was μ chains only.

From a conceptual point of view, one could consider the mildly reduced IgM_s, which was constructed of two μ chains and one light chain, as a three-quarter molecule of IgM_s. It was interesting to note that it migrated electrophoretically between unreduced IgM_s and free μ chains (Figure 8).

In the IgM molecule, then, there seems to be populations of disulfide bonds of measurable differences in susceptibility to reduction by MEA. The most labile bonds are the intersubunit, inter-μ-chain disulfide bridges. Following these is one of the two μ-light-chain pair bonds in the subunits, IgM_s. An intrasubunit, inter-μ-chain disulfide bond exhibits similar lability. The remaining μ-light-chain pair bond appeared to be the least labile of those demonstrated in the present work. Additional disulfide bridges were reduced when high concentrations of mercaptan were employed. Presently, one has not specifically demonstrated cleavage of the presumed second inter-μ-chain disulfide bond. Research in that direction is in progress, however.

Although experiments were not done with IgM of known antibody activity, the results presented here may help explain certain earlier reports. It is not known whether antibody IgM_s bears one or two functional antigen binding sites within the intact subunit. An interesting finding reported recently by Onoue *et al.* (1968) was that each subunit of rabbit anti-hapten IgM may have carried a high-affinity and a low-affinity antigen binding site. From a structural point of view, the explanation for this was not known. Based on the results now reported, one could speculate that the affinity of the binding sites may have something to do with the degree of complementarity of the two μ-light-chain pairs. Perhaps the μ-light-chain pair which is joined by the more labile interchain disulfide bond fails to form an antigen binding site which is as good as that of the other μ-light-chain pair in the unreduced antibody-active IgM_s molecule.

Acknowledgments

The plasma from patient Ov. was the gift of Dr. Ronald Roberts of the Marshfield Clinic, Wisconsin. Amino acid analyses were done by Mrs. Sara Cliett. Excellent technical assistance was performed by Mrs. Samira Hazen.

References

Beale, D., and Feinstein, A. (1969), *Biochem. J. 112*, 187.
Cecil, R., and Wake, R. G. (1962), *Biochem. J. 82*, 401.
Chaplin, H., Cohen, S., and Press, E. M. (1965), *Biochem. J. 95*, 256.
Crumpton, M. J., and Wilkinson, J. M. (1963), *Biochem. J. 88*, 228.
Inman, F. P., and Hazen, S. R. (1968), *J. Biol. Chem. 243*, 5598.
Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241*, 3228.
Miller, F., and Metzger, H. (1965a), *J. Biol. Chem. 240*, 3325.
Miller, F., and Metzger, H. (1965b), *J. Biol. Chem. 240*, 4740.
Miller, F., and Metzger, H. (1966), *J. Biol. Chem. 241*, 1732.
Morris, J. E., and Inman, F. P. (1968), *Biochemistry 7*, 2851.
Onoue, K., Grossberg, A. L., Yagi, Y., and Pressman, D. (1968), *Science 162*, 574.
Palmer, J. L., Nisonoff, A., and Van Holde, K. E. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 314.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Williamson, A. R., and Askonas, B. A. (1968), *Biochem. J. 107*, 823.