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Isolation of the Monomeric Subunit of Immunoglobulin M with Its Interchain Disulfide Bonds Intact*

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ABSTRACT: Immunoglobulin M (IgM) consists of five monomeric subunits, IgM_s, each apparently comprising two μ chains and light chains. The distribution of disulfide bonds between μ chains within IgM_s and those joining the μ chains of one IgM_s to another is not known with certainty. Our goal was to obtain IgM_s with its original inter-μ-chain and μ-light-chain disulfide bonds intact, and to determine whether one or two disulfide bonds join each IgM_s to another subunit. The amount of IgM_s released from IgM by reduction could be controlled by adjusting the concentration of mercaptoethylamine. In our hands, reduction of IgM with 0.015 M mercaptoethylamine resulted in a mixture containing about 50% IgM_s. The subunit, isolated by filtration of the mixture through Bio-Gel P-200, had a sedimentation coefficient of 6.17 S at a concentration of 6.21 mg/ml, electrophoretic mobility similar to its parent IgM, maintained the specific antigenic sites on the μ chain, and comprised both μ and light chains. Regardless of the time of reduction from 5 to 40 min with 0.015 M mercaptoethylamine, IgM_s contained about two carboxymethylcysteine residues per molecule. In addition, IgM_s isolated after 30-min reduction failed to dissociate in propionic acid into μ and light chain until it subsequently was reduced with 2-mercaptoethanol. In conclusion, the data indicated the IgM_s has maintained its original interchain disulfide bond integrity, and each IgM_s is bound to another by a single disulfide bond.

Considerable progress has been made in the last several years toward elucidation of the structures of the immunoglobulins. IgG,[1] which normally occurs in the largest quantity in serum, comprises two pairs of polypeptide chains. Two γ chains in rabbit IgG are joined covalently by one highly labile disulfide bond (Palmer and Nisonoff, 1964; Hong and Nisonoff, 1965) and by noncovalent forces in the Fc region (Marler *et al.*, 1964; Inman and Nisonoff, 1966). A single light chain is attached by one disulfide bond and noncovalent forces (Fleischman *et al.*, 1963) to each γ chain.

IgM occurs in small quantities in normal serum. It appears to be composed of five monomeric subunits held to one another by disulfide bonds (Miller and Metzger, 1965a,b; Lamm and Small, 1966). Miller and Metzger, (1965a,b) have proposed that the structural architecture of IgM subunits (IgM_s) resembles that of intact IgG, *i.e.*, IgM_s also comprises two pairs of polypeptide chains. The light chains apparently are very similar or identical with those of IgG (Miller and Metzger, 1965a; Lamm and Small, 1966), but the other pair of chains, μ, differs from the γ chains in several respects such as a higher molecular weight (Lamm and Small, 1966), and different amino acid composition (Chaplin *et al.*, 1965), antigenic analysis (Cohen, 1963; Miller and Metzger, 1965a), and carbohydrate content (Chaplin *et al.*, 1965; Lamm and Small, 1966). In addition, each μ chain appears to be involved with four interchain disulfide bonds (Miller and Metzger, 1965b). One of these bonds covalently links a μ and a light chain (Chaplin *et al.*, 1965; Miller and Metzger, 1965b). The remaining three bonds are intrasubunit inter-μ-chain and intersubunit inter-μ-chain. Since it was not possible under the conditions used to reduce selectively these bonds, it is not known whether there are one or two disulfide bonds between subunits.

Investigators have used several different chemical

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Tris–NaCl, 0.05 M Tris–0.5 M NaCl (pH 8.2); 0.32 SB, 0.32 M NaCl buffered with 10^{-3} M sodium borate (pH 8); MEA, mercaptoethylamine; 2-ME, 2-mercaptoethanol, redistilled. The nomenclature used is that recommended by the World Health Organization Committee on Nomenclature for Human Immunoglobulins (*Bull. World Health Organ. 30*, 447 (1964)).

agents to reduce the disulfide bonds in IgM. When cysteine was used (Miller and Metzger, 1965b), the moles of SH detected varied, and appeared to decrease with increasing time of exposure to reducing agent. Although the isolated IgM_s dissociated into μ and light chains little or none at all when gel filtered with propionic acid as solvent, the number of cleaved disulfide bonds was inconclusively determined.

We now wish to report our results concerning characterization of a Waldenstrom's macroglobulin and its subunit, IgM_s, released by partial reduction of the IgM with mercaptoethylamine.

Materials and Methods

Preparation of IgM. IgM was prepared from 40 ml of plasma from a patient suffering from Waldenstrom's macroglobulinemia by slowly dripping it into 560 ml of distilled water at room temperature. When necessary, lipoproteins were removed prior to precipitation of the euglobulin by adjusting the density to 1.2 with saturated NaBr solution. The high-density plasma was centrifuged at 48,000*g* in a Sorvall RC2-B centrifuge at about 22° for 30 min. The euglobulin precipitate was recovered by centrifugation at 12,000*g*, and was taken up in 30–40 ml of 0.16 M NaCl containing 10^{-3} M sodium borate buffer at pH 8. The IgM was precipitated one or two more times, and finally the precipitate was dissolved in not more than 40 ml of 0.05 M Tris–0.5 M NaCl (pH 8.2) (Tris–NaCl) or 0.32 M NaCl buffered with 10^{-3} M sodium borate (pH 8) (0.32 SB).[1] Some preparations then were purified by gel filtration on G-200 columns equilibrated with 0.32 SB. A column of G-200 (2.5 × 125 cm) was charged with 200–600 mg of the IgM preparation. The recovered protein was free of IgG and other serum proteins, but invariably contained a heavier component in addition to IgM. The former was assumed to be a dimer of IgM. For certain experiments, IgM free of the dimer was required. This could be accomplished by filtering the precipitated IgM on a column of Bio-Gel P-200 equilibrated with 0.32 SB (see Results).

The IgM or IgM_s routinely was concentrated in a Diaflo ultrafiltration cell (Amicon Corp.). The cell utilized a UM-1 membrane and was operated with house compressed air.

Determination of $s^0_{20,w}$ Values. IgM was purified on P-200 and dialyzed against a large volume of 0.32 SB in the cold. The IgM recovered from the dialysis bag was allowed to warm to room temperature, and any insoluble material was removed by centrifugation. Samples were prepared which ranged from 2.75 to 0.75 mg of IgM/ml. The concentration of the IgM was determined with a Hitachi Perkin-Elmer 139 spectrophotometer. We used $E^{1\%}_{280\,m\mu}$ 12 for both IgM and IgM_s (Miller and Metzger, 1965a). Analytical ultracentrifugation was performed in the Beckman Model E ultracentrifuge equipped with Schlieren optics and electronic speed control, and operated at 56,000 rpm and 20°. An An-H rotor and the same single-sector cell were used for each determination. s_{obsd} was corrected to $s_{20,w}$ according to Schachman (1957).

Reduction of IgM. IgM was reduced with the concentrations of mercaptoethylamine indicated in the text. Unless otherwise stated, the vials containing protein were incubated for 30 min at 30° with an atmosphere of N_2. The reduction mixtures were alkylated with a twofold excess of neutralized iodoacetic acid for 1 hr in the refrigerator. The IgM, reducing agent, and iodoacetic acid were made in Tris–NaCl buffer. The reduced-alkylated IgM was dialyzed against 0.32 SB before further treatment.

Determination of Carboxymethylcysteine. Duplicate samples of IgM_s were hydrolyzed in evacuated, sealed glass tubes 18 hr at 110° in 6 N HCl. The hydrolysates were recovered quantitatively, dried *in vacuo* over NaOH pellets, and taken up in amino acid analyzer diluting buffer. Analysis was done on a Beckman Model 120C amino acid analyzer. For analysis of carboxymethylcysteine (CM-cysteine), the long column was slightly overloaded, essentially according to the technique reported by Palmer and Nisonoff (1964). The separation of the CM-cysteine peak from the peaks due to methionine sulfoxide was facilitated by applying about 8 ml of sodium citrate buffer (pH 3.20) before switching to the buffer adjusted to pH 3.30.

Immunoelectrophoresis and Gel Diffusion. Immunoelectrophoresis was done with glass plates (11.5 × 5.7 cm) coated with 0.85% agarose in 0.1 M barbital (pH 8.2). Electrophoresis was carried out for 90 min with 8 mA/plate and 0.05 M barbital as the conducting buffer. The antisera to human IgM and human serum were obtained from Hyland Laboratories.

Ouchterlony double-diffusion analysis was carried out using the same gel system as electrophoresis.

Dissociation of Protein into μ and Light Polypeptide Chains. After dialysis overnight against Tris–NaCl buffers, IgM, or IgM_s was treated with 0.2 M 2-mercaptoethanol at 30° for 1 hr. The reduced protein then was alkylated with 0.4 M neutralized iodoacetic acid for 1 hr in the refrigerator prior to dialysis against 1 M propionic acid. After overnight dialysis the reduced-alkylated protein was applied to a column of Bio-Gel P-150 (2.5 × 35 cm) which had been equilibrated with propionic acid. Small volumes (about 2 ml/tube) were collected, and the optical density at 280 mμ was measured.

Results

Characterization of the IgM. IgM readily precipitated when the plasma was dripped slowly into water. After one or two reprecipitations, the IgM frequently contained very small amounts of the lighter component which was observed in the ultracentrifuge. Filtration through Sephadex G-200 or Bio-Gel P-200 allowed recovery of a large percentage of the column charge which was free of the lighter component (see Figures 1 and 5).

The IgM exhibited cryoglobulin properties. After dialysis in the cold room or storage in the refrigerator, the immunoglobulin always precipitated, but most of the precipitate went back into solution when warmed to room temperature. Insoluble material was removed by centrifugation (12,000*g*).

To obtain IgM free of the dimer, the protein prepared

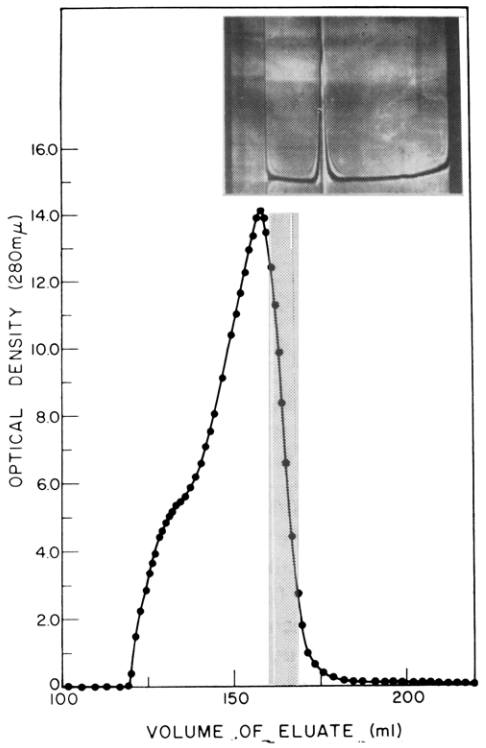


FIGURE 1: A typical elution profile of a euglobulin precipitate filtered through Bio-Gel P-200 equilibrated with 0.32 SB. The shaded area indicates the fraction of total eluted protein used for determination of $s^0_{20,w}$ of IgM. The insert is a typical schlieren pattern of the IgM represented by the stippled area. The schlieren pattern was photographed 16 min after reaching speed of 56,000 rpm at 20°.

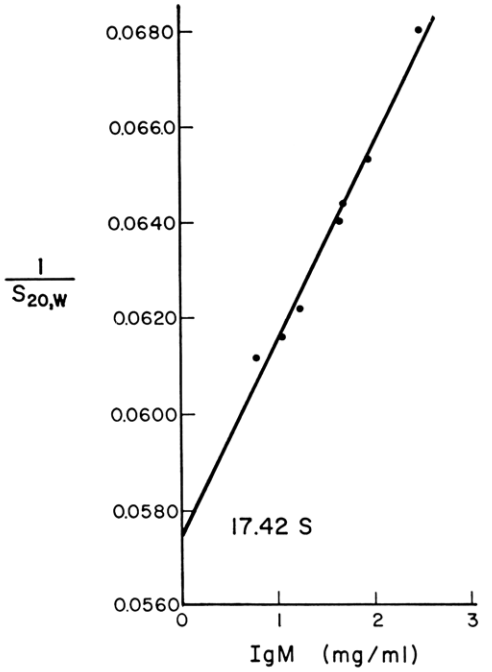


FIGURE 2: Determination of $s^0_{20,w}$ of IgM purified by filtration through Bio-Gel P-200 (Figure 1). Experimental conditions are described in Materials and Methods. The ordinate is the reciprocal of the $s_{20,w}$ and the abscissa is the concentration of IgM in milligrams per milliliter.

by euglobulin precipitation was filtered through P-200 equilibrated with 0.32 SB. A typical elution pattern is shown in Figure 1. The tubes containing eluted protein comprising the descending side of the peak were pooled, as indicated by the shaded area, and concentrated by pressure dialysis. The protein was analyzed in the analytical ultracentrifuge to determine purity. A schlieren pattern used for one of the $s_{20,w}$ determinations is shown in the inset of Figure 1. The IgM was free of both larger and smaller protein components.

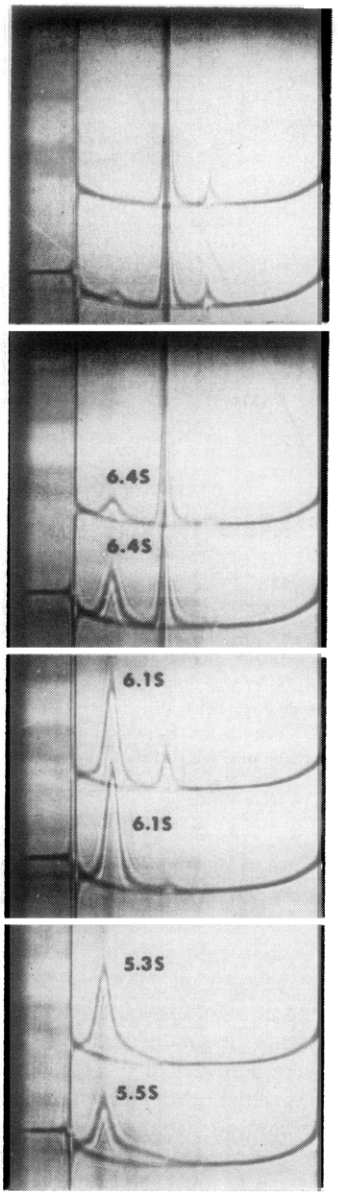
The $s^0_{20,w}$ of the IgM was ascertained as shown in Figure 2. When the reciprocal of the $s_{20,w}$ was plotted against the concentration of IgM, a value for $s^0_{20,w}$ of 17.42 S was obtained by extrapolation. A very similar value of 17.32 S was arrived at by plotting the $s_{20,w}$ against concentration. The average value of $s^0_{20,w}$ obtained by the two methods is 17.37 S.

Subunit (IgM$_s$) of IgM. Subunits of IgM have been isolated and characterized by Miller and Metzger (1965a,b). In the following experiments we were attempting to reduce IgM under gentle conditions with the hope of recovering IgM$_s$ which had its inter-μ-chain and its μ-light-chain disulfide bonds intact.

Each of several samples of IgM was reduced with a certain concentration of MEA and then was alkylated with iodoacetic acid. After each sample was dialyzed against 0.32 SB, aliquots were prepared at 5–6 mg/ml for analytical ultracentrifugation. The results are shown in Figure 3. The control was treated exactly as the experimental samples, but was not subjected to MEA. The major component seen in the schlieren pattern is IgM; no slower constituent appeared. The amount of the component sedimenting slower than IgM grew as the concentration of MEA was increased from 0.004 to 0.04 M. The sedimentation coefficient of this substance was about 6.1–6.4 S and represents the subunit, IgM$_s$. If the concentration of MEA were adjusted to 0.015 M, one would expect about 50–60% of the IgM to be reduced to the subunit form. This condition arbitrarily was assumed to be the more favorable one for the isolation of minimally reduced IgM$_s$ in a utilizable yield.

When the concentration of MEA was 0.06 M or greater, varying degrees of apparent aggregation of IgM$_s$ were evident. A small amount of a more slowly sedimenting component also could be observed but this is not demonstrated in Figure 3. It is noteworthy that intermediates between IgM$_s$ and unreduced IgM were not observed except when the higher concentrations of MEA were utilized.

Using 0.015 M MEA, similar samples of IgM were reduced for 5–40 min (Table I). Control specimens were incubated for 5 or 40 min, and except for the absence of MEA they were treated exactly like the experimental samples. IgM$_s$ from the reduced and alkylated samples was isolated by gel filtration through P-200 using about 1 mg of protein/4 ml of packed column volume. A typical elution profile is shown in Figure 4. Tubes containing IgM$_s$ were pooled as indicated by stippling. The protein was concentrated and examined by ultracentrifugation. A typical example is shown as the insert to Figure 4. Each preparation of IgM$_s$ analyzed appeared as a single symmetrical peak in the schlieren pattern. At a



MEA Concn (moles/l.)	% of IgM_s
0.000	
0.004	
0.008	
0.010	37
0.020	84
0.040	96
0.060	
0.080	

FIGURE 3: The relationship of the concentration of MEA to the amount of IgM_s released from IgM. The photographs of the schlieren patterns were made 24 min after reaching 60,000 rpm. IgM was incubated with the given concentration of MEA for 30 min at 30°. The mixture was alkylated with iodoacetic acid and then dialyzed against 0.32 SB before centrifugation. The concentration of protein in each schlieren pattern was 4 mg/ml.

concentration of 6.21 mg/ml the $s_{20,w}$ of isolated IgM_s was 6.17 S.

The highest rate of reduction resulting in release of IgM_s from the IgM occurred during the first 5 min (Table I). At this time, 27% of the mixture consisted of IgM_s. Additional reduction occurred at a much slower rate in the interval of 5–30 min; IgM_s increased only about 5% for each 5-min period. After 30 min, about 46% of the protein was in the form of IgM_s, and incubation beyond this time resulted in little additional conversion into IgM_s. Components sedimenting in the region between IgM_s and unreduced IgM were not seen, regardless of the time of reduction. Although the amount of

TABLE I: Carboxymethylcysteine Analysis of IgM_s.[a]

Time of Reduction (min)	IgM_s in Reduced Mixture (%)[b]	CM-cysteine Content of IgM_s (moles of CM-cysteine/mole of IgM_s)[c]
5 C[d]	0	T[e]
5	27.3	2.3
10	32.6	2.3
15	38.5	2.2
20	41.6	2.4
30	45.8	2.2
40	47.2	1.8
40 C[d]	0	T[e]

[a] The samples of IgM were reduced at 30° with 0.015 M MEA for the indicated time. The reaction mixtures then were alkylated with iodoacetic acid. After dialysis against 0.32 SB, the samples were examined in the ultracentrifuge. [b] The per cent IgM_s in the reduction mixture was determined by measuring the areas of the peaks observed in schlieren patterns. [c] The molecular weight of IgM_s was taken as 180,000 (Miller and Metzger, 1965a, Lamm and Small, 1966). [d] Represents control samples which were treated like the experimental ones except that no MEA was present. [e] Trace.

IgM_s formed was less than that anticipated (*ca.* Figure 3), the shape of the curve (Table I; per cent IgM_s *vs.* time) was similar to others we have found.

Only trace amounts of CM-cysteine were evident when either of the two control proteins was analyzed (Table I). The CM-cysteine contents of the isolated IgM_s samples were strikingly similar. Regardless of the length of time of reduction of the IgM, approximately the same number of free sulfhydral groups appeared on the corresponding IgM_s. The data are consistent with the thesis that each IgM_s is bound by a single disulfide bond to another IgM_s on either side.

Antigenic Analysis of IgM and IgM_s. IgM which had been purified by passage through G-200 was reduced with 0.015 M MEA. IgM_s was separated from IgM in the mixture by filtration through P-200. A typical schlieren pattern was observed in the analytical ultracentrifuge (see Figure 4). The IgM and IgM_s were examined by immunoelectrophoresis. Best results were obtained when the agarose-coated plates were incubated at 37° during immunodiffusion. Antihuman serum antiserum revealed only a single precipitin line against both IgM and IgM_s (Figure 5). This was consistent with the assumption that the faster component frequently observed in schlieren patterns of IgM is a dimer of IgM and not an antigenically different substance. In addition, no contaminating serum proteins were detected in the IgM preparation. When the plates were developed with anti-IgM antiserum, single precipitin arcs were observed again (Fig-

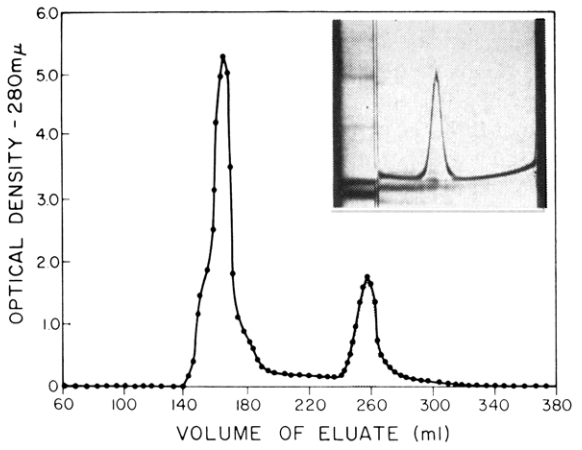


FIGURE 4: The isolation of IgM_s from partially reduced IgM. Experimental conditions are described in Results. That portion of the second peak indicated by stippling was pooled as IgM_s. The insert is a photograph of the schlieren patterns of the pooled material. The concentration of the protein was 6 mg/ml and the photograph was taken 40 min after reaching 60,000 rpm.

ure 5). In each case, the mobility of IgM_s was very similar to that of IgM.

In analysis by double diffusion, a line of identity formed between IgM_s and IgM. The plates were observed even after prolonged development for spur formation, and none appeared. IgM_s evidently had lost none of its μ-chain antigenic sites as detected by our antisera.

When IgM was tested by gel diffusion using anti-κ and anti-λ specific antisera, precipitation occurred only with the former (Figure 5). It was difficult to obtain precipitation of IgM_s with either anti-κ or anti-IgM antisera. A faint line which identifies IgM_s as a κ-type subunit may be seen (Figure 5). The precipitin line between anti-IgM antiserum and IgM_s was not dense enough to appear in the photograph, and it was added artistically (Figure 5).

Dissociation of IgM_s. The data to this point indicate that the IgM_s contains intact the disulfide bonds linking the μ and light chains. To test this, the following experiment was performed.

A sample of the same IgM from which IgM_s was derived was reduced with 0.2 M 2-ME and alkylated. A small portion (23.8 mg) was applied to a column of P-150 equilibrated with 1 M propionic acid. The elution profile may be seen in Figure 6 (reduced IgM). The identity of the peaks was established by gel diffusion of the components of each peak against anti-IgM and anti-κ antisera. The first peak was attributed to μ-polypeptide chains and the second to light chains. The yield of protein in experiments of this type ranged from 26.9 to 29.0% for light chains and 72.0 to 73.1% for μ chains.

When the IgM_s (18.8 mg) was similarly filtered through the same column, a peak corresponding in position to the light chains did not appear (Figure 6, IgM_s). However, when a portion of the IgM_s was reduced and alkylated, and applied to this column (19.7 mg) (Figure 6, reduced IgM_s) peaks corresponding to both μ and light chains were evident. The light chains accounted for 21.8–23.0% of the eluted protein, and 77–78.2% was attributable to μ chains. This clearly

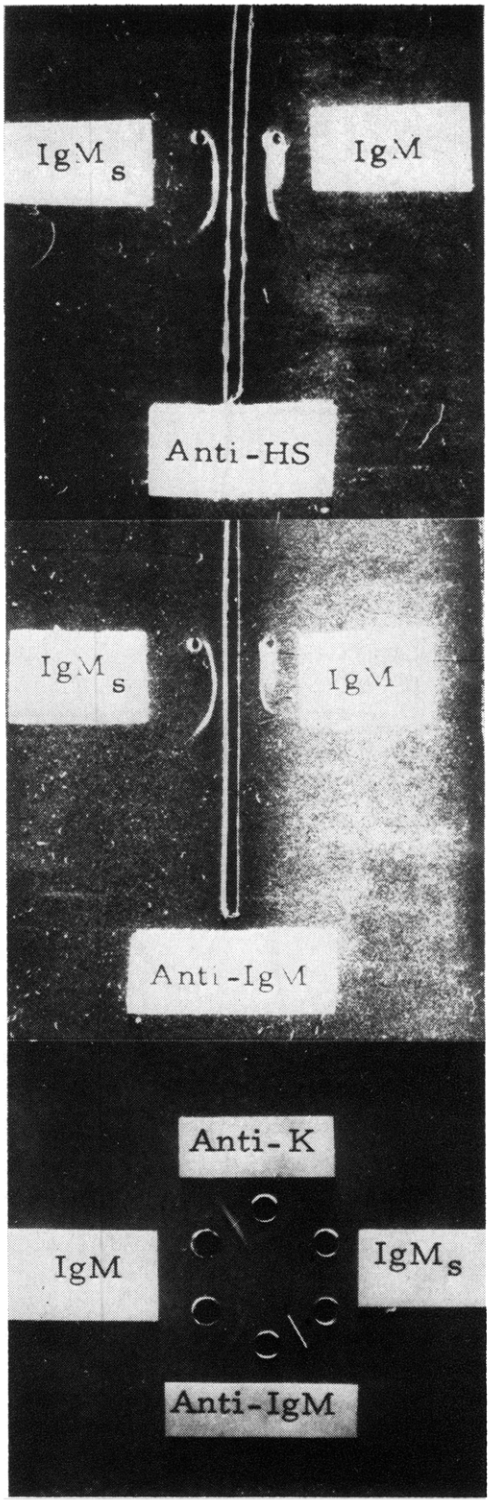


FIGURE 5: Immunoelectrophoresis and Ouchterlony analysis of IgM and IgM_s. Experimental conditions are given in Materials and Methods. Anti-HS, anti-human serum antiserum; Anti-IgM, antiserum specific for IgM; Anti-κ, anti-κ antiserum. The precipitin line shown between Anti-IgM and IgM_s in the Ouchterlony analysis was added artistically since the real line did not appear in the photograph.

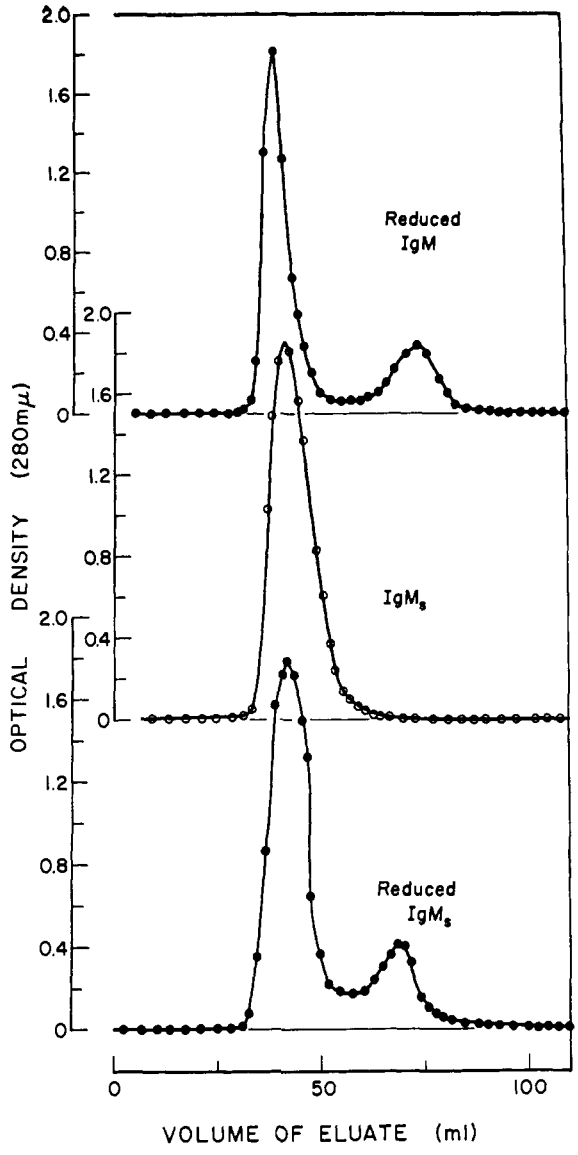


FIGURE 6: Reduced IgM, unreduced IgM$_s$, and reduced IgM$_s$ were filtered through the same column of Bio-Gel P-150 in 1 M propionic acid. The first peaks correspond in position to the μ chains of reduced IgM, and the second peak corresponds to light chains of IgM.

supported the notion that our IgM$_s$ contains most, if not all, its interchain disulfide bonds intact.

Discussion

It was shown previously (Miller and Metzger, 1965a,b; Lamm and Small, 1966) that IgM comprises five monomeric subunits (IgM$_s$) held by disulfide bonds. Although the evidence indicated each IgM$_s$ is composed of two μ chains and two light chains (Miller and Metzger, 1965a,b), it recently was announced (Suzuki and Deutsch, 1967) that the basic subunit of IgM consists of two μ chains and three light chains. The architecture of IgM$_s$ generally is visualized as resembling that of IgG. If this were so, then each subunit derived from antibody-IgM would be expected to be bivalent. The reports concerning the valency of IgM have been contradictory. Rabbit antibody-IgM directed against *p*-azobenzenearsonate appeared to have univalent subunits (Onoue *et al.*, 1965). Although a human macroglobulin, when intact, bound about 5 moles of IgG/mole of IgM, the activity of the F(ab')$_{2\mu}$ fragments was consistent with potentially ten combining sites (Metzger, 1967). On the other hand, Cooper (1967) reported that the 7S subunits of a cold agglutinin were bivalent, and Merler *et al.* (1968) recently demonstrated that a human IgM with antibody activity against *S. typhi* had ten combining sites per molecule.

It is difficult to reconcile these reports with the currently acceptable model of IgM structure. From our point of view, additional information regarding the structure of IgM is needed. It is felt that the antibody function of IgM$_s$ might best be studied with subunits which have their interchain disulfide bonds intact.

Miller and Metzger (1965b) used 0.02 M cysteine to dissociate IgM into IgM$_s$. The results were difficult to interpret because although the amount of subunit increased with the time of exposure to the mercaptan, the moles of new SH per mole of IgM reached a maximum early in the experiment and then began to decrease. The IgM$_s$ failed to dissociate when chromatographed in the presence of propionic acid. Although this indicated the subunit did have at least one of its two possible inter-μ-chain disulfide bonds intact, the distribution of these bonds between μ chains within the monomer and intermonomer μ chains could not be established conclusively.

In the present work, we used a different mercaptan to dissociate IgM. The amount of dissociation was shown to depend upon the molarity of MEA in the reduction mixture (Figure 3). That concentration of MEA which resulted in about 50% breakdown of the macroglobulin arbitrarily was chosen as most likely to give useful results. Hence, reduction of IgM was carried out with 0.015 M MEA. In keeping with the previously designated nomenclature for subunits resulting from mild reduction of IgM (Miller and Metzger, 1965a), we referred to this product as IgM$_s$.

Our IgM$_s$ compared favorably with others with respect to its sedimentation coefficient, antigenic analysis, and complement of polypeptide chains. The sedimentation coefficient was 6.17 S at 6.21 mg/ml, and was concentration dependent. The electrophoretic mobilities of IgM$_s$ and its parent protein were similar. Antigenically, the subunit maintained the μ-chain class-specific site, and the κ site on the light chains. The IgM$_s$, like that resulting from reduction of IgM with cysteine (Miller and Metzger, 1965b), failed to dissociate into its constituent μ and light chains when gel filtered with a dissociating solvent. This indicated the IgM$_s$ maintained the integrity of its μ-light-chain disulfide bond, and at least one inter-μ-chain bond. This was substantiated by reducing the subunit with 2-ME, and demonstrating it did indeed contain μ as well as light chains prior to this reduction.

Since the experiments involving IgM$_s$ produced by reduction of IgM with cysteine for various periods of time displayed some aberrant features (Miller and Metzger, 1965b), we proceeded to make a similar study

with the MEA system. Several samples of IgM were reduced with 0.015 M MEA for different lengths of time. IgM_s was separated from the incompletely reduced mixture, and the content of CM-cysteine in each subunit was determined. The number of free sulfhydral groups on each IgM_s was similar regardless of the time of reduction within the limits of the experiment. Since there were two CM-cysteine residues per mole of IgM_s, we interpreted this to mean each IgM_s is bound on either side to another IgM_s by a single disulfide bond. It was postulated earlier (Miller and Metzger, 1965b), that IgM must exist in a ring form stabilized by covalent (disulfide) bonds. If the μ chain is involved in four interchain disulfide bonds and one of these is a μ-light-chain bond (Chaplin *et al.*, 1965; Miller and Metzger, 1965b), it was implied that there are two intramonomer inter-μ-chain bonds.

It was noted that components intermediate in size between IgM and IgM_s were not observed in schlieren patterns. Similarly, when IgM was digested under certain conditions with papain (F. P. Inman and S. R. Hazen, unpublished observations), components sedimenting between the monomeric subunit and undigested IgM were never seen. It may be that the lability of the intermonomer disulfide bond in a given IgM molecule is increased after the initial cleavage to the extent that MEA preferred to attack these bonds rather than those in another unreduced IgM molecule.

Acknowledgments

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