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# Production of a Noncovalently Bonded Pentamer of Immunoglobulin M: Relationship to J Chain

**(2-mercaptoethylamine/disulfide cleavage)**

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**ABSTRACT** **Human immunoglobulin M was reduced with concentrations of 2-mercaptoethylamine chosen so that approximately 40% of the immunoglobulin M was reduced to 7S subunits. Under these conditions, which selectively cleave intersubunit disulfides, J chain was released. The 7S subunits of immunoglobulin M so produced did not contain J chain. The high-molecular-weight immunoglobulin M remaining after treatment with 2-mercaptoethylamine had a sedimentation coefficient of 18.0 S and molecular weight of 1 million, and dissociated in 4 M guanidine into subunits similar in size to the 7S subunits. J chain was found in the 18.0S, noncovalently linked immunoglobulin M from which it was released only after more complete reduction with 10 mM dithiothreitol. The results are consistent with the hypothesis that J chain participates in the formation of the intersubunit linkages. However, it need not necessarily be directly involved in all of the intersubunit disulfides. It may play an important role in modulating the assembly of immunoglobulin M subunits, perhaps by inducing conformational changes that lead to noncovalent interactions between the subunits.**

Immunoglobulin M (IgM) has been shown to contain five subunits which are linked in a circular pentamer. The IgM molecule does not dissociate in solvents such as guanidine, urea, or acid, which inhibit secondary (noncovalent) forces, but upon treatment with mild reducing conditions, the 19S molecule is cleaved into five subunits each with a sedimentation coefficient of approximately 7 S. The above observation suggests that the subunits are disulfide bonded in the intact molecule and that once these linkages are cleaved, the subunits do not possess secondary bonds of sufficient force to hold them together during sedimentation analysis.

Halpern and Koshland (1) have recently described a new polypeptide chain (J chain) in rabbit secretory IgA. This chain is also present in other polymeric immunoglobulin molecules, such as IgM (2) and polymeric serum IgA of several species (3, 4). The function of J chain is unknown, but because it has been found only in polymeric immunoglobulin molecules, it has been postulated to be involved in the intersubunit linkage. Attempts at reaggregation of the reduced IgM molecule in the presence and absence of J chain have led to conflicting results regarding the requirement for J chain in the polymerization reaction (5, 6).

This study examines the role of J chain in polymer formation in the IgM system. A unique noncovalently bonded 18S IgM molecule is described which contains J chain. It is produced by reduction with low concentrations of 2-mercaptoethylamine. The results are consistent with the hypothesis that although the J chain participates in the formation of the intersubunit linkages, it need not necessarily be involved directly in all of the intersubunit disulfide bonds. J chain may also serve to modulate the assembly of the IgM subunits ($IgM_s$).

Abbreviations: IgM, immunoglobulin M; $IgM_s$, 7S subunits of IgM.

## MATERIALS AND METHODS

Four human IgM's were isolated from the serum of patients with Waldenström's macroglobulinemia by repeated (3–5 times) euglobulin precipitation followed by chromatography on BioGel P200 in 0.14 M NaCl. Two lambda and two kappa IgM's were isolated and determined to be pure by gel diffusion in Ouchterlony plates, immunoelectrophoresis, polyacrylamide gel electrophoresis, and analytical ultracentrifugation.

The IgM was reduced according to Morris and Inman (7) at a protein concentration of 5 mg/ml, with 10–25 mM mercaptoethylamine for 1 hr at 30°, followed by alkylation with 12–30 mM iodoacetamide for 15 min at 4°. The reducing conditions were chosen so that approximately 40% of the IgM was reduced to 7S subunits ($IgM_s$) as determined by analytical ultracentrifugation. Under these conditions, it was previously established that the intersubunit disulfides are selectively split. That only intersubunit linkages were cleaved was verified by polyacrylamide gel electrophoresis in 8 M urea and chromatography on BioGel P200 in 4 M guanidine, both of which failed to show dissociation of $\mu$ and L chains.

Polyacrylamide gel electrophoresis was performed in 5% gels according to the method described by Ornstein and Davis (8, 9). In some experiments, the gels were subjected to electrophoresis in 8 M urea; in others, the samples were applied in 8 M urea but the gel buffer did not contain urea. Both methods gave essentially identical results. The gels were stained with Coomassie blue in 12.0% trichloroacetic acid and scanned in a recording densitometer (Clifford Instruments).

Analytical ultracentrifugation was performed in a Spinco model E at 52,000 rpm at 20°, with Schlieren optics. In some experiments, a positive wedge window was used. The relative amount of $IgM_s$ produced by reduction was measured by integrating the areas under the ultracentrifuge boundaries with a Nikon comparator. $s^0_{20,w}$ were calculated by correcting sedimentation coefficients to water at 20° and extrapolating to zero concentration. Molecular weights were determined by the high-speed sedimentation equilibrium method described by Yphantis (10) using the polymetric scanner.

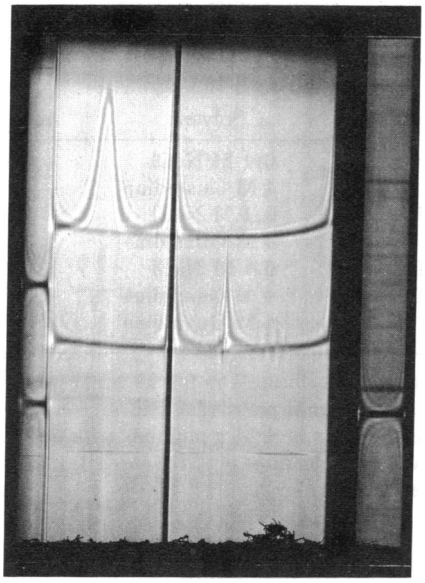

FIG. 1. Analytical ultracentrifugation pattern of IgM after treatment with 20 mM mercaptoethylamine, alkylation with 30 mM iodoacetamide, and dialysis against 0.1 M NaCl (*upper frame*); the same protein unreduced (*lower frame*). Sedimentation was from *left* to *right*. Picture was taken after 32 min of centrifugation at 52,640 rpm (Spinco model E Ultracentrifuge).

A partial specific volume of 0.717 was assumed in accordance with previously reported values for IgM (11).

An anti-J chain antiserum was produced in a rabbit by immunization with the J-chain bands cut from unstained, frozen polyacrylamide gels containing a reduced (10 mM dithiothreitol), alkylated IgA myeloma. J-chain bands from 10 gels were injected in complete Freund's adjuvant three times, 2 weeks apart. The antiserum was absorbed with a reduced, alkylated Fab fragment obtained by peptic digestion of the same myeloma protein. The antisera reacted in gel diffusion with reduced, alkylated IgM and IgA but not with the unreduced proteins. When unstained polyacrylamide gels of reduced, alkylated IgM or IgA myeloma proteins were placed in agar and the anti-J chain antisera in lateral troughs, a reaction occurred only with the J-chain bands. The locations of the L and H chains in these gels were marked with specific antisera to these chains.

## RESULTS

An analytical ultracentrifugation pattern of an IgM protein before and after reduction with 20 mM mercaptoethylamine for 30 min at 30° is shown in Fig. 1. As shown in this figure, treatment with mercaptoethylamine completely dissociated the higher polymers (26 S) although considerable amounts of what was interpreted initially to be unreduced 19S IgM remained. By measuring the areas under the 19S and 7S boundaries at various dilutions, we calculated that about 40% of the protein was reduced to $IgM_s$. Chromatography of the protein reduced by mercaptoethylamine on Sephadex G-200 in 0.14 M sodium chloride is shown in Fig. 2. Fractions were pooled as illustrated. The column had been previously marked with blue dextran by use of proteins of known size, including 19S IgM, 7S IgG, and 2S L-chain monomers, and the pools were made in accordance with the distribution of the markers.

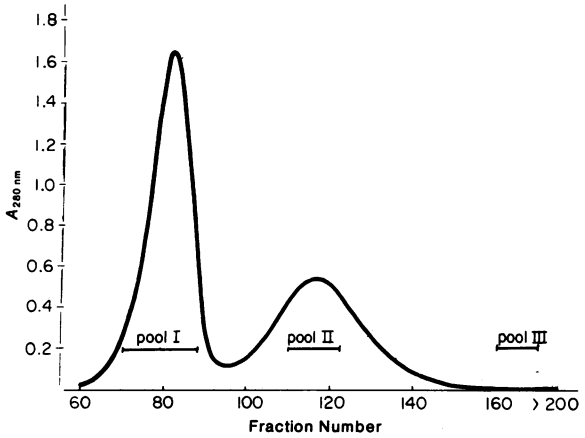


FIG. 2. Sephadex G-200 chromatograph in 0.14 M saline of reduced, alkylated IgM (20 mM mercaptoethylamine–30 mM iodoacetamide). Pool I corresponds to the position of the 19S marker, pool II the 7S marker, and pool III the 2S (L-chain) marker.

Pool I consisted of the "19S", pool II the "7S", and pool III the "2S" (L–J) regions of the column.

Polyacrylamide gel electrophoresis (Fig. 3) showed that electrophoresis in urea did not separate the L and H chains from either pool I or II. Pool III, which included the "L-J chain regions," contained very little protein, and L chains could not be detected immunologically. This observation verified that the initial mercaptoethylamine reduction did not cleave L–$\mu$ or inter-$\mu$ bonds and was apparently selective

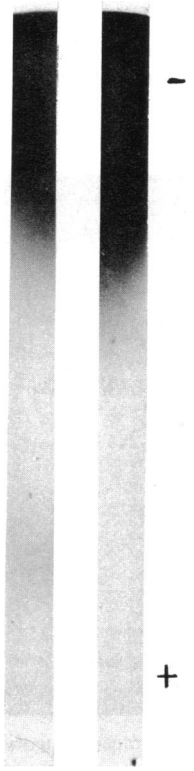


FIG. 3. 5% polyacrylamide gel electrophoresis in 8 M urea of material from pool I (*right*) and pool II (*left*). Pools as shown in Fig. 2. Anode on bottom.

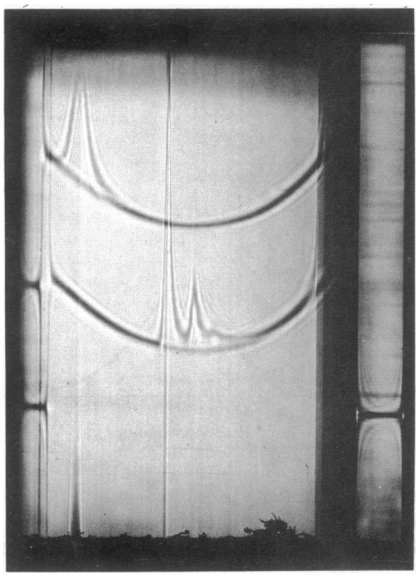

FIG. 4. Analytical ultracentrifugation pattern of material in pool I (see Fig. 2) (*upper frame*) and unreduced IgM (*lower frame*) centrifuged in 4 M guanidine. Sedimentation was from left to right. Picture was taken after 64 min of centrifugation at 52,640 rpm.

for the intersubunit linkage as reported by Morris and Inman (7). However, it was noted that the pool-I pattern was similar to that of pool II in that some migration into the gel had occurred (Fig. 3). This was quite different from the pattern obtained with the unreduced IgM, which did not penetrate the gel significantly.

The material in pool I was subjected to analytical ultracentrifugation at four different protein concentrations and was found to have an $s^0_{20,w}$ of 18.0 compared to 18.5 S for the unreduced IgM. The molecular weight of the IgM in pool I was 1 million.

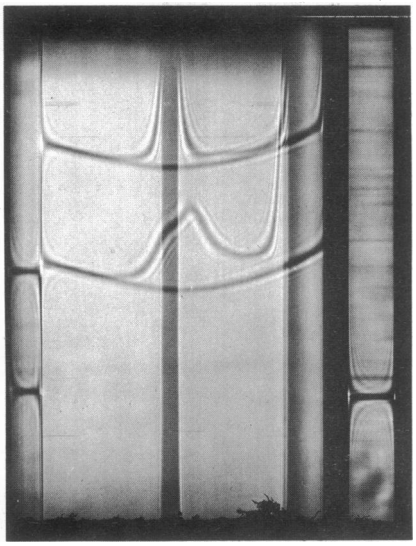

FIG. 5. Analytical ultracentrifugation pattern of Cohn fraction II (mainly 7S IgG) (*upper frame*) and pool I (see Fig. 2) (*lower frame*) centrifuged in 4 M guanidine. Sedimentation was from left to right. Picture was taken after 304 min of centrifugation at 52,640 rpm.

TABLE 1. *Sedimentation coefficients of IgM and mercaptoethylamine reduction products*

| Protein | Solvent | Sedimentation coefficient (S)* |
|---|---|---|
| Native IgM | 0.1 M NaCl | 18.5 |
| Native IgM | 4 M Guanidine | 6.9 |
| Pool I† | 0.1 M NaCl | 18.0 |
| Pool I | 4 M Guanidine | 2.7, 2.1 |
| Pool II† | 0.1 M NaCl | 7.4 |
| Pool II | 4 M Guanidine | 2.8 |
| IgG | 4 M Guanidine | 2.3 |

* Sedimentation coefficients in 0.1 M NaCl were extrapolated to infinite dilution from plots of $s$ against concentration. Sedimentation coefficients in 4 M guanidine were not corrected but were all measured at the same protein concentration (6 mg/ml).

† Pools as shown in Fig. 2.

However, when the material in pool I was centrifuged in 4 M guanidine (Fig. 4), it was apparent that the IgM dissociated to smaller subunits compared with the native IgM in the same solvent. Dissociation also occurred in 1 M propionic acid. These data suggested that the 19S IgM remaining after mercaptoethylamine treatment, initially considered to be unreduced IgM, consisted of noncovalently linked subunits with a sedimentation coefficient close to that of IgG. The boundary of pool I in 4 M guanidine split into two peaks late in centrifugation (Fig. 5). The various sedimentation coefficients measured are shown in Table 1. It is important to note that the sedimentation coefficients of both components present in pool I in the 4 M guanidine ($s$ = 2.7 S, 2.1 S) are close to that of the 7S marker ($s$ = 2.3 S) and considerably lower than the native unreduced IgM ($s$ = 6.9 S).

In order to investigate the relation of J chain to polymer formation, polyacrylamide gel electrophoresis was performed on the material in the pools (Fig. 6). In these experiments, the samples were reduced with 10 mM dithiothreitol, alkylated with iodoacetamide, and applied to the top of the gel in 8 M urea. Note that typical J-chain bands, usually two (occasionally three), were found in material from pool I, but were not present in pool II which contained $IgM_s$. Material from pool III, after lyophilization, reduction, and alkylation, showed a J-chain banding pattern similar to that of material from pool I and native IgM. It should be emphasized that J chain was released from the material in pool I only after reduction with dithiothreitol, and treatment with 8 M urea without prior reduction did not result in the appearance of J-chain bands. This result suggested that the J chain was disulfide bonded in the 18S (pool I) IgM. Densitometer tracings of polyacrylamide gels of material from pool I obtained from four different IgM proteins after reduction with 10 mM dithiothreitol showed that a mean of 5.7% (range 4.6–6.3) of the protein was contained in the "J-chain area". This was compared to a mean of 6.1% (range 4.5–6.8) when the starting IgMs were reduced under the same conditions. These results indicated that the content of J chain in the mercaptoethylamine polymer (pool I) and native IgM are very similar and probably identical within the experimental errors of the technique.

That the fast bands in the material from pools I and III were indeed J chain was verified by placing the gel in agar

(see *Methods*). An arc of precipitation appeared that was localized to the J-chain region after reaction with an anti-J chain antisera. Material from pool II was similarly studied and showed no reaction with the anti-J chain antisera. These results suggest that J chain was present in the noncovalently linked 18S polymer (pool I) and is not present in $IgM_s$ (pool II). Essentially identical results were obtained with three other monoclonal IgM proteins.

## DISCUSSION

Morris and Inman (7) reported that $IgM_s$ produced by 15 mM mercaptoethylamine not only had the L–$\mu$ bonds intact but that the $IgM_s$ contained two carboxymethyl cysteine residues per mol. This finding was interpreted to mean that in the intact IgM molecule each $IgM_s$ is bound on either side to another $IgM_s$ by a single disulfide bond. The possibility has been suggested (3) that a single J chain per IgM pentamer is disulfide bonded to each of the five IgM subunits. Since J chain has 10–12 half-cystine residues per molecule (3), there are sufficient numbers of sulphydryl groups to complex with each of the five $IgM_s$. This is also supported by the observation that 10 of the 12 half-cystines in J chain must be reduced before appreciable amounts were released from IgM (3). The release of J chain when the intersubunit bonds are selectively cleaved, as found in this study as well as in a recent report (12), is consistent with this thesis. These results would also suggest that once the interchain bonds are cleaved, the noncovalent interactions between J and $IgM_s$ are not of sufficient strength to hold them together during chromatography on Sephadex G-200 in aqueous buffers.

The results obtained in this study suggest an alternative, although admittedly speculative, interpretation. J chain interacts with the 7S monomer or perhaps links two such subunits together as a dimer. Complexing with J chain so alters the conformation of the subunits that they are able to interact with other subunits not containing J chain through secondary forces to form the noncovalently bonded 18S IgM polymer. The observed sedimentation coefficient of 18 S and molecular weight of 1 million is consistent with a pentamer. It is postulated that the noncovalent interactions between the subunits initiated by complexing of J chain are responsible for bringing the subunits into close enough apposition so that the intersubunit disulfides are formed. According to this hypothesis, J chain need not be directly involved in all of the intersubunit linkages although from the present data it cannot be determined whether J chain is directly linked to each of the subunits. This speculation seems reasonable in view of the likelihood that before disulfide-bond formation, close apposition of the subunits would be required to initiate the correct pairing of the sulhydryl groups on adjacent subunits. The necessity of close apposition would, in turn, require secondary forces probably extending over a significant length of the polypeptide chains. Such noncovalent interactions are common in disulfide-bonded chains, as for example, between L and H polypeptide chains.

The present observations would also be consistent with the reports (3, 13) that there is one J chain per IgM pentamer and one per IgA dimer. It should be noted, however, that the percentage of total protein attributable to J chain in the material of pool I and native IgM (5.7 and 6.1, respectively) is somewhat higher than those previously reported (2, 3) and calculate to two to three J chains per mol of IgM. However, because accurate data on the molecular weight of the J chain are not available, together with other technical difficulties such as variation in the uptake of stain by the different chains present in IgM and the presence of bound albumin which, after reduction, migrates in the J-chain region (Tomasi, unpublished observations), these results are at most, rough approximations. Assuming that IgM contains a single J chain, it may be that during intracellular synthesis, J chain initially links two subunits in IgM and IgA and the remaining subunits in the higher polymers in both systems are bonded first noncovalently and subsequently by disulfide linkages. Reduction with low concentrations of mercaptoethylamine could preferentially split interchain bonds not involving J chain. However, some of the J-chain disulfides are cleaved, as shown by the formation of 7S $IgM_s$ and the release of free J chain. Since the 18S noncovalently bonded IgM probably contains less than five J chains per mol and completely dissociates in guanidine, it seems likely that some of the IgM subunits must contain J chain and that others do not. Thus far, all attempts to separate the two molecular species observed on ultracentrifugation of the noncovalently bonded polymer (Fig. 5) have failed.

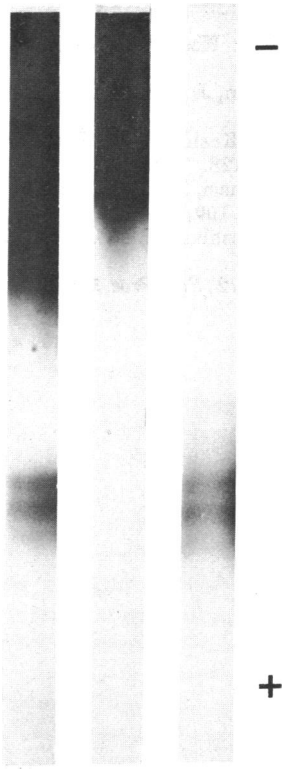


FIG. 6. 5% polyacrylamide gel electrophoresis in 8 M urea after reduction (10 mM dithiothreitol) and alkylation (25 mM iodoacetamide) of material in pool I (*left*), pool II (*center*) and pool III (*right*). Note J chain bands from pool I and pool III, but not pool II. Anode on bottom.

The thesis presented is open to further experimental testing with both IgM and IgA. For example, it should be possible, using appropriate techniques, to isolate a 7S monomer or dimer containing J chain that would form noncovalent complexes with 7S $IgM_s$ lacking J chain.

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